PLATELET-INSPIRED APPROACHES IN VASCULAR NANOMEDICINE

By:

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proprietary material contained therein.
Dedication

I would like to dedicate my dissertation to the many friends and family members who supported me through the process of obtaining my PhD, especially my labmates (Alyssa, Madhu, Michael, Tim, Lewis, Vic, Clarissa, Preethi, Greg, Kyle, Nat, Hassan, Gurbani, and Kavya), my parents and sisters, and my loving husband Donny.
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Figure 3.1: Events in the hemostatic action of platelets.

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Figure 6.2: Platelet-mimetic construct adhesion with varying extent of VBP decoration: Representative fluorescence microscopy images of varying extent of unmodified, VBP-decorated (2.5, 5, and 10 mol %), and heteromultivalent (VBP plus CBP at 2.5 mol % each) liposomes adhering to a VWF-collagen substrate after 30 mins of flow at 5, 30, and 55 dyn/cm².
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Quantitative analyses of fluorescence intensity of adhered liposomes at the 30 min time point at 5, 30, and 55 dyn/cm² show enhanced adhesion of VBP-decorated liposomes with increasing shear stress, compared to unmodified liposomes (*p<0.001). Increasing the total mol % of ‘VBP only’ decoration did not further enhance adhesion; however, heteromultivalent liposomes (VBP plus CBP) showed significantly higher adhesion and retention to the VWF-collagen surface compared to any ‘VBP only’ decorations (**p<0.001).

Figure 6.4: Platelet-mimetic construct adhesion with varying extent of CBP decoration:
Representative fluorescence microscopy images of varying extent of unmodified, CBP-decorated (2.5, 5, and 10 mole %), and heteromultivalent (VBP plus CBP at 2.5 mol % each) liposomes adhering to a VWF-collagen substrate after 30 mins of flow at 5, 30, and 55 dyn/cm².

Figure 6.5: Platelet-mimetic construct adhesion with varying extent of CBP decoration:
Quantitative analyses of fluorescence intensity of adhered liposomes at the 30 min time point at 5, 30, and 55 dyn/cm² show enhanced adhesion of CBP-decorated liposomes compared to unmodified liposomes (*p<0.001). Increasing the total mole % of ‘CBP only’ decoration did not further enhance adhesion; however, increasing CBP from 2.5 to 5 and 10 mole % resulted in enhancement of adhered construct retention (45 min time point) at high shear (55 dyn/cm²) (**p<0.05). In addition, heteromultivalent liposomes (VBP plus CBP) showed significantly higher adhesion and retention to the VWF-collagen surface compared to any ‘CBP only’ decorations (**p<0.001).

Figure 6.6: Construct adhesion for varying VBP: CBP ratios at fixed total peptide decoration:
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the liposome-coated coverslip surface. When the cRGD decoration was increased from 2.5 mole \% to 5 mole \% or from 5 mole \% to 10 mole \%, there was no statistical difference in extent of liposome-promoted platelet aggregation. Interestingly, a statistical increase was observed between samples with 2.5 mole \% cRGD versus those with 10 mole \% cRGD (**p<0.05). 

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Figure 6.10: Physical models of possible hemostatic mechanisms of the peptide-decorated liposomes: (A) VBP-CBP-decorated liposomal constructs can adhere to the vascular injury site and then promote further binding and multimerization of soluble VWF to the available exofacial VBP decorations on the liposome, which in turn can promote further binding of platelets at the site. (B) FMP-decorated liposomal constructs can result in clustering of active platelets onto the liposomes via FMP-to-GPIIbIIIa interactions and attachment of these clusters at the injury site can be mediated via direct adhesion of the platelets. (C) functionally integrated constructs show significantly higher hemostatic efficacy possibly because of their capability to undergo enhanced adhesion at the injury site and then promote enhanced platelet recruitment and aggregation at that site by both FMP-mediated and VBP-mediated mechanisms.

Figure 7.1: (A) Schematic of normal vascular endothelium and subsequent endothelial injury leading to VWF secretion, its shear-induced conformational change and multimerization on subendothelial collagen, and platelet adhesion, activation and aggregation on VWF/collagen matrix; (B) Schematic showing shear-induced conformational unraveling of VWF multimers leading to self-association along with atomic force microscopy (AFM) images of (i) globular and (ii) stretched VWF (adapted with permission from Marchant RE et al., Current Protein and Peptide Science 2002; 3: 249-74); (C) A closer schematic look at the various domains of VWF with specific bioactive functions.

Figure 7.2: A schematic of the envisioned mechanism of action of the VBP-cRGD-co-decorated liposomal constructs interacting with VWF and platelets to enhance the primary hemostatic processes of platelet recruitment and aggregation. In the schematic, ‘Fg’ stands for Fibrinogen. The VWF-binding peptide (VBP) peptide is the sequence TRYLRHLPQSWVHQI and the Fibrinogen-mimetic peptide (cRGD) containing the Arginine-Glycine-Aspartic Acid (RGD) sequence is cyclo-CNPRGDY(OEt)RC.

Figure 7.3: Representative fluorescence microscopy images (scale bar 100 μm) and quantitative fluorescence intensity data of interaction of calcein-stained (green) platelets to glass coverslip-adsorbed FVIII-free VWF in presence or absence of ristocetin (Risto) treatment with additional
presence or absence of glycocalicin (Glyco) pre-incubation. Platelets were found to significantly bind to Risto treated VWF compared to binding in absence of Risto; the platelet-binding to Risto-treated VWF was significantly reduced by pre-incubation with Glyco (p < 0.002) and this reduction was comparable to low platelet-binding on negative control BSA surface. Figure 7.4: Representative fluorescence microscopy images (scale bar 100 μm) and quantitative fluorescence intensity data of interaction of calcein-stained (green) platelets to glass coverslip-adsorbed FVIII-free VWF in presence or absence of ristocetin (Risto) treatment with additional presence or absence of glycocalicin (Glyco) pre-incubation. Platelets were found to significantly bind to Risto treated VWF compared to binding in absence of Risto; the platelet-binding to Risto-treated VWF was significantly reduced by pre-incubation with Glyco (p < 0.002) and this reduction was comparable to low platelet-binding on negative control BSA surface. Figure 7.5: Representative fluorescence microscopy images (scale bar 100 μm) and quantitative fluorescence intensity data of interaction of calcein-stained (green) platelets to glass coverslip-adsorbed FVIII-free VWF in presence or absence of ristocetin (Risto) treatment with additional presence or absence of glycocalicin (Glyco) pre-incubation. Platelets were found to significantly bind to Risto treated VWF compared to binding in absence of Risto; the platelet-binding to Risto-treated VWF was significantly reduced by pre-incubation with Glyco (p < 0.002) and this reduction was comparable to low platelet-binding on negative control BSA surface. Figure 7.6: (A1-D3) Representative fluorescence microscopy images (along with envisioned mechanistic schema) of peptide-decorated rhodamine-labeled (red) constructs and calcein-stained (green) platelets incubated simultaneously on Risto-treated VWF adsorbed on glass coverslips. (E) Quantitative overall fluorescence intensity data of platelets (green) adhered and aggregated on the VWF-adsorbed coverslips. A1, B1, C1 and D1 represent construct binding; A2, B2, C2 and D2 represent platelet binding; A3, B3, C3 and D3 represent merged results to exhibit co-localization in yellow. The conditions tested were undecorated (Unmod-Lipo), VBP-decorated (VBP-Lipo) and VBP-cRGD-co-decorated (VBP-cRGD-Lipo) liposomal nanoconstructs incubated with predominantly inactive platelets (Platelet) and ADP-activated platelets (Act Platelet). Undecorated constructs showed minimal VWF-binding and platelet co-localization, VBP-decorated constructs showed concomitant VWF-binding with platelets but minimal platelet co-localization and VBP-cRGD-co-decorated constructs showed substantial VWF-binding as well as platelet co-localization, especially if platelets were already in a pre-activated state. Figure 8.1: Illustration of cell-cell and cell-matrix interactions of platelets in thrombosis and possible restenotic and inflammatory processes; platelet hyperactivity and aggregation is mediated by GPIIbIIIa-to-Fg interaction and P-selectin-to-PSGL-1 interaction; P-selectin based interactions also lead to recruitment and adhesion of leukocytes. Figure 8.2: Representative results of platelet-relevant and platelet-inspired therapeutic approaches in the diagnosis and treatment of thrombotic and restenotic events; (A) exhibits research data regarding liposomes that target activated platelets, showing one-to-one correspondence between [i] liposome fluorescence and [ii] phase contrast images of active
platelets in vitro, [iii] enhanced binding of ligand-decorated liposomes to activated platelets compared to quiescent platelets depicted by flow cytometry, [iv] active binding of fluorescently labeled platelet-targeted liposomes to a carotid artery injury site in vivo in a rat model. Adapted with permission from [21] Copyright 2009 Wiley Periodicals, Inc.; (B) shows illustration of [i] fabricating VWF-binding gelatin nanoparticles loading tPA and [ii] efficacy of enhanced thrombolysis in myocardial infarction or TIMI with the tPA-loaded gelatin nanoparticles along with ultrasound-triggered release, compared to tPA alone or tPA-plus-ultrasound. Adapted with permission from [18] Copyright 2012 Elsevier; (C) shows [i] schematic illustration of CREKA-peptide modified micelles for targeting to fibrin-rich clot site and [ii] enhanced in vivo targeting of the micelles to a vascular clot site indicated by green fluorescence label of the micelles. Adapted with permission from [14] Copyright 2009 National Academy of Sciences, USA; (D) illustrates [i] the design of a lipid-polymer complex nanoparticle surface-decorated with peptides that targeted sub-endothelial matrix at injury site and [ii] efficacy of the targeted nanoparticle to target an angioplastastic vascular injury site in vivo. Adapted with permission from [19] Copyright 2010 National Academy of Sciences, USA; (E) shows the design of [i] PLGA-based nanoparticles and [ii] microscale conglomerate nanoparticles loaded with thrombolytic drug, that when injected in a vascular occlusion scenario, can undergo shear-induced disintegration and drug release, resulting in removal of occlusion as depicted in going from [iii] to [iv]. Adapted with permission from [20] Copyright 2012 American Association of the Advancement of Science.

Figure 9.1: Design of liposomal constructs surface-functionalized with two types of ligands for simultaneous targeting of GPIIb-IIIa integrin and P-selectin expressed at high quantity on the membrane surface of activated platelets involved in thrombotic and inflammatory events in vascular disease.

Figure 9.2: MALDI-TOF mass spectrometric characterization of the GSSGGRGDSPA and DAEWVDVS peptides synthesized via FMoc-based solid phase chemistry on Knorr resin using Applied Biosystems solid phase synthesizer.

Figure 9.3: Process for fabricating the heteromultivalent liposomal constructs for dual targeting of activated platelets; bottom panel shows representative DLS characterization data for the constructs before and after extrusion; after extrusion the constructs are largely monodisperse with an average diameter of 150 nm.

Figure 9.4: Fluorescence microscopy experiment to establish the specific binding of FMP-modified liposomes to activated platelets; top panel shows the experiment procedure; middle panel shows representative microscope images of control and test conditions (A through E); the quantitative analysis of fluorescence intensity from multiple images (n=10 per test or control condition) at these conditions are shown in the graph at the bottom, confirming the enhanced binding of activated platelets by FMP-modified liposomes compared to unmodified liposomes.

Figure 9.5: Fluorescence microscopy experiment to establish the specific binding of SMP-modified liposomes to activated platelets; top panel shows the experiment procedure; middle
panel shows representative microscope images of control and test conditions (A through E); the quantitative analysis of fluorescence intensity from multiple images (n = 10 per test or control condition) at these conditions are shown in the graph at the bottom, confirming the enhanced binding of activated platelets by SMP-modified liposomes compared to unmodified liposomes.

Figure 9.6: Specific receptor blocking studies analyzed by fluorescence microscopy to establish that FMP-modified liposomes bind to GPIIb-IIIa on activated platelets; top panel shows the experiment procedure; middle panel shows representative microscope images of control and test conditions (A through E); the quantitative analysis of fluorescence intensity from multiple images (n=10 per test or control condition) at these conditions are shown in the graph at the bottom, confirming that pre-incubation of activated platelets with FMP-modified liposomes results in significant blocking of fluorescent antibody specifically binding to activated platelet GPIIb-IIIa; this result in combination with results shown in Figure 3 confirm that the FMP-modified liposomes can specifically target and bind to GPIIb-IIIa on activated platelets.

Figure 9.7: Specific receptor blocking studies analyzed by fluorescence microscopy to establish that SMP-modified liposomes bind to P-selectins on activated platelets; top panel shows the experiment procedure; middle panel shows representative microscope images of control and test conditions (A through E); the quantitative analysis of fluorescence intensity from multiple images (n=10 per test or control condition) at these conditions are shown in the graph at the bottom, confirming that pre-incubation of activated platelets with SMP-modified liposomes results in significant blocking of fluorescent antibody specifically binding to activated platelet P-selectins; this result in combination with results shown in Figure 4 confirm that the SMP-modified liposomes can specifically target and bind to P-selectins on activated platelets.

Figure 9.8: Schematic representation of experimental set-up and procedure for the PPFC experiments to establish the enhanced binding and retention of peptide-modified liposomes to activated platelets under hemodynamic flow relevant shear stress ranges over time; the bottom left also shows representative SEM images of albumin-coated surface area and collagen-coated surface area after incubation with activated platelets, confirming that the collagen-coated surface has a significantly high density of active platelets; allowing the test and control liposomes to interact with platelet-rich (collagen-coated) and platelet-deficient (albumin-coated) surface regions on the same slide under various shear stress ranges in the PPFC set-up for effective analysis of liposome binding and retention.

Figure 9.9: Representative flow cytometry results showing (A) the gated activated platelet population in whole blood aliquots under analysis and (B) the fluorescence histograms from platelet population interacting with unmodified (non-targeted), FMP- or SMP-modified (singly targeted) and simultaneous FMP- and SMP-modified (dual-targeted) fluorescently labeled liposomes; it is evident that though the singly-targeted liposomes are capable of binding activated platelets significantly more than the non-targeted liposomes, the dual-targeted liposomes have even higher extent of binding activated platelets when compared to the singly-targeted liposomes.
Figure 9.10: Representative fluorescence microscopy images and quantitative data from PPFC experiments to study binding and retention of test (single or dual-targeted) and control (non-targeted) liposomes to activated platelet-coated surface versus platelet-deficient surface under flow at three different shear stress values (low-to-high shear) over a period of 45 min (30 min liposomal suspension flow + 15 min 1X PBS flow); it is evident that in a dynamic flow environment, dual-targeted liposomes are capable of binding and staying retained on target cells (activated platelets) at significantly enhanced levels over time at all shear stress values compared to non-targeted and even singly-targeted liposomes.

Figure 10.1: Design components of a delivery system that can specifically target and bind the site of vascular disease via simultaneously binding to activated platelet GPIIb-IIIa and P-selectin can allow site-selective delivery of various bioactive and therapeutic agents via PLA2-triggered payload release.

Figure 10.2: Active platelet binding of constructs with varying extent of FMP and SMP decoration. (A) Representative fluorescence microscopy images of varying extent of FMP-decorated (2.5, 5, and 10 mol %), SMP-decorated (2.5, 5, and 10 mol %), and heteromultivalent (FMP plus SMP at 2.5 mol % each) liposomes binding to active platelets at 5, 25, and 55 dyn/cm². (B-D) Quantitative analysis of fluorescence intensity of bound liposomes at 5, 25, and 55 dyn/cm² show enhanced platelet binding (30 min time point) and retention (45 min time point) of heteromultivalently-modified liposomes compared to FMP- and SMP-only liposomes, even when the total peptide content was less. Increasing the mol % of FMP- or SMP-only constructs from 2.5 to 5 mol% did not have a significant impact on the degree of binding, but demonstrated higher particle retention at high shear. Increasing from 5 to 10 mol% generally resulted in less particle binding and retention with the exception of SMP liposomes at 5 dyn/cm²...

Figure 10.3: Platelet binding of heteromultivalently-modified constructs containing varying FMP:SMP ratios at fixed total peptide decoration. (A) Representative fluorescence images for platelet binding (30 min time point) and retention (45 min time point) of liposomes for the varying ratios of FMP:SMP at total peptide decoration of 5 mol %. (B-D) Quantitative analysis of fluorescence intensity of bound liposomes at 5, 25, and 55 dyn/cm² for the varying ratios of FMP:SMP. Under medium (25 dyn/cm²) to high (55 dyn/cm²) shear conditions, liposomes containing 60% FMP and 40% SMP demonstrate the highest degree of platelet binding (30 min time point) and retention (45 min time point)....

Figure 10.4: (A) and (B) show, respectively, the carboxyfluorescein fluorescence and absorbance calibration curves based on which the encapsulation efficiency (EE) of CF in the liposomes was determined. (C) is the EE analysis from three batches of liposomes, showing that the average EE was ~ 75%.

Figure 10.5: (A) Schematic representation of CF release study. (B) Shows the CF release profile from liposomes in the presence or absence of PLA2. Enzymatic action of PLA2 on the liposome membrane leads to significantly enhanced CF release.

Figure 10.6: (A) Schematic representation of PPFC clot lysis experiment. (B) Representative fluorescence images of the clot surface (green) and particles bound to the clot (red) at various
time points. (C) Quantified analysis of clot lysis based on the change in clot (green) fluorescence over time shows significant clot lysis up to ~70% by the thrombus-targeted, SK-loaded particles in the presence of PLA2 compared to the saline control condition. (D) Quantitative analysis of particle (red) fluorescence intensity shows an increase in amount of bound particles over the course of the experiment.  

Figure 10.7: (A) Schematic representation of ferric chloride-induced carotid artery thrombosis formation in mice. (B) Representative grayscale snapshots of real time intravital microscopy video shows platelet-targeted enhanced particle accumulation at the site of thrombosis compared to non-targeted particles. (C) Representative ex-vivo fluorescence microscopy images of the excised carotid artery show enhanced targeted-particle binding (green fluorescence) to the thrombus both 1 and 2 hours post-occlusion compared to non-targeted control particles.
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Platelet-inspired Approaches in Vascular Nanomedicine

Abstract

by

CHRISTA LYNN MODERY-PAWLOWSKI

Nanomedicine strategies of packaging drugs within nanovehicles that are engineered to preferentially localize at a disease site for site-selective drug delivery have shown great promise in cancer therapies but have shown only limited potential in treating vascular diseases. Ideally for vascular nanomedicine, once injected into the systemic circulation, the vehicles must be able to marginate across bulk blood flow towards the vessel wall, anchor specifically onto target cells or molecules at the vessel wall via active binding mechanisms to allow disease site-selective accumulation and stable retention under flow, and then allow delivery of the drug in a controlled fashion. Meeting these multiple design requirements under the complex vascular hemodynamic flow environment can become quite challenging. To address these requirements, my research has considered blood platelets as a unique design paradigm since these cells have an innate ability to perform all of the above-stated functions. In this context, the primary focus of the current dissertation is on the functional mimicry of platelet’s cell-cell and cell-material biointeractions, under the overall hypothesis that surface-engineering of drug delivery vehicles with platelet-inspired heteromultivalent biointeractions can enable site-specific anchorage and retention of the vehicles under a hemodynamic environment in the vascular compartment. I have tested this hypothesis in two vascular nanomedicine designs: (i) a platelet-inspired synthetic hemostat for management of bleeding...
complications and (ii) a platelet-inspired vehicle for clot-targeted delivery of thrombolytic agents. Beyond validating the hypothesis in these two systems via integrating platelet-inspired heteromultivalent biointeractions on model liposomal platforms, I have also carried out pilot studies in the other components of the platelet-inspired design, namely engineering of particles that have enhanced margination to the vessel wall and engineering of particles that can be triggered by in situ stimuli to render disease site-selective controlled drug delivery. In summary, my research establishes the potential of platelet-inspired design approaches for future applications in vascular nanomedicine.
Chapter 1: Nanomedicine

1. Introduction

Nanomedicine is a broad and interdisciplinary field in which nanoscale materials, or materials containing at least one characteristic dimension between $1 \times 10^{-9}$ m (1nm) – $1 \times 10^{-6}$ m (1000nm), are used for medical applications such as monitoring, diagnosing, preventing, repairing, or curing diseases and damaged tissues. The distinction of the field of nanomedicine compared to other areas of nanoscale science and materials (e.g. colloid science, molecular biology, quantum physics, etc.) is that in nanomedicine, because of introduction of nanoscale materials into the human body, there exists several complex engineering criteria for materials design such as biocompatibility, biodistribution, clearance, etc. The term nanomedicine was first coined in the book *Unbounding the Future: The Nanotechnology Revolution* in 1991 [1]. Over the past two decades, the field of nanomedicine has seen an explosion in research activity, especially in the area of nanoparticle-mediated, localized drug delivery [2], [3]. This application involves packaging pharmaceuticals, bioactive molecules, and/or imaging agents within nanosized materials for controlled delivery at a target site inside the body to provide focused diagnostics and/or therapy, while minimizing systemic drug distribution and side-effects. Nanomedicine-based localized delivery has demonstrated substantial pre-clinical and some clinical success in the area of cancer therapy [4–7], but this approach has great potential for applications in other areas, specifically in cardiovascular pathologies [8]. This chapter will highlight the clinical significance of nanomedicine, discuss the ideal
design criteria for nanomedicine-based drug delivery systems, and review the current state of investigational and approved nanomedicine products, especially in the context of vascular applications.

2. Significance of Nanomedicine

Although new and improved therapeutic compounds continue to be introduced into the clinic, the method of administration of such compounds has remained essentially unchanged, where drug molecules in their native form are introduced systemically into the body [9]. This conventional administration approach presents several limitations, as summarized in Table 1.1. First, systemic administration can lead to dilution of the drug in the blood as well as deactivation or modification of the drug in the plasma, such that the amount and potency of the drug fraction that reaches the target site may be insufficient to have a clinical effect [10], [11]. Additionally, the biodistribution and other pharmacokinetic properties of the native form of the therapeutic compound can be very poor. Lastly, a lack of selectivity in drug accumulation and drug action can lead to potentially harmful and debilitating side effects [10], [11]. These issues with current therapies can potentially be resolved by localizing the delivery (and action) of the drugs specifically to the target site. This is where drug delivery strategies utilizing the nanomedicine approach have raised significant clinical interest.

Using the tools of nanotechnology, drug delivery systems can be developed to alter both pharmacological and therapeutic effects of drug molecules. Therapeutic molecules can be packaged within nano-sized materials, thereby protecting them from rapid degradation in plasma or clearance from circulation [10], [12]. Furthermore, these drug
delivery systems can be engineered to enhance drug accumulation in target tissues, thereby decreasing the dose required to have a therapeutic effect while reducing side effects [10], [12]. Therefore, these properties of nanomedicine strategies can not only increase the therapeutic efficacy of a drug but can also reduce costs of drug discovery, design, and development [13].

Limitations of Current Systemic Drug Delivery

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<th>Limitations</th>
<th>Design Criteria for an Ideal Nanomedicine Drug Delivery System</th>
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<tr>
<td>i. Dilution of drug in blood and drug deactivation or modification in plasma</td>
<td>i. Package drug at appropriate doses and protect drug from plasma deactivation</td>
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<td>ii. Poor biodistribution and pharmacokinetics</td>
<td>ii. Distribute through body without rapid clearance and cross biological and physical barriers</td>
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<tr>
<td>iii. Insufficient drug concentration at target site</td>
<td>iii. Home and accumulate/stay retained at target site</td>
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<tr>
<td>iv. Indiscriminate drug action and harmful side effects</td>
<td>iv. Release payload at target site in appropriate quantities and time frame</td>
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This clinical need yields a sizable global market for nanomedicine. According to a report by Wagner et al., in 2006, there were 207 companies that visibly pursue nanomedicine activities, accounting for ~$6.8 billion in sales, with drug delivery systems accounted for more than 75% of the total [3]. Furthermore, BCC research analysis has estimated that, with the explosive growth in the field over the past few years, the nanomedicine market will reach $96.9 billion by 2016 [14]. Therefore, there is great interest in the utilization of nanotechnology in drug delivery applications.
3. Engineering an Ideal Nanomedicine Drug Delivery System

In order for a drug delivery system to resolve the issues otherwise presented by current systemic delivery approaches, the system should have the following characteristics (Table 1.1): (i) ability to package therapeutic or diagnostic cargo at appropriate doses and keep cargo encapsulated while circulating in the body, (ii) ability to distribute throughout the body without getting rapidly cleared and cross many biological barriers, (iii) ability to home to and accumulate/stay retained at a specific target tissue site, (iv) ability to release payload in adequate quantities within an appropriate therapeutic window of time. Nanotechnology can provide the necessary tools for engineering such a delivery system, and to this end over the past few decades, many nanomedicine approaches have been investigated for a variety of therapeutic and diagnostic applications. The most common and promising nanomedicine strategy for targeted drug delivery involves the use of nanoparticles, and therefore nanoparticle systems will be the focus for the rest of this section. The unique and tunable properties of nanoparticles give them the potential to become an ideal drug delivery system, as outlined in the next few paragraphs.

In order to meet design criterion (i), drugs and other therapeutic/diagnostic cargo can be loaded at high capacities within or onto nanoparticles. Drug loading or entrapment via nanoparticles not only protects the drug from plasma deactivation, but it also prevents indiscriminant drug distribution in the body, thereby reducing harmful side-effects [10], [11]. Furthermore, nanoparticles can improve pharmacokinetic properties of a drug by increasing drug solubility in blood and increasing circulation times by reducing rapid clearance [10], [11]. Although the volume available for drug entrapment in nanoparticles
is limited by their small size, high concentrations of drug molecules can be loaded in each nanoparticle [10], [11]. Additionally, since drug loading and entrapment efficiency depends on a number of factors including drug solubility, molecular weight, drug-particle interactions, and hydrophobicity [15–18], the flexible chemistries and various fabrication methods of nanoparticles allow for specific tailoring to achieve maximum loading for a specific drug.

Therapeutic molecules can be loaded into nanoparticles at several locations based on the type of nanoparticle system, and each has its advantages and disadvantages. Nanocapsules are vesicular systems in which drug is entrapped in a cavity surrounded by a polymer membrane (ie. liposomes and micelles) [15]. Nanocapsules typically consist of self-assembly nanoparticles, and the drug is usually incorporated at the time of nanoparticle formulation. Drug encapsulation in nanocapsules is largely influenced by the hydrophobicity of the drug and the particle (void) size, and the encapsulation efficiency varies with the method of formulation [19]. Nanospheres are matrix systems in which the drug is physically and uniformly dispersed, again usually at the time of nanoparticle formulation (ie. poly(lactic-co-glycolic acid) (PLGA) nanoparticles) [15]. Nanosphere drug entrapment is largely influenced by drug-polymer interactions and drug solubility, and they typically demonstrate less protection of the drug from degradation and faster release compared to nanocapsules [20]. Lastly, solid nanoparticles can also carry drug by physically adsorbing or chemically conjugating drug molecules to the surface after particle fabrication (ie. gold nanoparticles) [15]. Loading of drugs onto the surface of solid nanoparticles is limited by the surface area of the particles, resulting in lower
loading efficiencies compared to both nanocapsules and nanospheres [15]. Furthermore, solid nanoparticles provide much less protection of the drug from plasma deactivation.

To address design criterion (ii), the small physical dimensions of nano-sized materials enable them to circulate in the blood and penetrate many biological and physiological barriers that are normally impermeable to larger particulate systems [21]. Additionally, most nanoparticle surfaces can be chemically modified quite easily to alter and improve their pharmacokinetic and pharmacodynamic properties [22]. The most common example is surface modification with hydrophilic polymers, such as poly(ethylene glycol) (PEG), which has been shown to significantly increase circulation time by sterically protecting nanoparticles from interaction with plasma proteins (e.g. with opsonins) and reducing the non-specific uptake by the reticuloendothelial system (RES) [23–30].

Nanoparticles can be engineered to specifically home in and accumulate at a specific target tissue site (design criterion (iii)) by two main mechanisms: 1) passive uptake regulated by nanoparticle physical properties in combination with physiological properties of the target area, and/or 2) active molecular targeting mechanisms via surface modification of the nanoparticles [31].

**Passive targeting:** Under certain pathological conditions, especially cancer, the endothelial lining of the blood vessels becomes permeable such that it allows large molecules and particles (ranging from 10-500nm) to leave the vascular space and accumulate in the interstitial space [31–34]. Additionally, the lymphatic drainage in these areas can be inadequately developed, leading to enhanced retention of particles [35], [36]. Therefore, drug-carrying nanoparticles can be engineered within this size range to
facilitate the passive accumulation and retention of drug at such disease areas, a phenomenon referred to as the enhanced permeation and retention (EPR) effect [37], [38]. However, this passive targeting strategy is only effective if the concentration of particles in the bloodstream is higher than that in the target tissue, since the process is diffusion driven. Once the particles are cleared from the blood, resulting in higher tissue concentrations, the nanoparticles could efflux back into the bloodstream [39].

Furthermore, in most pathologies, the integrity of the vascular endothelium remains unaffected and there is no opportunity to utilize the EPR effect for targeting [31]. Additionally, nanoparticle retention at the target tissue may be influenced by physical and mechanical forces, such as the shear stresses of blood flow [40]. Hence, nanoscale drug carriers have been further refined to actively bind to target tissue and thereby facilitate drug localization and retention at disease sites. This is referred to as ‘active targeting’ and is achieved via ligand-based surface modification of nanoparticles.

*Active targeting*: The versatile surface chemistry of nanoparticles provides the ability to conjugate biological moieties such as antibodies, peptides, and oligonucleotide sequences, to enable the particles to bind to specific antigens on target cells and diseased tissues, thereby increasing localization and retention [41]. Surface modification with disease-specific targeting ligands can allow the particles to selectively accumulate at a certain target tissue site, and modulation of the targeting moiety provides an easy way to tailor a drug delivery system for a specific disease condition. For example, many cancerous cells overexpress the epidermal growth factor receptor (EGFR) compared to healthy cells; therefore, nanoparticles decorated with EGFR-targeting ligands have demonstrated enhanced localization to such tumors compared to unmodified particles.
Furthermore, unlike the pseudo-steady state conditions in tumor interstitium, many target tissue sites for various pathological conditions are under a physiological flow environment, such as any target site within the vasculature. Therefore, active targeting can not only enhance accumulation of drug-loaded nanoparticles at the target disease site, but can also enable the particles to stay retained at the target site under flow conditions.

As an example, nanoparticles decorated with ligands that bind to active platelet and endothelial cell P-selectin at sites of vascular disease demonstrated higher binding and retention compared to unmodified particles under physiologically-relevant fluid flow.[43–46]

Once the drug-loaded nanoparticles accumulate at the target tissue, the drug needs to be released in adequate quantities within an appropriate therapeutic window of time (design criterion (iv)). The ideal drug release profile, therefore, varies with each specific drug molecule and disease. Drug release rate from nanoparticles is governed by several properties including drug solubility, desorption or cleavage of drug from the particle surface, drug diffusion through nanoparticle matrix or membrane, nanoparticle matrix.

![Figure 1.1: Nanoparticle components that meet the design criteria for an ideal drug delivery system.](image-url)
erosion or degradation, or some combination of these properties [15]. In nanocapsule systems, drug release is mainly governed by the diffusion of drug through the membrane coating [15]. When drug is uniformly distributed within the particle, as is the case of nanospheres, drug release occurs by both diffusion of the drug through the matrix and erosion of the matrix. Therefore, the relative rates of diffusion and erosion govern the release profile for the drug. Furthermore, an initial ‘burst’ release is seen in some nanoparticle systems where weakly bound or entrapped drug is released rapidly due to the particles’ large surface area [47]. Additionally, nanoparticles can be further engineered such that release of the drug can be triggered by other factors such as pH and enzymatic action or external sources such as heat, light, and energy [48], [49].

To summarize, in theory, nanoparticles can be engineered to meet all of the design criteria of an ideal drug delivery system (Figure 1.1). In order to achieve this, nanoparticle composition, size, and surface properties in combination with therapeutic cargo choice must be carefully optimized. Over the past few decades, researchers have been investigating the balance of these properties towards to goal of creating ideal delivery systems for a variety of disease conditions.


Because the field of nanomedicine is so vast, a comprehensive review of all nanomedicine strategies for all disease types currently under investigation is beyond the scope of this thesis. However, this section will highlight the current state of nanomedicine strategies in the process of clinical translation for the purpose of understanding the current challenges and future directions of the field.
Nanoparticle systems currently under investigation include, but are not limited to, self-assembling polymeric nanoconstructs (ie. liposomes and micelles), functionalized carbon nanotubes, buckyballs, inorganic nanoparticles (ie. colloidal gold and silver, iron oxide nanoparticles), quantum dots, etc. In 2013, Etheridge et al. released a comprehensive analysis of the state of investigational and approved nanomedicine products [50]. The results from their review paint a clear picture of the current status and future directions in the applications of nanomedicine strategies. As of last year, there were 141 unique nanomedicine applications and products undergoing clinical trials (Table 1.2). Of the nanomedicine strategies already approved for clinical use, 7 are classified by the FDA as biologicals, 38 as devices, and 32 as drugs. Of those still under clinical investigation, 26 are biologicals, 21 devices, and 91 are drugs. Thus, the majority of nanomedicine products are used for drug delivery. It should also be noted that the majority of nanomedicine strategies under clinical investigation consist of liposomes and emulsions, demonstrating the promise of the nanocapsule design. Lastly, when classified by intended use, about two-thirds of the investigational applications identified are focused on cancer treatment (Figure 1.2). This is largely because the majority of therapeutic nanomedicine strategies have relied on passive targeting via the EPR effect, with only 1 approved product and 19 applications under clinical study exploiting active targeting. Still, all of the actively targeted products under investigation are aimed at diagnosing or treating various forms of cancer.

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*Table 1.2: Number of nanomedicine applications and products in development, sorted by phase. Adapted with permission from Etheridge et al. Ref #[50].*
From this analysis, it is clear that packaging drugs within nanoparticles is widely established with several clinical successes in the field of cancer therapy. However, the clinical use of nanoparticles in other disease areas has been limited. One field that has great potential for nanomedicine applications is that of cardiovascular diseases [8], [40], [51–53]. However, the complex hemodynamic environment can present several challenges for nanoparticle-mediated drug delivery in the vascular compartment.

5. Challenges of Vascular Nanomedicine and Inspiration from Platelets

Since most of the focus in nanomedicine has been in cancer treatment, the vascular system has been simply viewed as a path for the nanoparticles to reach their target malignant site. As such, the nanoparticle systems have been designed to interact in the most passive way possible with the vascular system and its components [52]. Therefore,
the engineering principles for successful navigation, site-specific targeting, and drug delivery need to be reevaluated for therapies directed at sites within the vascular system.

The first challenge in vascular nanomedicine is designing the nanoparticles such that they are able to traverse across the bulk blood flow and marginate to the vascular wall (Figure 1.3). Secondly, the nanoparticles must bind specifically to the vessel wall at the target site via active targeting strategies so that they can not only successfully accumulate at the disease site, but also stay retained there under the hemodynamic flow conditions. Lastly, once stably anchored to the vascular disease site, the particles must be able to deliver the drug in a sustained way.

To address the above criteria for engineering of vascular nanomedicine systems, blood platelets provide a unique design paradigm. Natural platelets have the innate ability to (i) marginate across bulk blood flow towards the vascular wall, (ii) interact actively

![Figure 1.3: Vascular nanomedicine design challenges. In order to render effective drug delivery in the vasculature, upon system administration (e.g. IV injection), nanoparticles must be able to marginate to the vessel wall under bulk fluid flow, bind stably to the target site under hemodynamic shear stresses via active targeting, and deliver drug in a site-specific and sustained manner.](image-url)
with specific cellular antigens and matrix proteins at the vascular wall to undergo stable anchorage under the hemodynamic flow environment, and (iii) release a variety of cytoplasmic granule contents to modulate physiologic (e.g. in hemostasis) or pathologic (e.g. in thrombosis) mechanisms. Therefore, elucidating the natural cues of platelets that allow these capabilities can lead to unique platelet-inspired design of drug delivery systems for vascular applications. To this end, the current dissertation has investigated the functional mimicry of various platelet properties, to achieve the design of vascular drug delivery systems.

6. References


Chapter 2 : The Platelet Paradigm for Vascular Nanomedicine

Some content based on:


1. Introduction

Platelets are megakaryocyte-derived anucleated cell fragments found in the blood, and although their biology is simpler than many other types of mammalian cells, platelets play diverse roles in various physiological and pathological processes including hemostasis, atherosclerosis, thrombosis, inflammation, immune response and cancer metastasis. The combination of platelets’ unique physical, mechanical, and biological properties yields their remarkable ability to marginate and bind to target sites in the vasculature and site-specifically release bioactive agents, making platelets an interesting paradigm for nanomedicine strategies in the vasculature. The current chapter will highlight basic concepts in platelet biology and how several of platelets’ properties can provide inspiration to engineer smarter and more effective vascular nanomedicine strategies.

Platelet Biology

Platelet formation
Platelets are produced by mature megakaryocytes, which are a myeloid cell type that reside primarily in bone marrow, but can also be found in the lung and peripheral blood [1], [2]. Megakaryocytes prepare their cytoplasm and membrane systems for platelet production by increasing in size to ~100μm as well as by filling with high concentrations of ribosomes for production of platelet-specific proteins [3]. Additionally, megakaryocytes significantly amplify their DNA without undergoing cell division, a process known as endomitosis [4–7]. Furthermore, granules and organelles are assembled in bulk through the formation of an expansive network of tubules called the demarcation membrane system (DMS) [8–10]. During this process, platelet-specific proteins are either sent to the megakaryocyte surface or packaged within granules such as alpha (α) and dense granules [11], [12]. These fully matured megakaryocytes then form long, beaded pseudopodal extensions called proplatelets, which are eventually completely fragmented, resulting in the release of platelets (Figure 2.1) [13–17]. Each megakaryocyte can give rise to thousands of platelets during this process at a rate of 20-50 platelets per hour [18–20]. The normal platelet count in the blood is between 150 and 350 x 10⁹ per liter [21], and each platelet has an average lifespan of ~8-10 days [22]. Disorders in platelet production, such as Bernard-Soulier syndrome, MYH9-related disorders, and gray platelet syndrome, can lead to severe thrombocytopenia (low platelet counts) and bleeding complications [1].
Platelet activation

The resultant platelets produced by the aforementioned process are biconvex discoid in shape with diameters between 2-5µm and thickness of ~0.5µm [23]. In the blood stream, platelets travel singly as these smooth-surfaced discs; however, in response to a range of biological and mechanical stimuli, platelets undergo an activation and shape change process (Figure 2.2) [24]. A variety of physiologic stimuli, including matrix proteins such as collagen, proteolytic enzymes such as thrombin or trypsin, and low
molecular weight compounds such as ADP, serotonin, and epinephrine, can activate platelets via binding to specific platelet surface receptors [24]. Additionally, platelets can become activated under pathologic conditions. For example, activated leukocytes produce platelet activating factor (PAF), which stimulates platelets, even at low
concentrations [25], [26]. Exposure to elevated shear stress over time, as seen in occlusive cardiovascular diseases, has also been shown to induce platelet activation mechanically [27].

Upon activation, platelets change from discoid to a stellate, or star-like, morphology by rearranging cytoskeletal components and extending very fine (0.1µm) pseudopodia from the rim of the disc, increasing the total platelet surface area [28]. This shape change and cytoskeletal rearrangement facilitates several important biological changes such as platelet granule content release and changes in expression of several platelet membrane receptors. Microtubule bundles lie beneath the rim of the disk to surround the platelet granules, concentrating them toward the center of the platelet [29]. Here, the granules fuse with one another and with the platelet membrane to release their contents into the extracellular environment [30–32]. The contents of the granules, summarized in Figure 2.2B, play various roles in platelet biology, such as activating nearby platelets and endothelial cells, propagation of the coagulation cascade, and promoting angiogenesis. Additionally, an important membrane protein, P-selectin, is stored in the membrane of alpha granules, and it redistributes to the platelet plasma membrane when activated [33], [34]. P-selectin can then act as an adhesion receptor to recruit other active platelets and neutrophils to the sites of tissue injury [35] and facilitate acute inflammation [36]. Also upon platelet activation, cytoskeletal rearrangements cause a conformational change in the GPIIb-IIIa complex, allowing it to bind to blood protein fibrinogen and facilitate platelet aggregation [37]. All of these physical and biological changes that occur upon activation significantly change the functionality of platelets.
Therefore, platelet activation acts as an ‘on switch’ that is triggered site-specifically by precise physiological or pathological cues.

**Platelet functions**

Platelets have many functions in the body, and several of their functions will be discussed in detail in later chapters of this dissertation. However, this section will highlight the key physiological and pathological functions of platelets in the body and how platelets’ biology facilitates these functions.

The main role of platelets in the body is to regulate hemostasis, or blood clotting upon injury. Platelets regulate this process through a series of coordinated steps. First, platelets adhere to the site of an injury by binding to von Willebrand Factor (vWF) secreted by injured endothelial cells via their GPIba and GPIb-IX-V receptors, in addition to platelet GPIa-IIa and GPVI binding to subendothelial collagen [38]. This adhesion process in combination with stimulation by agonists (e.g. ADP and thrombin) secreted by the injured endothelium results in platelet activation [39]. Activation-induced conformational changes in GPIIb-IIIa allow platelets to aggregate together via binding to blood protein fibrinogen and form a primary platelet plug [40]. Furthermore, a regulated flip-flop mechanism on the membrane of aggregated active platelets causes negatively charged phosphoserine lipid to become exposed on the outer surface of the platelets, which in turn leads to colocalization and activation of multiple coagulation factors [41]. This pro-coagulant process leads to the formation of thrombin, which converts fibrinogen into fibrin, arresting the platelet plug and other blood components to form the final, stable clot [42]. Dysregulation or hyperactivity of these hemostatic mechanisms can lead to the
formation of an occlusive thrombus, which can result in cardiovascular events such as myocardial infarction, stroke, and peripheral vascular diseases [43].

Beyond hemostasis and thrombosis, there is growing evidence that platelets have multiple mechanistic roles in other pathologies such as inflammation, immune response, and cancer metastasis via various ligand-mediated interactions as well as secretion of molecules that influence the disease microenvironments [44], [45]. In any inflammatory response, the process of leukocyte homing to the inflamed tissue involves leukocyte recognition of the injury site and migration toward the endothelium, initial tethering and rolling of leukocytes along the endothelium, activation of leukocytes causing expression of various cell adhesion molecules, firm adhesion followed by diapedesis through the endothelium, and migration through the tissue to the source of injury/infection along with transformation into the macrophage phenotype. Platelets have been shown to orchestrate many of these steps through direct interaction with inflammatory cells (e.g. P-selectin binding to PSGL-1) and release of soluble factors (e.g. PF4, IL-8, PDGF, and TGF-β) [46–49]. Similarly, platelets have also been shown to modulate innate and adaptive immune responses by recruiting immune cells to transplanted organs or microorganisms [50–52]. Lastly, there is compelling experimental and clinical evidence that suggests a significant role of platelets in the process of cancer metastasis, or spread of cells from a primary tumor to a distal site in the body through the blood [53–55]. This process is again mediated by both direct binding between platelets and cancer cells and platelets’ secretion of various pro-metastatic biomolecules.

2. The nanomedicine paradigm
Platelets are able to play many diverse roles in the body because of their innate ability to (i) marginate across bulk blood flow towards the vascular wall, (ii) interact actively with specific cellular antigens and matrix proteins at the vascular wall to undergo stable anchorage under the hemodynamic flow environment, and (iii) release a variety of cytoplasmic granule contents to modulate physiologic or pathologic mechanisms. Therefore, elucidating the natural cues of platelets that allow these capabilities can lead to unique platelet-inspired design of drug delivery systems for vascular applications.

**Margination across bulk blood flow toward vessel wall**

The margination of platelets is significantly influenced by their interactions with the RBC volume, which in turn is influenced by their respective shapes, sizes and moduli. Natural quiescent platelets are biconvex discoid in shape, about 2-5 μm in diameter and 0.5 μm thick, while natural RBCs are biconcave discoid in shape, about 8-10 μm in diameter and 1-2 μm thick [56], [57]. Also, the elastic modulus of natural quiescent platelets is 2-5 times higher than natural RBCs; that is, they are much stiffer than RBCs in the quiescent state [58–61]. Additionally, RBCs occupy a much larger volume fraction (~40%) of blood than platelets (~0.7%). These physico-mechanical parameters result in expulsion of platelets from bulk RBC flow in the center of a blood vessel into a relatively cell-free plasma layer closer to the vessel wall, a phenomenon known as margination [58], [62–64]. This enhances the platelets’ probability to collide and interact with the vessel wall. The margination behavior of platelets is a combination of a convection-diffusion process and a collision-enhanced lateral drift [65]. While platelets are transported in the direction of blood flow as a result of convective transport, they are simultaneously dispersed laterally toward the vessel wall as a result of shear-induced
diffusion. This convection-diffusion process can be modeled, assuming blood flow in the x-direction between two fixed parallel plates (e.g. vessel wall considering z-direction to be symmetrical) with a parabolic velocity profile at steady state as follows:

$$u(y) = \gamma_w y \left(1 - \frac{y}{2H} \right) \text{ (Eq. 1)}$$

where $\gamma_w$ refers to the wall shear rate, $2H$ is the channel width, and $y$ is the channel height. The convective-diffusion equation is:

$$\frac{\partial P}{\partial t} + u(y) \frac{\partial P}{\partial x} = -\nabla (-D \nabla P) \text{ (Eq. 2)}$$

where $P$ is the platelet concentration and $(-D \nabla P)$ is the diffusive flux of platelet, $D$ being the diffusion coefficient. For a heterogeneous collection of particles in suspension under a laminar shear flow (e.g. different blood cells in hemodynamic flow), the diffusion coefficient is influenced by particle dimension, volume fraction and shear rate. Recently, several researchers have looked into developing mathematical models for enhanced multi-factorial diffusive flux coefficient, incorporating the relative presences of RBCs and platelets in blood flow as well as platelet’s own Brownian diffusion. One example model from Tokarev et al states [66]:

$$D = 0.15 \left(\frac{d_{RBC}}{2}\right)^2 \dot{\gamma} \phi_{RBC} (1 - \phi_{RBC})^{0.8} + 0.15 \left(\frac{d_p}{2}\right)^2 \dot{\gamma} V_p P + D_{Br} \text{ (Eq. 3)}$$

where $d$ corresponds to the cell diameter, $\phi_{RBC}$ is the RBC volume fraction, $V_p P$ is the platelet volume fraction, and $D_{Br}$ is platelet’s Brownian diffusion. From such models, it is important to note that the shear-induced platelet diffusion increases with increasing shear rate ($\dot{\gamma}$ in Eq. 3), which is highest near the vessel wall. Also, platelet’s own Brownian diffusion coefficient can be mathematically stated as:

$$D_{Br} = \frac{kT}{6\pi\mu r} \text{ (Eq. 4)}$$
where $k$ is the Boltzmann constant, $T$ is absolute temperature, $\mu$ is fluid dynamic viscosity, and $r$ is the platelet radius. It can be seen that since this diffusion is inversely proportional to particle radius (Eq. 4), platelets with small size have enhanced diffusivity compared to the larger RBCs. Therefore, in a laminar shear flow, by virtue of the combined effect of increasing shear rate and smaller size, the platelets undergo enhanced diffusive flux towards the wall.

Once marginated to the cell free plasma layer in close proximity to the vascular wall, further lateral drift of platelets towards the wall is due to rheological events which cannot be solely described by the traditional shear-enhanced diffusion coefficient [67], [68]. This drift is also significantly influenced by stiffer platelets’ collisions with flexible red blood cells (RBCs). As evident from previous equations, due to relatively larger size and reduced diffusivity, RBCs travel near the center of the parabolic blood flow profile leaving a platelet-enriched plasma layer closer to the vessel wall, a phenomenon referred to as the Fahraeus-Lindqvist effect [69]. Also, because RBCs are more deformable, collision of the stiffer platelets with RBCs acts in favor of expulsion of platelets away from the RBC volume in the center of the vessel [64], [70]. Additionally, RBCs that end up colliding with the vessel wall due to their normal diffusion (albeit lower than platelets) tend to ‘bounce’ back away from the wall as a result of their more flexible modulus, while stiffer platelets have less ability to do that. On top of this, platelets which do move away from the wall in the normal direction, end up colliding back with the RBC layer towards the center of the vessel and are deflected once more towards the wall. Therefore, platelets’ stiffer modulus in comparison to that of RBCs is a significant contributor to their lateral drift. Lastly, the biconvex discoid shape of platelets also influences their
Discoidal platelets have a greater propensity to marginate because as the aspect ratio increases, the rotational inertia of the cell increases. This inertia brakes up the symmetry of the longitudinal particle motion and causes transverse lateral drift. With this rationale, the net result of the combination of size, shape and modulus parameters on convection-diffusion processes in shear flow and collision-enhanced lateral drift is an enrichment of platelets in the close proximity of the vessel wall. Since platelet size, shape, and modulus play important roles in their hemodynamic margination properties [58], [62], [63], design of a vascular nanomedicine platform should incorporate these natural physical and mechanical cues for appropriate fluid dynamic interactions. Charoenphol et al. [71]
have demonstrated that spheroidal particles of platelet-mimetic size of 2-5μm diameter have a higher propensity to marginate to the wall under flow, compared to smaller (e.g. nanoscale) particles. Fujita et al. have studied the motion of rGPIbα-coated albumin spheres of various sizes in a model arteriole [72]. In these studies, large spheres (1900 ± 400 nm in diameter) resulted in a significantly higher localization of spheres near the vessel wall under flow shear rate of 1500 s⁻¹ compared to small spheres (240 ± 50 nm in diameter). They also observed back and forth motion of the larger particles in the lateral direction near the wall, which is advantageous towards enhancing binding interactions of particles. Gentile et al. have modeled various shaped silica particles and their behaviors under laminar fluid flow, showing that the largest number of marginated particles have a discoidal shape compared to quasi-hemispherical and spheroidal shapes (Figure 2.3A) [73]. Toy et al. have recently demonstrated similar phenomenon in vitro using a PDMS micro-channel device, showing that most favorable margination behavior occurs for oblate-shaped particles [74]. Several other theoretical and experimental studies [75], [76] have also suggested that anisotropic shapes (e.g. spheroids, ellipsoids, rods, etc) with microscale dimensions may have higher capabilities to migrate towards vessel walls and undergo enhanced adhesion in a physiologically relevant flow environment (Figure 2.3B-C). Such dimensional cues can become an important design parameter in engineering of platelet-inspired nanomedicine systems.

*Stable target-site anchorage under hemodynamic flow*

Anchorage of platelets to a specific site in the vasculature is facilitated by multiple, different but simultaneous ligand-receptor interactions and these ligand-receptor systems vary based on the physiological or pathological condition. These
interactions have been shown to increase the strength and stability of platelet binding under hemodynamic flow. In addition, the physical properties (e.g. size and shape) of platelets contribute to their stable target-site anchorage. Lastly, the mechanical properties of platelets, specifically deformability, lend to their remarkable ability to roll and bind to the vessel wall under high shear.

In vascular pathologies, the most prevalent pathological events are dysregulated hemostasis (leading to bleeding complications) or thrombosis/coagulation (leading to vascular occlusion), which involve a plethora of cellular (e.g. diseased endothelial cells (ECs), activated platelets, inflammatory leukocytes) and molecular (e.g. von Willebrand Factor or VWF, sub-endothelial matrix proteins like collagen, and various coagulation factors) components. The interactions between these components often occur concurrently, mediated by various receptors and ligands. For example, interactions between active platelets, diseased ECs, and inflammatory leukocytes can be mediated by simultaneous binding of integrins (e.g. GPIIb-IIIa on platelets and \( \alpha_v\beta_3 \) on ECs) to fibrinogen and VWF [46], [77–82], and binding of selectins to corresponding glycoprotein ligands and sulphatides [83–87]. In hemostasis, the adhesion of platelets to the injury site is mediated by simultaneous binding to VWF (by platelet surface glycoprotein receptor GPIb\( \alpha \) component) and sub-endothelial collagen (by platelet surface glycoprotein receptors GPIa-IIa and GPVI), while the aggregation of active platelets at the site is mediated by platelet GPIIb-IIIa binding to fibrinogen and P-selectin binding to P-selectin Glycoprotein Ligand-1 (PSGL-1) [88–94]. In these various disease pathologies, the potential target receptors or molecular entities that are spatio-temporally upregulated or uniquely expressed are usually multiple and quite heterogenous in
distribution. Hence, it can be rationalized that simultaneously targeting multiple types of receptors and proteins by utilizing multiple types of ligands may enhance the disease-specific binding affinity and selectivity of a drug delivery vehicle. Furthermore, such multi-targeting may also enhance the strength and retention stability of ‘bound’ vehicles at the disease site, especially in a dynamic blood flow environment. This multi-targeting strategy can be described as heteromultivalent, where ‘hetero’ refers to utilizing different types of ligand-receptor interactions, and ‘multivalent’ refers to the ability to undergo multiple binding interactions simultaneously by virtue of the surface modification of the nanoparticle drug delivery vehicles with multiple copies of the various targeting ligands.

In fact, the earliest reports regarding heteromultivalent targeting/binding of multiple receptor types were in the area of vascular targeting. To this end, Trubetskoy et al were the earliest group of researchers that demonstrated that when a mixture of avidin-modified antibodies were directed towards surfaces coated with a mixture of

![Figure 2.4](image.png)

**Figure 2.4:** Schematic of experimental design and representative results from the studies carried out by Trubetskoy et al. where plastic surfaces were coated with various antigens (e.g. fibronectin, fibrinogen, LDL etc.), avidin-modified specific antibodies were directed towards these antigens, and subsequently biotinylated liposomes bearing 14C label were directed to bind these antibodies. The resultant binding was measured by radioactivity units of disintegrations per minute (DPM) and as shown in adjoining graph, biotinylated liposomes directed simultaneously to multiple antibodies resulted in significantly higher binding activity compared to liposomes directed to any one type of antibody. Adapted and modified with permission from Ref. [95].
corresponding vascularity-relevant antigens (e.g. fibronectin, fibrinogen, low-density-lipoprotein etc.), followed by incubation with biotin-decorated liposomes, the assay resulted in an overall enhancement of liposome accumulation on the surfaces compared to antibody-plus-liposome strategies directed to a single type of antigen (Figure 2.4) [95]. Eniola (currently Eniola-Adefeso) et al. have reported on developing polymeric nanoparticles that were surface co-decorated with sialyl Lewis X (sLeX) moieties that can bind P-selectins and anti-ICAM-1 antibodies that can bind intercellular adhesion molecule-1 (ICAM-1) [96], [97]. This heteromultivalent design was inspired by the mimicry of leukocyte interactions with active platelets and diseased endothelium via simultaneous binding to selectins (e.g. via P-selectin glycoprotein ligand-1, PSGL-1) and ICAMs (e.g. via β2 integrins), in vascular pathologies (e.g. leukocyte-endothelium interactions shown in Figure 2.5A). The resultant particles were perfused over surfaces coated with 50:50 P-selectin and ICAM-1 (Figure 2.5B) in parallel plate flow chambers.

Figure 2.5: Schematic of biological phenomena of leukocyteendothelial interactions, experimental design of mimicking these interactions on a synthetic particle system and representative results from the studies carried out by Eniola et al. where polymeric particles heteromultivalently surface-decorated with anti-ICAM-1 antibodies and sLeX motifs were exposed to surfaces coated with 50:50 ICAM-1: P-selectin or only P-selectin, under flow. As evident from the adjoining graph, the heteromultivalently decorated particles showed significantly high binding to the mixed coated surface compared to only sLeX-decorated particles or only P-selectin-coated surface. Adapted and modified with permission from Ref. [97].
at controlled flow rates (wall shear), and the extent of particle binding was monitored. The results indicated that the selectin-binding mediated rolling adhesion of the particles aided in slowing down the particles for subsequent firm adhesion via ICAM-1-binding, altogether leading to a cumulative enhancement of number of bound particles (Figure 2.5C). The results also demonstrated that without such co-operative binding interactions, for example with only ICAM-1-targeted particles, the extent of bound particles was significantly reduced. Following similar rationale, Ferrante et al. have reported on the development of microbubble-based ultrasound contrast agents, heteromultivalently decorated with sLeX (for P-selectin targeting) and an antibody directed against Vascular Cell Adhesion Molecule-1 (VCAM-1) [98]. The ligand-decorated microbubbles were allowed to flow over P-selectin- and/or VCAM-1-coated surfaces at 1.5-6 dyn/cm² shear stress values. These studies demonstrated that the dual-targeted microbubbles were capable of binding to the target surface at levels twice that of singly-targeted microbubbles (Figure 2.6A), thereby emphasizing the benefit of co-operative binding mechanisms. Gunawan et al. have reported on similar studies but on binding to activated endothelial and smooth muscle cells (instead of receptor-coated surfaces) using liposomes heteromultivalently decorated with antibodies directed to ICAM-1, VCAM-1 and Endothelial-Leukocyte Adhesion Molecule (ELAM) [99–101]. In these studies, the lipid components of the liposomes were varied to influence membrane rigidity/fluidity and thereby influence the presentation and flexibility of the decorated antibodies towards their target receptors. The results demonstrated that the nanoparticle (in this case liposome) flexibility and synergistic mechanisms of dual receptor targeting enhance targeting efficacy (Figure 2.6B). In a recent interesting study by Kibria et al, it has been
Figure 2.6: (A) Representative results from studies carried out by Ferrante et al. where microbubbles heteromultivalently decorated with an anti-VCAM-1 monoclonal antibody (MVCAM) and a sialyl Lewis X polymer (PAA-sLeX) showed double the extent of adhesion on VCAM-1 and P-selectin coated surface under flow, compared to microbubbles decorated with MVCAM only or PAA-sLeX only (adapted with permission from Ref. [98]); (B) Representative results from studies reported by Gunawan et al. where immunoliposomes heteromultivalently decorated with an anti-ICAM-1 antibody and an anti-ELAM-1 antibody showed enhanced binding on activated endothelial cells (EC) and this enhancement was more prominent on liposomes made with the more flexible DOPC lipid than that with the rigid DPPC lipid (adapted with permission from Ref. [100]); (C) Representative quantitative results along with fluorescent images from studies reported by Kibria et al. where Rhodamine-labeled (red fluorescence) liposomes heteromultivalently surface-decorated with an αVβ3-binding RGD peptide and a cell-penetrating R8 peptide showed enhanced targeting and internalization in activated endothelial cells, compared to liposomes bearing RGD peptide only or R8 peptide only (adapted with permission from Ref. [102]); (D) Representative quantitative results along with fluorescent images from studies reported by Singh et al. where PLGA nanoparticles heteromultivalently surface-decorated with Transferrin (Tf) and RGD peptides resulted in high delivery of an anti-VEGF intraceptor plasmid Flt23K in choroidal neovascular cells, red (nanoparticles containing Nile red as a tracking dye), green (green fluorescent protein expression on transfection of retinal cells) and blue (cell nuclei stained with DAPI) with arrowheads showing particle and transfected cell overlap (adapted with permission from Ref. [103]).

demonstrated that a liposome surface can be co-decorated with a cell-targeting Arginine-
Glycine-Aspartic Acid (RGD) peptide (in this case targeting of integrin αvβ3 on endothelial cells) and a cell-penetrating octaarginine (R8) peptide, to enable enhanced targeting, internalization and transfection of cells (Figure 2.6C)[102]. Singh et al. have reported on poly-lactide-co-glycolide (PLGA) nanoparticles that are heteromultivalently decorated with transferrin and an RGD peptide and loaded with anti-vascular endothelial growth factor (anti-VEGF) intraceptor for targeted gene therapy of choroidal neovascularization (CNV) lesions in age-related macular degeneration [103]. Targeting of the particles was studied in a rat model of laser-based CNV induction, and the studies showed that the RGD-decorated particles resulted in higher localization in the lesions compared to transferrin-decorated particles or dual-targeted particles (which were statistically equivalent in localization) (Figure 2.6D). This may not necessarily negate the advantages of dual-targeting, but may be a reflection of the difference in expression levels of RGD-binding versus transferring-binding receptors in the in vivo lesions, since the corresponding expression levels were not correlatively quantified in these studies.

In addition to influencing fluid dynamic behavior and margination, shape and size properties can also influence the binding interactions of synthetic constructs. Pillai et al. have reported on shape and resultant adhesive properties of particles intended for adhesion in the vasculature [104]. Hydrodynamic shear stresses at the vascular walls act to dislodge adherent particles, and this can be modulated by altering particle orientation (aspect ratio of length to width), the overall volume exposed to the hydrodynamic forces, and the surface area available for adhesive ligands to interact with desired target receptors. High aspect ratio particles have a higher probability to adhere firmly to the vascular wall when compared to volume-matched spherical particles. Doshi et al. have
observed this phenomenon in vitro in synthetic microvascular networks, showing enhanced adhesion of elongated and flattened particles compared to spheres with the highest aspect ratio rods showing the greatest adhesion (Figure 2.7A) [105]. Not only does a higher aspect ratio enhance the degree of particle adhesion, but it can also enhance the rate at which the particles adhere under shear as demonstrated by Okamura et al (79).

The group has fabricated 3µm PLGA disk-shaped nanosheets decorated with an H12, fibrinogen γ-chain dodecapeptide. While keeping the surface area and H12 conjugation number consistent, they compared binding of the disk-shaped particles to spheres to collagen-adhered activated platelets under shear stress using a flow chamber. Disk-shaped particles specifically interacted with the activated platelets at twice the rate as the spheres. The mechanism behind the enhanced rate and degree of adhesion of high aspect ratio particles under shear could be due to the increased area in contact when the particle is on its longitudinal axis.

Furthermore, platelet deformability significantly affects the rolling and tethering of the platelets on the vascular surface by facilitating the ease with which multiple points

![Figure 2.7: (A) In a flow chamber model simulating microcirculatory pattern, microscale ellipsoid disks [•] showed higher localization at the wall compared to spherical [o] microparticles under shear flow (reproduced with permission from [105]); (B) By modulating rigidity of the liposome membrane, it was found that ‘softer’ liposomes surface-decorated with vWF-binding rGPIba motifs had a longer duration of adhesion to vWF surfaces under flow as a result of enhanced deformability and contact area of interaction, compared to rigid liposomes (reproduced with permission from [107]).](image-url)
of contact are formed for firm attachment [106]. To investigate the effect of mechanical modulus/deformability on binding properties under flow, Takeoka et al. have studied rGPlbα-decorated liposomes having various lipid membrane fluidities [107]. The degree of membrane fluidity influences the membrane flexibility, and therefore the deformability of the particles. Adhesion of these particles to vWF was tested under flow using a circulating chamber. The results showed that the rolling speed of the particles was directly correlated to the membrane fluidity, whereby “soft” liposomes rolled slowly, suggesting better adhesion, while “hard” liposomes rolled faster (Figure 2.7B). This effect was attributed to possibility that more deformable particles could be flattened to increase the area of contact (and hence adhesion), similar to the phenomenon seen with natural platelets.

**Triggered bioactive molecule delivery**

As mentioned previously, platelets undergo an activation process in response to specific physiological and pathological stimuli, which leads to the release of proteins and other small biomolecules contained in intracellular granules. For example, P-selectin becomes expressed for interaction with leukocytes during inflammation, ADP is released for activation of neighboring platelets, several cytokines are released for pro-angiogenic activity, and several enzymes are released to influence matrix remodeling. The contents of platelets’ granules are not released unless they become activated; therefore, while circulating in the vasculature, platelets act as ‘carrier particles’ for these bioactive molecules. Upon site-specific stimuli, platelets ‘deliver’ specific granule content based on the physiological or pathological need. Therefore, platelets have the innate ability to act as on-demand drug delivery systems.
Although nanoparticle systems have yet to be engineered in a manner to mimic platelet’s biomolecule release via granule secretion, several mechanisms of site-specific, triggered drug release have been investigated. The concept of stimuli-responsive drug delivery was first evaluated in the late 1970’s with temperature or thermo-responsive liposomes [108]. In the past several decades, nanoparticles have been engineered to change their properties and release drug in response to endogenous stimuli (e.g. pH changes, enzymes, shear stress) or exogenous stimuli (e.g. heat, light, ultrasound, electric/magnetic fields) [109]. For example, ultrasound-responsive, poly(vinyl alcohol) (PVA)-based bubble structures were reported that can be loaded with the vasodilatory and antithrombotic bioactive gas nitric oxide (NO), for simultaneous imaging and NO delivery to vascular disease tissues (Figure 2.8A) [110]. Ultrasound-sensitive bubbles that allow cavitation-induced payload release have also been reported for delivery of DNA, double stranded RNA and oligonucleotides, recombinant proteins, growth factors and thrombolytic agents (e.g., Streptokinase, tPA, etc.) (Figure 2.8B-C) [111–115]. Terminologies like ‘sonothrombolysis’ has been coined to emphasize the combined effect of ultrasound-induced mechanical cavitation and site-specific thrombolytic drug release to enhance clot dissolution properties. One interesting approach in utilizing platelet-mimicry in drug delivery is demonstrated in the recent report from Korin et al., who fabricated microscale aggregates of polymeric nanoparticles that disintegrated to their nanoscale components under high shear to allow shear-induced drug delivery (Figure 2.8D) [116]. This design is inspired by the fact that natural platelets are known to get activated at high shear and release granule contents.
Altogether, using platelets as a unique paradigm and rationalizing from the above-described functional physico-mechanical and biointeractive parameters of platelet action, the design of a vascular nanomedicine platform can be envisioned that incorporates surface-mediated biological interactions, margination influencing particle shape/size/mechanical properties, and site-specific triggered drug release mechanisms to ensure maximum performance efficacy. Of these three design attributes, the current dissertation will focus principally on platelet-inspired heteromultivalent biointeractions, while providing partial demonstrations of utilizing stimuli-responsive drug delivery approaches in refining vascular delivery platform. To this end, the central hypothesis of this dissertation is that utilizing platelet-inspired heteromultivalent ligand interactions on
a synthetic drug delivery vehicle will result in enhanced specific binding and retention of the vehicles at a vascular target site under hemodynamic flow conditions. This hypothesis will be tested in two specific vascular nanomedicine applications: (i) the design of platelet-inspired synthetic hemostats for management of bleeding complications and (ii) the design of platelet-inspired vehicles for targeted delivery of thrombolytic agents at a clot site.

4. References


Chapter 3: Platelet-inspired Hemostatic Nanomedicine

Based on:

1. Introduction

Transfusion of natural platelets is used in the clinic for emergency or prophylactic hemostasis in patients suffering from hematological complications arising from malignancies, including leukemia and myeloma, chemo/radiotherapy-induced bone marrow suppression, trauma, and surgery, among others. The gamut of treatments that make use of platelet transfusion ranges from staunching trauma or surgery-associated heavy bleeding [1], [2] to prevention of malignancy or myelosuppression-associated internal bleeding and thrombocytopenic complications [3]. According to the American Red Cross, a total of 30 million blood components are transfused every year in the U.S. All of these transfusions are currently carried out using natural blood-based products [4]. However, less than 38% of the U.S. population is eligible to donate blood components, and only 85.2 units were donated per thousand individuals in the donor age between 18 and 64 in 2008 [5]. It is estimated that every 2 seconds, someone in the U.S. needs blood, and therefore the current level of supply is not enough to keep up with the high demand. In addition to short supply, products based on natural blood suffer from limited storage life. For example, platelet suspensions can only be stored for about 5 days [2], [4]. These short storage periods arise due to risks of biological/pathological contamination. In recent years, certain photochemical pathogen reduction technologies (e.g. psoralen-based and riboflavin-based UV treatment) have been clinically established to reduce contamination
risks [6–8], but these have succeeded in increasing the shelf-life only up to ~ 7 days [9], [10]. In vivo, natural platelet-based transfusion products present risks of several negative side effects, like febrile non-hemolytic reactions, alloimmunization-induced refractoriness, graft-versus-host disease, immunosuppression, and acute lung inflammation/injury [11–14]. These negative side effects can be only partially reduced by extensive screening and serological testing of donor blood and leukoreduction processes, and the associated procedures considerably increase the cost of the transfusion products. These issues limit the therapeutic utility of not only the clinically used allogeneic natural platelet transfusions, but also of other platelet-derived investigational hemostatic products like frozen (-80°C) platelets, cold-stored (4°C) platelets, lyophilized platelets, platelet-derived microparticles and infusible platelet membranes (IPM).

The aforementioned issues with products based on natural platelets have led to a significant clinical interest in synthetic platelet analogues that can mimic and amplify the biological hemostatic functions of platelets, while simultaneously providing advantages of easy manufacturing, high reproducibility, ability to scale-up, potential for sterilization, and long storage life (Table 3.1). Also, such a synthetic substitute reduces the need for

<table>
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<tr>
<th>Disadvantages of Natural Platelet Products</th>
<th>Advantages of Synthetic Platelet Products</th>
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<tbody>
<tr>
<td>Shortage in supply (donor scarcity)</td>
<td>Long shelf-life</td>
</tr>
<tr>
<td>Limited shelf-life (3-7 days)</td>
<td>Portable</td>
</tr>
<tr>
<td>Inconvenient storage conditions</td>
<td>Reduced risk of biological contamination</td>
</tr>
<tr>
<td>Biological/pathological contamination</td>
<td>Ability to sterilize</td>
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<td>Negative side effects from allogenic</td>
<td>Reduced need for blood typing</td>
</tr>
<tr>
<td>transfusion</td>
<td>Large-scale production</td>
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<tr>
<td>Need for blood typing/matching</td>
<td>Reproducible quality</td>
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<td>Expensive</td>
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Table 3.1: Limitations of natural platelet-based products and potential advantages of synthetic platelet analogs.
blood typing, making all products available to any patient in need. A synthetic platelet analogue should be able to mimic the hemostatic properties of natural platelets to allow site-selective clotting, staunching of bleeding, and healing over time. To this end, various designs of synthetic platelet substitutes have been proposed over the past 20 years (Table 3.2), and a few reviews have been published previously on this topic [2], [15–18]. This chapter will address the past and current state-of-the-art approaches in synthetic platelets in terms of design components of biological properties. In addition, I will provide insight and opinion from my own research towards optimizing the design of synthetic platelets, to help refine future research approaches.

<table>
<thead>
<tr>
<th>Platelet-mimetic Synthetic Hemostat Designs</th>
<th>Design Components</th>
<th>References</th>
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<tbody>
<tr>
<td><img src="image" alt="Platelet surface glycoproteins isolated and reconstituted within liposomal membranes (plateletosomes)" /></td>
<td>Platelet surface glycoproteins isolated and reconstituted within liposomal membranes (plateletosomes)</td>
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<td><img src="image" alt="RBCs surface-decorated with fibrinogen-derived RGD peptides (Thromboerythrocytes)" /></td>
<td>RBCs surface-decorated with fibrinogen-derived RGD peptides (Thromboerythrocytes)</td>
<td>Collier et al (48)</td>
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<td>Heat-stabilized albumin microparticles surface-coated with fibrinogen (Synthocytes)</td>
<td>Levi et al (49)</td>
</tr>
<tr>
<td><img src="image" alt="Liposomes, albumin particles or latex particles surface-decorated with GPIba, GPIa/IIa, or fibrinogen-derived RGD or H12 peptides" /></td>
<td>Liposomes, albumin particles or latex particles surface-decorated with GPIba, GPIa/IIa, or fibrinogen-derived RGD or H12 peptides</td>
<td>Takeoka et al (34,35), Nishiyama et al (37, 39), Okamura et al (36,63-67)</td>
</tr>
<tr>
<td><img src="image" alt="PLL-PLGA nanoparticles surface-decorated with fibrinogen-derived RGD peptides" /></td>
<td>PLL-PLGA nanoparticles surface-decorated with fibrinogen-derived RGD peptides</td>
<td>Bertram et al (52)</td>
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<td><img src="image" alt="Liposomes heteromultivalently surface-decorated with VBP, CBP and cRGD peptides" /></td>
<td>Liposomes heteromultivalently surface-decorated with VBP, CBP and cRGD peptides</td>
<td>Ravikumar et al (60)</td>
</tr>
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</table>

Table 3.2: Past and current state-of-the-art design approaches for platelet-mimetic synthetic constructs.
2. Mechanisms for Hemostatic Action of Platelets

In the body, platelets are primarily responsible for hemostasis at sites of vascular injury. The hemostatic function of platelets involves a series of coordinated steps including: (1) margination to the vascular wall from bulk blood flow, (2) stable adhesion to the injury site at the vascular wall in spite of hemodynamic flow via specific interactions with vascular matrix proteins such as von Willebrand Factor (vWF) and collagen, (3) concomitant activation through action of agonists such as Adenosine Diphosphate (ADP) and thrombin, (4) subsequent aggregation in the active state at the adhesion site via specific ligand-receptor activity involving fibrinogen, and (5) co-localization of coagulation factors on the active platelet membrane, triggering the coagulation cascade (Figure 3.1) [19].

Figure 3.1: Events in the hemostatic action of platelets.

Properties of Platelets Influencing Hemostasis

Once marginated to the wall, the adhesion of platelets at the vascular injury site primarily occurs by shear-dependent interactions of the GPIbα extracellular domain of the platelet surface glycoprotein GPIb-IX-V complex with vWF secreted and deposited locally from the injured endothelium [20], [21]. This adhesion mechanism is further supplemented by shear-independent binding of platelet surface glycoproteins GPIa-IIa and GPVI with sub-
endothelial collagen [22], [23]. The vWF-binding helps in arrest and rolling of platelets at the injury site, while collagen-binding stabilizes the adhered platelets under flow. Therefore, mimicking the property of adhesion to vWF and collagen under flow conditions is an important design criterion for a synthetic platelet substitute. Following adhesion, platelets undergo activation by action of agonists like ADP and thrombin which results in inside-out signaling mechanisms that cause platelet morphology to change from its ‘quiescent’ discoid form to a ‘spread’ form with a spheroidal center and pseudopodal membrane protrusions. The activation and signaling also results in conformational changes of the platelet surface integrin GPIIb-IIIa into a ligand-binding form that can bind the native blood protein fibrinogen (Fg). Fg has multiple GPIIb-IIIa-binding peptide domains, namely RGD and HHLGGAKQAGDV (also known as H12) in its α and γ chains, respectively, on both termini, and hence it can bridge multiple active platelets [24]. The active platelets also secrete more ADP to further activate more platelets locally, and the Fg-mediated inter-platelet bridging leads to accelerated platelet aggregation to form the primary hemostatic plug. Subsequently, coagulation factors localize on the membranes of these activated and aggregated platelets, stimulate the coagulation cascade, and ultimately lead to the formation of cross-linked fibrin (secondary hemostasis) that arrests the primary platelet plug and other blood components at the injury site to result in the final clot. Over time as the injury heals, the clot is digested through regulated enzyme action. Therefore, mimicking or amplifying the Fg-GPIIb-IIIa interaction-mediated aggregation mechanism of active platelets is another critical design component for a synthetic platelet substitute.
With the aforementioned rationale, efficient mimicry of platelet's biological properties are required to design an ideal platelet-mimetic synthetic analogue. The following sections will provide a comprehensive review of the various research approaches towards imparting platelet-mimetic properties on various synthetic platforms.

3. Mimicking the Properties of Platelets with Synthetic Particles

Mimicking Platelet Adhesion

To mimic mechanisms of platelet adhesion, researchers have created synthetic particles that can adhere to vWF or collagen under flow. For example, Takeoka et al. have fabricated liposomes, latex beads and albumin particles surface-decorated with recombinant GPIbα (rGPIbα) fragments [25–27]. The rGPIbα-modified particle constructs were found to adhere to vWF-coated surfaces under flow in vitro, and the number of adhered constructs increased with shear rate, mimicking the shear-dependent platelet adhesion to vWF (Figure 3.2 A). However, since the vWF-binding is strengthened at higher shear, utilizing only this mechanism on synthetic platelet constructs may not allow effective performance in low shear conditions.

![Figure 3.2](image.png)
Therefore, an additional mechanism of platelet-mimetic adhesion has been investigated for synthetic platelet design, i.e., mimicking the strong, irreversible adhesion mechanism of platelet GPIa-IIa and GPVI surface proteins to collagen. To this end, Nishiya et al. have investigated decorating liposomes with recombinant GPIa-IIa (rGPIa-IIa) [28]. Their results showed strong binding of the rGPIb-IIa–liposomes to collagen-coated surfaces in the presence and absence of soluble vWF under low shear. However, the liposomes adhered more effectively at low shear rates because of a slow rate of bond formation with immobilized collagen and a low resistance of the bond to tensile strength (Figure 3.2 B). Wada et al. have researched the adhesive properties of polymerized albumin particles conjugated with rGPIa-IIa using atomic force microscopy, showing that the adhesive force between the particles and collagen was 52% that of natural platelets [29]. These results suggest that mimicking adhesion to only vWF or only collagen may not be adequate to efficiently mimic platelet adhesion under physiologically relevant flow conditions, and a synergistic combination of these adhesions may perform better.

Based on this rationale, Nishiya et al. have investigated modifying liposomes with both collagen and vWF binding motifs to achieve stable adhesion especially at low-to-high shear rates. These liposomes were surface-decorated with both rGPIbα and rGPIa-IIa, and interaction of these constructs to a collagen surface in the presence of soluble vWF under flow was studied. At low shear values, the interaction of the liposomes with the surface was mediated by GPIa-IIa binding to collagen. At high shear, interaction between GPIbα and vWF reduced the velocity of the liposomes, promoting adhesion and arrest such that supplemental irreversible binding of GPIa-IIa to collagen could be achieved (Figure 3.2 C). Comparing adhesion of these particles to natural platelets,
liposomes covered 5.41 ± 0.45% of the protein surface and platelets covered 7.01 ± 1.51% at a low shear of 600 s\(^{-1}\), while liposomes covered 28.57 ± 2.13% and platelets covered 22.85 ± 2.06% at a high shear rate of 2400 s\(^{-1}\). Thus, at high shear, the surface-engineered liposomes actually demonstrated a higher surface coverage than natural platelets, establishing efficient mimicry of platelet adhesion [30].

It is to be noted that the use of recombinant high molecular weight protein fragments like rGPIbα and rGPIa-IIa may raise issues of immunogenicity and may also result in high cost regarding scale-up towards translation. Moreover, there may be steric limitations regarding decoration of multiple copies of such big protein fragments on micro- or nanoscale particle surfaces.

*Mimicking Platelet Aggregation*

Based upon the previously described mechanism of Fg-mediated active platelet aggregation, mimicry of this mechanism toward a synthetic platelet analogue has mainly included surface-decoration of particle platforms with Fg or Fg-mimetic peptides, such that each particle can cluster multiple active platelets. One of the earliest designs in this approach involved surface-decoration of RBCs with Fg [31] or Fg-derived peptides (e.g. RGD) [32]. In the work reported by Agam and Levine, constructs were created with an Fg surface density of 58-1400 molecules per RBC by crosslinking the Fg on RBCs using formaldehyde or transglutaminase. These cells increased platelet aggregation in a manner dependent on the Fg density. Tail bleeding times of thrombocytopenic rats injected with 1 x 10^9 of these cells had bleeding times reduced from 18 min to ~5 min. It is interesting to note that a decreased bleeding time was observed in RBCs conjugated with as little as 58
Fg molecules per cell. However, a limitation of this design is the biocompatibility and immunogenicity issues associated with the source of Fg (e.g. bovine origin). An additional issue may be the limited storage stability of Fg, that may give rise to reduced shelf-life of such Fg-coated products. In the work reported by Coller et al. [32], constructs termed “thromboerythrocytes” were fabricated by decorating RBC surface with an Fg-mimetic synthetic peptide CGCRGDF that can bind to platelet GPIIb-IIIa. These thromboerythrocytes interacted specifically with activated platelets and demonstrated minimal binding with resting platelets. Studies with monoclonal antibodies to GPIIb-IIIa and soluble RGD peptides confirmed that the RGD sequence on the thromboerythrocytes interacted with the ligand-binding conformation of GPIIb-IIIa receptors on activated platelets. This interaction was found to occur even in the presence of natural levels of plasma fibrinogen. To better simulate an in vivo environment, it was shown that the thromboerythrocytes could also bind to activated platelets that are adherent to collagen and promoted formation of large platelet aggregates. It is to be noted that, since thromboerythrocytes could only aggregate preexisting activated platelets, the number of available active platelets needed by a patient to observe a positive therapeutic effect as a result of thromboerythrocyte administration was difficult to estimate. Also, RBC-based semi-synthetic platelet designs are not feasible in patients where sufficient autologous RBC populations are not available. Although the use of RBCs from universal donors may render higher availability, the general shortage of blood donors all across the ABO groups still remains a significant limitation regarding availability of RBCs that can be further engineered into platelet-mimicking constructs. To address such limitations, some research efforts are being directed towards creating ‘universal donor-like RBCs’ by
masking RBC surface antigens with synthetic polymer coatings, or enzymatically converting the A and B antigens into O type, or stimulating stem cells into becoming universal donor type RBCs [33]. These research efforts are currently ongoing, and there are no clear conclusions regarding scale up, efficacy and safety of these. Hence, alternative designs to mimic platelet aggregation have investigated several synthetic, non-biological particle platforms instead of RBCs.

Another fibrinogen-based approach that has undergone pre-clinical research and has progressed into clinical trials in the US and Europe is Fibrocaps™ (ProFibrix, The Netherlands). Fibrocaps™ are a solution of fibrinogen and thrombin that are separately spray-dried and combined to produce a mixture of soluble free-flowing microparticles. In clinical trials for treatment of bleeding during surgery and trauma-related injury, time-to-hemostasis was measured after direct administration of Fibrocaps™ to the surgical wound (in a Gelatin Sponge) to be 1.9 ± 1.3 minutes compared to 4.8 ± 3.1 minutes for a Gelatin Sponge alone. ProFibrix is currently recruiting for a Phase III clinical trial [34]. One limitation of this product is that it is directly applied to the wound (sprayed or applied in a sponge) and has not been investigated for I.V. administration, which severely limits the range of clinical applications of the product.
Levi et al. have reported on a synthetic platelet substitute termed Synthocytes™

**Figure 3.3:** Representative data from various design approaches mimicking the aggregation functionality of natural platelets: (A) Fg-coated albumin microcapsules (Synthocytes), when administered in thrombocytopenic rabbits, resulted in significant reduction [Δ and ○] of surgical abdominal incision wound bleeding, compared to administration of uncoated microcapsules [□] or just saline [✓] [reproduced with permission from (51)]; (B) Injection of polymeric nanoparticles surface-decorated with various RGD peptides linked via 4600 MW PEG spacers significantly reduced bleeding time in a rat femoral artery injury model, compared to injection of saline or coagulation factor FVII [reproduced with permission from (55)]; (C) Liposomal vesicles decorated with H-12 peptide showed enhancement of aggregation [○] of active platelets on collagen from thrombocytopenic blood, compared to vesicles without peptide modification [●] or just saline [▲] [reproduced with permission from (61)]; (D) Further refinement of the H-12-decorated liposome design by encapsulating platelet-agonist ADP in the liposomes enabled enhancement of platelet aggregation [○] compared to ADP-loaded liposomes with no H-12 decoration [□] [reproduced with permission from (58)]; (E) Injection of albumin microparticles decorated with Fg-mimetic H-12 peptide showed reduction [○] of tail vein bleeding time in rats compared to injection of saline [●] [reproduced with permission from (59)].
(ProFibrix, The Netherlands), created by coating human albumin microcapsules with Fg [35]. The albumin spheres had a median diameter of 3.5-4.5 μm, and Fg was immobilized onto the surface by incubation for up to 4 hours at a controlled ionic strength and pH. The Synthocytes™ were tested in rabbits with antibody-induced thrombocytopenia, and they effectively reduced the bleeding time in an ear wound, as well as in a surgical abdominal incision wound, in a dose-dependent manner (Figure 3.3 A). In another test in rabbits with chemotherapy-induced thrombocytopenia a normal bleeding time of 18.6 ± 2.1 minutes was shortened to 5.3 ± 2.7 minutes after administration of specific doses of Synthocytes™ [35]. Similar in vivo results have also been reported by another variation of Fg-coated albumin microparticle design called Thrombospheres™ (Advanced Therapeutics & Co., Anaheim CA) [36]. Compared to Synthocytes™, the Thrombospheres™ demonstrated a longer duration of hemostatic effect, with decreased bleeding times observed up to 72 hours after injection. However, the mechanism for this prolonged duration is poorly understood, as the hemostatic effects persist even after the Thrombospheres™ are no longer detectable in blood [37]. Further evolution of this design has led to a product called Fibrinoplate™ (Advanced Therapeutics & Co., Anaheim CA), which is a polymerized human albumin microsphere coated with human Fg. This product has undergone Phase II and III clinical trials in patients with low platelet count (<30*10⁹ per liter). Results showed a decrease in bleeding time compared to albumin microparticles alone. Advanced Therapeutics plans to submit Fibrinoplate™ to the FDA for evaluation in the future [38].

In a similar approach, Nishiya et al. have studied the effects of liposomes coated with Fg on aggregation of platelets in vitro [30]. In a perfusion chamber, a mixture of Fg-
coated liposomes and platelets were allowed to flow over a collagen surface at various shear rates. Surface coverage of adhered platelets to the collagen surface increased from 8.61 ± 0.79% to 19.87 ± 1.76% with an increase in the shear from 600 to 2400 s⁻¹. Also, the % surface coverage of the Fg-liposomes increased with increasing platelet adhesion. Additionally, Fg-liposomes enhanced platelet aggregation in a dose-dependent manner as measured by aggregometry.

Recently, Bertram et al. have reported on polymeric nanospheres (~170 nm in diameter) surface-decorated with multiple copies of the GRGDS peptide sequence tethered via PEG spacers [39]. In these studies, various PEG spacer lengths were investigated and the nanospheres bearing peptides tethered via 4600 MW PEG spacers showed the greatest decrease in bleeding times (by almost 50% compared to controls), when administered at a dose of 20 mg/mL in a rat femoral artery acute injury model (Figure 3.3 B). This synthetic platelet design was also reported to have better performance than the clinically used pro-coagulant hemostatic agent NovoSeven® (recombinant coagulation factor VIIa).

It is important to note that the RGD-containing peptide sequences used by Coller et al. (CGCRGDF) and Bertram et al. (GRGDS) are not specific in their bioactivity to active platelet GPIIb-IIIa. In fact, these and similar sequences can bind different integrins on other types of cells (e.g. integrin αVβ3 on endothelial cells) and have been reported to even bind integrins on quiescent/resting cells to activate them [32], [39], [40]. These scenarios may result in systemic platelet activation or heterotypic cellular reactivity by such sequences. Therefore, in vivo, the therapeutic benefit and hemostatic efficacy of synthetic platforms decorated by such peptides may become substantially reduced.
Rationalizing from the design concept of utilizing Fg-mimetic peptides that are specific for active platelet GPIb-IIIa interaction, Okamura et al. have studied the hemostatic capabilities of liposomes and albumin microparticles modified with Fg γ-chain derived H12 peptide tethered directly or via PEG spacers [27], [41–44]. The H-12-decorated liposomal vesicles exhibited enhancement of platelet aggregation on collagen from thrombocytopenic blood (Figure 3.3 C) [44]. Further refinement in design of H12-decorated liposomes involved loading of platelet agonist ADP in them, which promoted significant enhancement in platelet aggregation due to ADP release (Figure 3.3 D) [41]. Analogous designs developed on a polymerized albumin particle platform with or without PEG-linker mediated decoration of H12 peptides, termed H12-PEG-polyAlb and H12-polyAlb, respectively, were administered to thrombocytopenic rats in order to measure the half-life of the particles in vivo and to assess their efficacy in reducing tail bleeding times. Through flow cytometry, it was shown that the particle bound specifically to activated platelets. Compared to H12-polyAlb constructs, the PEGylated constructs were more stable in vitro and had a much longer half-life in vivo. Also, the H12-PEG-polyAlb constructs showed significant reduction in tail vein bleeding time in thrombocytopenic rats compared to control saline injection when administered at 5, 60, 180 and 360 minutes before injury (Figure 3.3 E).

Integrating Platelet-mimetic Adhesion and Aggregation Functionalities on a Single Particle Platform

For natural platelets, the interplay between adhesion and aggregation functionalities is critical in promoting hemostasis [45–47]. Designing synthetic platelet constructs with
adhesion properties alone may enable them to attach to sites of vascular injury, but will not provide a mechanism for them to recruit and cluster sufficient number of natural platelets necessary to render hemostasis. On the other hand, designing synthetic platelet constructs that only mimic and amplify active platelet aggregation without any mechanism for vascular injury site-selective matrix adhesion, may cause free-floating platelet aggregates in circulation that can potentially increase the risk of thromboembolic complications. Therefore, several recent research approaches have investigated designing synthetic platelet constructs where both adhesion and aggregation capabilities are integrated on a single particle platform. To achieve such functional integration, the earliest research approaches involved using lyophilized platelets (reconstituted in saline), infusible platelet membrane (IPM), and liposomes bearing platelet membrane proteins incorporated within the liposome membrane (termed Plateletosomes).

The interest in lyophilized platelets dates back to the 1950s when pro-coagulant and hemostatic activities of freeze-dried platelet suspensions were tested in treating thrombocytopenia and hemophilia, but showed only limited therapeutic success [48–52]. The limited efficacy was traced back to the alteration and disintegration of the platelet membranes (hence of membrane-associated protein structures relevant to hemostasis) during the lyophilization processes. During the 1970s, it was demonstrated that the platelet membrane ultrastructures could be preserved by aldehyde-based crosslinking (i.e. fixing), and these fixed platelets could render hemostatic functionalities through surface interactions in spite of being metabolically inactive [53–56]. After membrane-crosslinked lyophilized platelets are reconstituted, they remain morphologically similar to fresh platelets, with only slight reductions in membrane protein reactivities [56–58]. These
platelet products were found adhere to vWF, but unlike actual platelets, did not aggregate in response to ADP or collagen. These findings have led to optimization of the crosslinking chemistry and testing the capability of rehydrated, lyophilized ‘fixed’ platelets in rendering bleeding time correction in thrombocytopenic rabbit and rat models and hemostasis in pre-surgical conditions using canine models [59–62]. For example, it was shown in one study that in thrombocytopenic rabbits, an infusion of reconstituted lyophilized platelets significantly reduced the ear bleeding time from >900 seconds to 234 seconds, although not as low as the 177 seconds when fresh platelets were administered. These pre-clinical studies have shown promising results [63], [64] and consequently studies in large animal models (dogs, baboons etc) have been intensified for translating this technology to veterinary, as well as, human clinical applications [65]. In this context, significant research efforts are being focused on refining the fixation and storage protocols for lyophilized platelets (e.g. Trehalose loading in the platelets prior to freeze-drying [66], [67], as well as, minimizing the biologic contamination and immunologic side-effect issues [68].

Infusible platelet membranes (IPM) are Cypress Bioscience’s (San Diego, USA) platelet microparticle product, prepared from outdated human platelet concentrates. These platelet microparticles are formed spontaneously during the storage of platelets [69]. IPMs have been shown in vivo to shorten ear bleeding time of thrombocytopenic rabbits, even 6 hours after injection. Because these products retain partial functionality of the GPIb-IX-V receptor, they retain binding capacity to collagen and vWF [69], [70]. Galan et al. have also shown that IPMs improve fibrin deposition and platelet adhesion and aggregation to a damaged vessel at thrombocytopenic platelet counts under a flow
environment \textit{in vitro} [71]. Phase II clinical trials of IPMs in thrombocytopenic patients have indicated improvement or cessation of bleeding in some cases [72]. Plateletsomes are another semi-synthetic hemostat design that involves reconstitution of 15 platelet membrane glycoproteins, including GPIb, GPIIb-IIIa, and GPIV, into the membrane of lipid vesicles (liposomes) [73]. A 42\% reduction in tail bleeding times of thrombocytopenic rats was observed after injection with Plateletsomes [74]. Although these products have shown favorable hemostatic properties, their primary limitation is the use of natural platelet-derived components in making the product. These natural sources pose the same issues of insufficient availability and biological contamination risks, as the natural allogeneic platelet suspensions currently used in the clinic.

In combining platelet’s biological properties on a single synthetic platform, Okamura et al. have reported the development of latex beads surface-decorated with either the Fg \(\gamma\)-chain H12 peptide (aggregation functionality only) or the rGPlb\(\alpha\) fragment (adhesion functionality only) or both (integrating adhesion and aggregation) [75]. Bead adhesion and platelet aggregation was observed in an \textit{in vitro} flow environment for these three samples. The H12 bead average surface coverage decreased with increasing shear (5.1\% at 150 s\(^{-1}\), 1.2\% at 800 s\(^{-1}\) and 1.0\% at 1600 s\(^{-1}\)), while the rGPlb\(\alpha\)-latex beads alone showed increased surface coverage with increasing shear (2.8\% at 150 s\(^{-1}\), 4.3\% at 800 s\(^{-1}\), and 5.0\% at 1600 s\(^{-1}\)). An even mixture of H12/rGPlb\(\alpha\) beads resulted in cooperative surface coverage of 3.5\% at 150 s\(^{-1}\), 3.7\% at 800 s\(^{-1}\), and 4.6\% at 1600 s\(^{-1}\) (\textbf{Figure 3.4}). These results establish that presence of both adhesion and aggregation functionalities together produce superior hemostasis compared to any of these functions alone. In these studies, it was also found that for beads simultaneously
bearing rGPIbα protein fragment and H12 peptides, it was necessary to conjugate these two motifs at two different spacer lengths from the surface to ensure minimum spatial interference (and therefore masking of bioactivity) between them. As mentioned previously, using only rGPIbα allows for just vWF binding which may be sub-optimal in ensuring binding stability at low-to-moderate shear. Moreover, use of recombinant protein fragments may pose issues of high cost in scale up and may have steric limitations regarding multivalent decoration on particle surfaces.

![Graph](image.png)

**Figure 3.4:** Representative data from a design approach integrating the platelet-mimetic properties of adhesion and aggregation. A mixture of latex beads surface-decorated with Fg-derived H-12 peptide and latex beads surface-decorated with vWF-binding rGPIbα motif showed higher hemostatic surface coverage [●] compared to just beads with H-12 peptide [△] or control unmodified beads [□] [reproduced with permission from (96)].

### 4. Discussion

The clinical need for a synthetic platelet analogue continues to be significant, as evident from companies like ProFibrix, Advanced Therapeutics, and Cypress Bioscience planning advanced clinical trials for their hemostatic products and various other designs undergoing *in vivo* pre-clinical studies. **Table 3.3** shows the various natural platelet-based, synthetic platelet-mimetic or coagulation promoting products that are currently

<table>
<thead>
<tr>
<th>Natural Platelet Products</th>
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<tr>
<td><strong>Product Name (Company)</strong></td>
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<td>---------------------------</td>
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### Synthetic Platelet-mimetic Products

<table>
<thead>
<tr>
<th>Product Name (Company)</th>
<th>Composition</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthocytes™ (Andaris Group Ltd.)</td>
<td>Albumin microsphere coated with human fibrinogen</td>
<td>Phase II</td>
</tr>
<tr>
<td>Fibrocaps™ (ProFibrix)</td>
<td>Fibrinogen and thrombin microparticles</td>
<td>Phase III</td>
</tr>
<tr>
<td>Fibrinoplast™ (Advanced Therapeutics &amp; Co.)</td>
<td>Albumin microsphere coated with human fibrinogen</td>
<td>Phase III</td>
</tr>
</tbody>
</table>

### Other Hemostatic Products

<table>
<thead>
<tr>
<th>Product Name (Company)</th>
<th>Composition</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemocomplettan P® and RiaSTAP® (CSL Behring)</td>
<td>Human fibrinogen concentrate</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Biostate® (CSL Behring)</td>
<td>Human Factor VIII concentrate</td>
<td>Clinical use</td>
</tr>
<tr>
<td>NovoSeven® (Novo Nordisk)</td>
<td>Recombinant human coagulation Factor VIIa</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Gelfoam® Plus and FloSeal Matrix™ (Baxter), BioFoam™ (CryoLife), Thrombi-Gel® (Pfizer)</td>
<td>Gelatin sponge with bovine thrombin</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Vitage™ (Orthovita)</td>
<td>Collagen matrix with bovine thrombin</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Evarrest™ (Johnson &amp; Johnson), TachoSil® (Takeda Pharmaceuticals)</td>
<td>Collagen matrix with human fibrinogen and thrombin</td>
<td>Phase II and III, Clinical use</td>
</tr>
<tr>
<td>HemCon® and ChitoFlex® PRO (HemCon Medical Technologies)</td>
<td>Chitosan matrix</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Surgicel® (Ethicon)</td>
<td>Cellulose matrix</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Sangustop®, Lyostypt® (B Braun)</td>
<td>Bovine collagen matrix</td>
<td>Clinical use</td>
</tr>
<tr>
<td>Nplate® (Amgen)</td>
<td>Thrombopoietin receptor agonist</td>
<td>Clinical use</td>
</tr>
<tr>
<td>QuikClot® (Z-Medica)</td>
<td>Zeolite</td>
<td>Clinical use</td>
</tr>
</tbody>
</table>

**Table 3.3:** Hemostatic therapies in clinical trials and applications.

either in clinical trials or approved applications [76]. The natural platelet based products
still suffer from the issues of availability, processing and storage expenses, contamination risks, and biologic or immunogenic side effects, and recent leukoreduction and pathogen reduction technologies have resolved these risks only to a limited extent. Some hemostatic products are clinically approved only for topical applications (e.g. QuikClot® [77]), but many bleeding scenarios require prolonged intravenous administration. Currently, the clinically approved intravenous hemostats are either coagulation factors (e.g. NovoSeven®, RiaSTAP® and Biostate® [78–82]) or thrombopoietic agonists (e.g. Nplate® [83]). The coagulation factors are prescribed only for selective indications, where the target patients have deficiencies in those specific factors but have normal platelet count and function. For example, for the action of NovoSeven®, a significant presence of Tissue Factor and activated platelet membrane is needed, which implies that it would require significant presence of active platelets. Hence none of the intravenously administered coagulation factors are meant to influence primary hemostasis and only correct secondary hemostasis. As for thrombopoietic agonists, they have shown therapeutic benefit in chronic thrombocytopenic conditions, but may not be suitable for acute bleeding scenarios where immediate primary hemostatic action is needed. Moreover, these agonists are recombinant protein based molecules that are highly expensive and can have adverse side effects since they act by affecting bone marrow cells. Currently, clinical studies are under way to determine the safety and therapeutic repertoire of these agents. Other hemostatic products involving coagulation-promoting fibrin- or thrombin-incorporated matrices, the application would be limited to open or accessible wounds and not in intravenous treatments. Hence, a synthetic construct that can adhere specifically and stably to a vascular injury site and promote
recruitment/aggregation of more platelets at that site can not only enhance primary hemostasis, but may possibly further enhance the action of pro-coagulant agents like NovoSeven®. Such platelet-mimetic constructs may enhance the therapeutic repertoire of synthetic hemostats beyond hemophilic patients, for example, in trauma, surgery, malignancy, myelosuppression and thrombocytopenia. To this end, the only design that has gone into advanced clinical trials is fibrinogen-coated albumin constructs (Synthocytes™) and further clinical data about this product is currently unavailable. As rationalized previously, platelet-mimetic products that only amplify platelet aggregation without directing platelet-mimetic adhesion, may have limited efficacy in treatment of bleeding condition. Hemostatic efficacy can be enhanced by integrating the adhesion and aggregation functionalities on a single platform. Regarding surface-modification, it is important to use motifs that allow interactions selectively to active platelets, such that in vivo, its performance is not affected by interaction with other cells or proteins.

It is also important to note that immunogenicity remains an important area of concern for both natural platelet-based as well as synthetically bioengineered hemostatic products. As a result, evaluation of immunogenic potential is listed as one of the critical components for clinical translation of such products, as per FDA guidelines [84]. For donor blood-derived, natural platelet concentrates and products obtained therefrom, screening procedures (e.g. HLA tests) and leukoreduction techniques may reduce but not completely eliminate auto- or allo-immunogenicity risks [85]. Such residual risks may not only affect the activity of donor platelets (e.g. refractoriness and fever), but also compound into causing sepsis-like symptoms and life-threatening respiratory failure [86–90]. The current knowledge about the underlying mechanisms of such negative effects is
very limited, and further research needs to be continued in elucidating and preventing these adverse events. For platelet-mimetic products based on synthetic particle platforms, it is to be considered that immunogenic reactions of synthetic particles are influenced by particle size, shape and surface physical and chemical properties [91], [92]. To this end, it is important to use biomaterials and surface-modification chemistries that reduce rapid macrophagic uptake and allow substantial circulation half-life, especially if the synthetic platelet analogues are to be used in a prophylactic fashion. Furthermore, the physical and mechanical properties of particles have been shown to modulate macrophagic uptake, and hence circulation time [93]. For example, spherical particles larger than 200nm are known to experience enhanced uptake, while ellipsoidal particles are more difficult phagocytize if contacted by a macrophage on their long axis compared to spherical particles. In addition, more flexible particles show decreased uptake compared to their rigid counterparts of same physical properties. Hence, ongoing and future research to identify the optimum design parameters that minimize immunogenicity of synthetic particles can lead to enhancement of safety and biocompatibility of synthetic platelet products. In the context of utilizing proteins, antibodies, antibody fragments or peptides to decorate particle surface for platelet-mimetic hemostatic functions, peptides may provide the most advantage since they are known to have much lower immunogenic risks compared to proteins or antibodies [94], [95]. Therefore, future research should be directed at identifying the optimum physico-mechanical parameters of particle design and the optimum densities of heteromultivalent surface modifications with pro-adhesive and pro-aggregatory small molecular weight ligands, that when integrated on a biocompatible
particle platform, will maximize the platelet-mimetic hemostatic performance of the particles.

Beyond rendering hemostasis via surface-based molecular interactions, natural platelets also modulate several biological processes in the body via secretion of a variety of granule contents, for example, P-selectin for interaction with leukocytes during inflammation, ADP for activation of neighboring platelets, several cytokines for pro-angiogenic activity and several enzymes to influence matrix remodeling. Based on this rationale, the synthetic particle platforms utilized for mimicking platelet surface biological functionalities for hemostatic action, can be further engineered to package platelet-mimetic granule contents (e.g. ADP and other agonists, cytokines, enzymes etc) to act as injury site-selective delivery vehicles for local modulation of hemostasis and healing activities. The possibility of such designs is demonstrated in the recent reports from Okamura et al., where ADP was encapsulated in liposomes surface-modified with Fg γ-chain derived H12 peptides [56]. This design not only enhanced platelet aggregation in vitro, but also showed significant hemostatic effect in thrombocytopenic rabbit model [41], [96]. Another demonstration is found in the work of Kona et al. who created PLGA nanoparticles surface-modified with GPIbα fragment and encapsulated a drug (dexamethasone) for use in repair of vascular injury via injury-targeted delivery [97]. Similarly, Doshi et al. have reported on discoidal-shaped protein particles surface modified with vWF-A1 domain fragments with the capability of encapsulating bioactive payload [98]. Another interesting approach in utilizing platelet-mimicry in drug delivery is demonstrated in the recent report from Korin et al., who fabricated microscale aggregates of polymeric nanoparticles that disintegrated to their nanoscale components.
under high shear to allow shear-induced drug delivery [99]. This design is inspired by the fact that natural platelets are known to get activated at high shear and release granule contents. Therefore, a platelet-mimetic paradigm can be utilized not only in development of advanced synthetic hemostat technologies, but also in vascularly targeted drug delivery systems.

6. Conclusion

Issues of limited availability, pathologic contamination risks, short shelf-life, and negative biological side-effects with natural platelet-based hemostatic products have led to a significant clinical interest in platelet-mimetic synthetic analogues that can allow long storage-life and minimum side-effects while providing efficient hemostasis via intravenous administration. Accordingly, several synthetic designs have undergone research, with the idea of decorating various synthetic particle platforms with motifs that promote platelet-mimetic hemostatic bioactivities. In the current review, we have provided a comprehensive discussion of these design approaches. Ongoing and future refinement of these strategies are focused on combining the platelet-mimetic adhesion and aggregation bioactivities on a single particle platform. The functionally integrated design of a synthetic platelet analogue should exhibit stable adhesion at the wall injury site under a shear flow environment and promotion of platelet aggregation (primary hemostasis) and fibrin formation (secondary hemostasis) selectively at the adhesion site, while avoiding systemic effects. Such a synthetic platelet analogue will be of high clinical value in hemostatic therapy.

7. References


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Chapter 4: Mimicking Adhesive Functionalities of Blood Platelets Using Ligand-Decorated Liposomes

Based on:

1. Introduction

Platelet transfusion plays a major role in the treatment of bleeding complications in patients with various hematologic or oncologic platelet disorders [1–6]. Additionally, platelet transfusion is often applied in pre-surgical settings and in traumatic injury for rapid staunching of heavy bleeding [5], [6]. The current clinical platelet transfusions consist of allogeneic platelet concentrates re-suspended in autologous plasma stored at ~22 °C, which have a shelf life of only 3–7 days [7–10]. Other products based on natural platelets under clinical investigation are frozen platelets (−80 °C), cold-stored platelets (4 °C), photo-chemically treated platelets, lyophilized platelets, platelet-derived microparticles, and infusible platelet membranes (IPM) [7–10]. Since all of these products are derived from pooled platelets of multiple donors, there is high risk of biologic (viral, protozoal, and bacterial) infections. The infection risk can be partly reduced by excluding high-risk individuals from the donor pool, serological testing of donor blood, and pre-storage leuko-reduction processes [9–14]. However, these further increase costs of platelet concentrate preparation. In addition, natural platelet-based products present risks of febrile non-hemolytic reactions, alloimmunization-induced refractoriness, graft-versus-host disease, acute lung injury risks, and immunosuppression [15–18]. Altogether, these factors severely limit the efficacy of natural platelet-based
transfusion products. Consequently, there is significant clinical interest in synthetic constructs that can mimic or amplify platelet’s hemostatic functionalities, while allowing large-scale preparation, reproducible quality, long storage life, and absence of biologic infections [19]. Past approaches on mimicking platelet’s hemostasis-relevant functions on synthetic platforms have mainly focused on amplifying platelet’s “aggregation” functionality by decorating synthetic particle surfaces with aggregation-promoting biomolecules like Fibrinogen (Fg) or Fg-derived peptide sequences. For example, synthetic particle platforms like liposomes, albumin spheres, and synthetic polymeric particles have been surface-decorated with platelet membrane-derived glycoproteins [20], [21], Fg [22], [23], Fg-derived Arginine-Glycine-Aspartic Acid (RGD) peptides [24], and Fg-derived H12 dodecapeptides [25], [26]. All of these are essentially various designs of “super-fibrinogen” particles that can amplify the aggregation of active platelets due to the increased surface-valency of their platelet-bridging motifs (i.e., Fg or Fg-derived peptides), compared to hexavalent Fg itself. However, in natural primary hemostasis, platelet aggregation is preceded by stable platelet adhesion at the injury site under blood flow as shown in Figure 4.1 [27], [28]. Platelet adhesion is mediated by shear-dependent binding of the GPIbα extracellular domain of the platelet surface glycoprotein GPIb/IX/V complex with von Willebrand Factor (vWF) secreted from the injured endothelium [29], [30], augmented by binding of platelet surface glycoproteins GPIa/IIa and GPVI [31], [32] to sub-endothelial collagen. The vWF-binding helps in the initial arrest and rolling of platelets at the injury site, while collagen-binding stabilizes the adhered platelets under the hemodynamic flow environment. These adhesion mechanisms result in platelet activation signaling, ultimately leading to a ligand-binding conformational change of the
platelet surface integrin GPIIb-IIIa, which then binds to Fg to promote aggregation of the activated platelets to form the primary hemostatic plug [33], [34]. A few research approaches have investigated the mimicry of platelet’s adhesion mechanisms by decoration of liposomes, albumin particles, or latex particles with adhesion-promoting motifs like recombinant GPIbα (vWF-binding) or GPIa/IIa (collagen-binding) fragments [35–38]. However, these designs have not demonstrated the integration of the dual adhesion mechanisms (simultaneous vWF-binding and collagen-binding). In addition, recombinant protein fragments are quite expensive for large-scale production, purification, and scale-up. Furthermore, they can also potentially present immunogenic risks.

Rationalizing from these attributes of past research, here we report on bioengineering of synthetic constructs where vWF-binding and collagen-binding ligand motifs are integrated on the same particle (Figure 4.2), and investigate their platelet-mimetic adhesive capabilities under physiologically relevant flow environment (wall shear stresses) in vitro, using a parallel plate flow chamber (PPFC). For vWF-binding, we have investigated a small synthetic peptide with amino acid sequence,
TRYLRIHPQSWVHQI, that is derived from the C2 domain (residues 2303−2332) of the coagulation factor FVIII, which is known to form a complex with vWF prior to thrombin or factor Xa catalyzed activation in the coagulation cascade [39]. We have compared the vWF-binding of liposomes surface-decorated with this vWF-binding peptide (VBP) to liposomes surface-decorated with the previously reported recombinant GPIbα fragment. For collagen-binding, we have investigated a short 7-repeat of the Glycine(G)-Proline(P)-Hydroxyproline(O) tri-peptide (−[GPO]7−), with helicogenic affinity to fibrillar collagen [40–42]. This small collagen-binding peptide (CBP) can promote adhesion to fibrillar collagen, but cannot activate quiescent platelets due to the absence of long triple-helical conformation. We have demonstrated that the vWF-binding constructs undergo enhanced adhesion under increasing wall shear, while the collagen-binding constructs undergo stable adhesion in an apparent shear-independent fashion. Furthermore, we have integrated simultaneous vWF-binding and collagen-binding motifs on the same liposome platform and have investigated their adhesion capability to a vWF/collagen mixed surface under flow, in vitro. In such heteromultivalent liposome surface-decoration, we have demonstrated that the platelet-mimetic dual adhesion mechanisms (simultaneous vWF-binding and collagen-binding) can be successfully achieved provided that the vWF-
binding and the collagen-binding ligand motifs do not spatially interfere with each other while conjugated onto the liposome surface. Altogether, by surface engineering of liposomes via decoration of specific ligands, we demonstrate efficient molecular mimicry of platelet’s dual adhesion mechanisms. This approach can be potentially adapted to various particle platforms to optimize the design of a platelet-mimetic synthetic bioconjugate construct.

2. Experimental Procedure

Materials

Cholesterol, dimethyl sulfoxide (DMSO), and collagen were purchased from Sigma Aldrich (Saint Louis, MO, USA). The lipids distearyl phosphatidyl choline (DSPC), 2000 MW polyethylene glycol-modified distearyl phosphatidyl ethanolamine (DSPE-PEG2000), and carboxy-terminated polyethylene glycol-modified DSPE (DSPE-PEG2000-COOH) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). N-Hydroxysuccinimide- modified fluorescein (NHS-Fluorescein) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). Human vWF (FXIII free) was purchased from Hematologic Technologies Incorporation (Essex Jn, VT, USA). The Parallel Plate Flow Chamber (PPFC) system was purchased from Glycotech (Gaithersburg, MD, USA).

Ligand Motifs.

vWF-Binding Motifs: For vWF binding, a recombinant GPIbα fragment (rGPIbα) containing the vWF binding sites (residues 1 to 302) or a short-chain vWF-binding peptide (VBP) was used. The GPIbα fragment was expressed in CHO cells and isolated,
adapting methods described by Murata et al [43]. The VBP, TRYLRIHPQSWVHQI, was synthesized using fluorenlymethyloxycarbonyl chloride (Fmoc)-based solid phase chemistry on a Knorr resin, and characterized using mass spectroscopy. Each vWF molecule has only one binding region for this peptide. Hence, we rationalized that a vWF-precoated surface or shear-enhanced multimerization of vWF on a collagen-coated surface will present multiple binding sites for multiple copies of this peptide decorated on the liposome surface, thereby providing a mechanism for enhanced adhesion of the liposomes with increasing shear.

Collagen-Binding Motifs: The CBP, [GPO]7, was also synthesized using Fmoc-based solid-phase chemistry on Knorr resin and characterized using mass spectroscopy. The GPO trimer is based on amino acid repeats found in the native collagen structure. It has been reported that the activation of platelets usually caused by interaction with collagen through GPVI and GPIa/IIa can also potentially occur when platelets interact with collagen-derived peptides [41], [42]. This can be a potential problem regarding decorating synthetic particle surfaces with collagen-derived peptides for binding of collagen, because in vivo, the constructs can potentially interact with quiescent blood platelets and systemically activate them, posing thromboembolic risks. However, Farndale et al. have reported that interaction of platelet receptors with collagen and the subsequent platelet activation mechanisms are dependent upon receptor clustering induced by multimeric long chain triple-helical fibrillar collagen and not by short collagen-mimetic peptide repeats [44]. In fact, it has been shown that GPO-trimer repeats as high as a 30-mer (10 repeats) only partially interact with platelet GPIa/IIa and GPVI integrins and are incapable of activating platelets, yet they can effectively bind to fibrillar
collagen via helicogenic interaction [45], [46]. Hence, we rationalized that our 7-mer short chain monomeric CBP will not activate quiescent platelets in circulation but can still allow binding of CBP-decorated liposomes to collagen-covered surface, under flow. The mass spectrometric characterization data of the peptides are shown in Figure 4.3. Additionally, aggregometry data confirming the inability of both VBP and CBP to activate platelets is shown in Figure 4.4.

![Figure 4.3: MALDI-TOF mass spectrometric characterization of the vWF-binding peptide (VBP) and the collagen-binding peptide (CBP) synthesized via FMoc-based solid phase chemistry on Knorr resin using Applied Biosystems solid phase synthesizer.](image)

The rGPIbα, VBP, or CBP were conjugated to DSPE-PEG-COOH using carbodiimide-mediated amidation chemistry to form DSPE-PEG-ligand molecules, utilizing previously reported methods [47], [48]. For lipid–peptide conjugation, the N-terminal amine of the peptide sequence was reacted with DSPE-PEGCOOH while all amino acid side-chains remained protected and the peptide itself still remained conjugated onto the solid-phase resin bead through its C-terminus. Hence, the carbodiimide-mediated conjugation reaction was selective to the N-terminus of the VBP and CBP peptides. Deprotection of
the side-chain groups and the cleavage of the lipid–peptide conjugate from the resin was carried out only after this selective conjugation. Hence, it was not necessary to confirm that the conjugation reaction occurred only at the N-termini and not at the side chain amine groups. We have utilized this method extensively in preparing ligand-modified liposomes, as reported previously [49], [50]. In the current studies, we followed exactly the same methods for the lipid–peptide conjugation reactions and assessed reaction efficiency using a ninhydrin assay. The assay showed peptide conjugation to have occurred at 95.7% ± 4.9% conjugation efficiency. To fluorescently label the liposomes, DSPE-Fluorescein (green fluorescence, λmax ≈ 530 nm) was synthesized by reacting the free amine (−NH2) termini of DSPE with NHS-Fluorescein at basic pH. Specific proportions of the DSPE-PEG-rGPIbα, DSPE-PEG-VBP, or DSPE-PEG-CBP were combined with unmodified DSPE-PEG, DSPC, cholesterol, and DSPE-Fluorescein to fabricate peptide-decorated green fluorescent liposomal constructs, using the standard reverse-phase evaporation and extrusion technique [51]. The liposome size distribution,
characterized by dynamic light scattering (DLS), was found to be \( \sim 150 \) nm diameter (Figure 4.5).

**Figure 4.5:** Dynamic Light Scattering (DLS) characterization of peptide-decorated liposomes before and after extrusion through nanoporous (200nm diameter) polycarbonate membranes. The average post-extrusion hydrodynamic diameter of the liposomes was \( \sim 150 \)nm.

**Parallel Plate Flow Chamber (PPFC).**

The PPFC setup is appropriate for bio-molecular interaction analysis under a dynamic shear flow environment [52], [53]. In the PPFC, by maintaining Renyold’s number in the “laminar” range (\( \sim 105 \)), the wall shear stress (\( \tau_w \)) can be modulated as a function of flow rate (\( Q \)) by

\[
\tau_w = 6\mu Q/bh^2 \quad (1)
\]

where \( \mu = \) fluid viscosity, \( b = \) width of the chamber, and \( h = \) distance between plates. For our experiments, \( b/h > 20 \) and \( Q \) was maintained to provide \( \tau_w \) in the range 5–55 dyn/cm², which covers a substantial range of physiological shear in blood flow [54].

Distinct circular areas on glass slides were coated with collagen, vWF, or 50:50 mixture of vWF/collagen (test surfaces) and bovine serum albumin (BSA, negative control surface with no adhesion specificity). The coated slides were vacuum-sealed into the PPFC for subsequent experiments.
**Platelet-Mimetic Adhesion Studies under Flow in Vitro.**

For studying platelet-mimetic vWF-adhesive functionality, 2.5 mol % DSPE-PEG-rGPIbα or DSPE-PEG-VBP was combined with DSPC (49 mol %), cholesterol (45 mol %), DSPE-PEG (2.5 mol %), and DSPE-Fluorescein (1 mol %) to form the final liposomal construct. The fluorescein- labeled (green fluorescent, λmax = 530 nm) liposomes, at a concentration of 10 μM total lipid, were allowed to flow through the PPFC in a closed loop over the vWF-coated and BSA-coated surface under various flow rates to produce wall shear stresses from 5 to 55 dyn/cm² for 30 min. After 30 min, flow of just PBS was maintained in an open loop for an additional 15 min in order to remove any loosely bound constructs and gain insight on the adhesion stability and retention of the constructs on the test and control surfaces. The slides were imaged at various time points (5, 15, 30, 35, and 45 min) of flow in the PPFC using an inverted epifluorescence microscope (Carl Zeiss Axio Observer D1) with a photometrics chilled CCD camera (Axiocam MRM) and a 63x objective. Images were collected using Axiovision software with fixed exposure times of 400 ms. From each image, extent of adhesion and retention was quantified by measuring surface-averaged fluorescence intensity using Adobe Photoshop CS5 software. Statistical analysis of fluorescence intensity was performed using ANOVA and significance was considered as p < 0.05. **Figure 4.6 A** shows a schematic view of the PPFC experimental setup and the expected interaction of the vWF-binding liposomal constructs with the test and control surface regions. In an additional experimental design, we aimed to investigate if soluble vWF could adhere to collagen and multimerize under high shear stress, and then induce adhesion of VBP-decorated
liposomes. This was inspired by the natural physiological mechanism where soluble vWF

![Image of experimental setup showing liposomes interacting with vWF-coated surface versus albumin surface.](image)

**Figure 4.6:** Representative results from PPFC studies using rGPIbα- or VBP-decorated liposomal constructs allowed flow over the vWF-coated surface versus the albumin surface. (A) Schematic of experimental setup. (B) Ligand-modified liposomes showed minimal adhesion and retention on the albumin surface and unmodified liposomes showed minimal adhesion and retention on the vWF surface. On the other hand, rGPIbα-modified and the VBP-modified liposomes both showed significant adhesion and retention on the vWF surface under flow. (C) Quantitative analysis of the adhesion (at 30 min) and retention (at 45 min) using surface-averaged fluorescence intensity shows that both rGPIbα-modified and VBP-modified liposomes undergo increasing adhesion and retention on vWF surface with increasing shear, mimicking the vWF-binding of platelets.
adheres to exposed subendothelial collagen, multimerizes under shear, and subsequently allows adhesion of platelets via interaction with platelet surface GPIbα. For this, green fluorescent VBP-modified liposomes and soluble FVIII-free human vWF were introduced into the PPFC and allowed to flow over collagen-coated surface or albumin-coated surface under high shear stress of 55 dyn/cm² for 5–30 min and the adhesion of the liposomes over time was imaged with epifluorescence microscopy using the PPFC setup described previously. For studying platelet-mimetic collagen-adhesive functionality, 2.5 mol % DSPE-PEG-CBP was combined with DSPC (49 mol %), cholesterol (45 mol %), DSPE-PEG (2.5 mol %), and DSPE-Fluorescein (1 mol %) to form the final liposomal construct. These fluorescently labeled liposomes were allowed to flow through the PPFC over collagen-coated and BSA-coated surfaces in the same way as VBP-decorated liposomes (30 min in closed loop followed by 15 min open loop circulation of just PBS). Imaging at various time points and image analysis were carried out as before. Figure 4.8 A shows a schematic view of the PPFC experimental setup and the expected interaction of the CBP-decorated liposomes with the test and control surfaces. For studying cumulative effects of simultaneous vWF and collagen-binding, 2.5 mol % DSPE-PEG-rGPIbα or DSPE-PEGVBP and 2.5 mol % of DSPE-PEG-CBP were combined with DSPC (49 mol %), cholesterol (45 mol %), and DSPE-Fluorescein (1 mol %) to form the final heteromultivalently decorated liposomal constructs. These fluorescently labeled liposomes were allowed to flow in the PPFC over a surface coated with 50:50 vWF/collagen (mixed coating) under 5–55 dyn/cm² shear stress, and the imaging and analysis were carried out as before. For all adhesion studies, besides testing the interaction of ligand-modified liposomes on negative control albumin surfaces,
additional control studies were also carried out by testing unmodified (no surface
decoration) liposomes on vWF-coated, collagen-coated, or mixed-coated surfaces.

3. Results


Figure 4.6 B shows a representative set of fluorescence images from PPFC experiments
using rGPIbα-decorated liposomes and VBP-decorated liposomes on vWF-coated
surfaces versus albumin-coated surfaces under flow. Although images were taken at six
shear values between 5 and 55 dyn/cm2 and at five time points during flow between 5
and 45 min for each of the liposomal constructs, representative fluorescent images are
shown at only two shear values (5 and 35 dyn/cm2) and two time points (30 and 45 min)
for convenience. Figure 4.6 C shows the quantitative analysis of surface-averaged
fluorescence intensity values from the adhesion of the various liposomal constructs on
vWF-coated surfaces over the entire shear stress range at 30 and 45 min. Statistical
analysis of fluorescence intensity values shows that rGPIbα-decorated liposomal
constructs undergo increased adhesion on the vWF-coated surfaces with increasing shear,
while on albumin-coated surfaces, these constructs showed only minimal adhesion
irrespective of shear stress values. VBP-decorated constructs also showed similar trends
of increased adhesion with increasing shear. However, beyond 45 dyn/cm2 shear stress
values, the level of adhesion of the VBP-modified constructs was not found to increase
further statistically. The unmodified liposomes showed minimal adhesion to vWF at all
shear stresses. Figure 4.7 shows representative fluorescent images from the additional
experimental design involving flow of soluble vWF and VBP-modified liposomes over
Collagen or albumin surface under high shear stress. Representative results are shown for the highest shear stress value of 55 dyn/cm² for three time-points of 5, 15, and 30 min of flow. As evident from the results, green fluorescent VBP-modified liposomes are able to adhere when introduced in flow along with soluble vWF, to a collagen-coated surface, but not to an albumin-coated surface. Furthermore, the VBP-modified liposomes seemed to undergo enhanced amount of adhesion (larger fluorescent “patch” areas) with time, under the high shear value.

**Platelet-Mimetic Collagen Adhesion of Ligand-Decorated Liposomes under Flow in Vitro.**
Figure 4.8: Representative results from PPFC studies using CBP-decorated liposomal constructs allowing flow over the collagen-coated surface versus the albumin surface. (A) Schematic of experimental setup. (B) Ligand-modified liposomes showed minimal adhesion and retention on the albumin surface, and the unmodified liposomes showed minimal adhesion and retention on the collagen surface. On the other hand, CBP-modified liposomes showed significant adhesion and retention on collagen surface under flow. (C) Quantitative analysis of the adhesion (at 30 min) and retention (at 45 min) using surface-averaged fluorescence intensity shows that the CBP-modified liposomes undergo enhanced adhesion and retention on the collagen surface in a shear-independent fashion.
using CBP-decorated liposomes on collagen-coated surfaces versus albumin-coated surfaces under flow. As before, the representative images are shown at only two shear values (5 and 35 dyn/cm²) and two time points (30 and 45 min) for convenience. **Figure 4.8 C** shows quantitative analysis of the surface-averaged fluorescence intensity values from the adhesion of the various liposomal constructs on collagen-coated surfaces over the entire shear stress range at 30 and 45 min. Statistical analysis of fluorescence intensity measurements shows the CBP-decorated liposomal constructs undergoing significant adhesion to collagen-coated surfaces under flow with no apparent shear-dependent effect, but minimal adhesion to albumin surfaces. In addition, the unmodified constructs showed minimal adhesion on collagen surfaces.

*Combining vWF-Adhesion and Collagen Adhesion on the Surface of Liposomes in Vitro.* **Figure 4.9 A** shows representative set of fluorescence images for adhesion of liposomes surface-decorated with both rGPIbα and CBP (2.5 mol % each), or both VBP and CBP (2.5 mol % each), onto a mixed coated (vWF/collagen 50:50) surface under flow in the PPFC. The adhesion of unmodified liposomes on these mixed coated surfaces and the adhesion of the ligand-modified liposomes on the negative control (albumin) surfaces are also shown in the figure for comparison. As before, the representative images are shown at only two shear values (5 and 35 dyn/cm²) and two time points (30 and 45 min) for convenience. **Figure 4.9 B** shows the quantitative analyses of the fluorescence intensity data from the adhesion of the various heteromultivalent liposomal constructs over the entire shear stress range, compared with the adhesion data for liposomes bearing rGPIbα-decorations only, VBP-decorations only, and CBP-decorations only, all on mixed coated
As evident from the fluorescent images as well as the quantitative data, liposomes bearing both rGPIbα and CBP showed enhanced adhesion on the mixed coated surface with increasing shear, but this was not statistically different for the vWF/collagen 50:50 surfaces. As evident from the fluorescent images as well as the quantitative data, liposomes bearing both rGPIbα and CBP showed enhanced adhesion on the mixed coated surface with increasing shear, but this was not statistically different.
from liposomes bearing rGPIbα modification alone. In comparison, liposomes bearing both VBP and CBP not only showed enhanced adhesion to the mixed coated surface under increasing shear, but the levels of adhesion were significantly higher than liposomes bearing VBP decoration or CBP decoration alone.

4. Discussion

In the current research, we have investigated the mimicry of platelet’s dual adhesion mechanisms on a model synthetic particle platform (liposome). Our results suggest that the shear-dependent enhancement in adhesion of the rGPIbα-decorated or VBP-decorated liposomal constructs were due to specific interactions of the ligands to the vWF surface, since the same liposomes showed only minimum adhesion on albumin surfaces. Furthermore, the results from experiments with VBP-decorated liposomes and soluble vWF under flow on collagen surfaces, indicate that possible multimerization of the soluble vWF with time on the collagen surface under high shear allows increased adhesion of VBP-decorated liposomes on the vWF-rich areas, as evident from the formation of larger fluorescent patches of liposomes binding to vWF with time. Altogether, these results demonstrate successful mimicry of shear-dependent platelet adhesion to vWF using surface-engineered liposomes. The results from interaction of CBP-decorated liposomes on collagen-coated surface indicate that the adhesion is mostly shear-independent and is due to specific helicogenic interaction of the CBP with collagen. Hence, these data establish successful mimicry of the collagen-binding property of platelets with CBP-decorated liposomes. Here, it is also interesting to note that our CBP-decorated constructs showed higher adhesion capabilities under shear flow
compared to GPIaIIa-modified liposomes reported previously [55]. We rationalize this observation by the fact that, because GPIaIIa is a big protein fragment, there are only a limited number of copies of it that can be decorated on a nanoscale particle surface (e.g., liposome), which would lead to a limited extent of binding interactions with a collagen substrate. On the other hand, since CBP is a small peptide, a lot more copies of it can be decorated on the liposome surface, leading to a higher extent of helicogenic interactions with collagen and hence a higher extent of adhesive property of the CBP-decorated constructs.

We would like to point out that, in all the in vitro experiments carried out in the current studies, the constructs were tested in the shear stress ranges of 5−55 dyn/cm² since this range covers the physiological shear conditions in most vasculature. The fluorescent images of construct adhesion are shown only for 5 dyn/cm² and 35 dyn/cm² as representative physiological low shear and high shear scenarios [54], although image data was collected over the whole shear range of 5−55 dyn/cm². This collective data over the entire range is shown in the histogram plots of Figure 4.6, Figure 4.8 and Figure 4.9. Also, in these figures the histogram plots are shown only for the 30 min and 45 min time points as representative scenarios for convenience, although the analyses were carried out at multiple time points of 5, 15, 30, 35, and 45 min. The rationale for showing the 30 min time point was that it was the last time point up until which the constructs were allowed to “bind” to their target substrate in a recirculating loop flow. The rationale for showing the 45 min time point was that it was the last time point up until which the bound constructs were exposed to open loop flow of PBS to test binding strength and retention in a hemodynamically relevant shear environment. Beyond the 45 min time point, no
further statistical variation in retention of constructs was observed in our experiments. We also point out that all the in vitro experiments had durations of ≤60 min, because in longer time periods, human blood tends to gradually clot in vitro and becomes unsuitable for experiments.

Analysis of the results from experiments with liposomes simultaneously bearing vWF-binding and collagen-binding motifs suggest that, when decorated simultaneously on the liposomal surface, the larger rGPIbα motif (∼300 amino acid residues) possibly masks the much smaller CBP motif (∼21 residues), thereby preventing the synergistic effect of simultaneous vWF-binding and collagen-binding by the liposomes. The resultant adhesion seems to happen primarily due to only rGPIbα–vWF interaction in a shear-dependent fashion. This observation is similar to the results demonstrated by Okamura et al.56 where simultaneous decoration of a particle surface with rGPIbα motifs and Fg-mimetic H12 peptide motifs showed apparent masking of the peptide by the bigger protein fragment. In contrast, when VBP (∼15 amino acid residues) is used in conjunction with CBP for liposome surface decoration, these two small peptides possibly do not mask each other’s specific interactions, and therefore, a combined effect of vWF binding and collagen binding becomes evident in the enhanced adhesion of the heteromultivalent liposomal constructs on the mixed coated surfaces under flow. Hence, we demonstrate that, by decorating a synthetic particle (liposome) surface with ligands binding simultaneously to vWF and collagen, and ensuring that the decorated ligands do not spatially mask each other, we can successfully mimic the hemostatically relevant dual adhesion mechanisms of platelets. Furthermore, simultaneous mimicry of these dual mechanisms of adhesion significantly enhanced the retention of the heteromultivalently
modified liposomal constructs on vWF/collagen-coated surface, compared to liposomes bearing only one adhesion mechanism functionality (homomultivalent) or no functionality (unmodified). This is indicative of a much enhanced mimicry of natural platelet adhesion where the initial adhesion through vWF interaction is stabilized by subsequent supplemental binding interactions with collagen. In the current studies, we have used a 50:50 mixture of vWF/collagen as a representative test surface for demonstrating feasibility of our platelet adhesion-mimetic design. In addition, we have also tested surfaces of 100% collagen with vWF in the soluble form under flow. Our ongoing and future studies are directed at modulating the relative peptide densities on the particle surface and the relative substrate ratios on the test surface, to optimize our design. Results from these studies will become part of a future research publications. We rationalize that the functional biomimetic design of a platelet-mimetic synthetic construct should incorporate both the “adhesion” functionalities and the “aggregation” functionalities of natural platelets. In fact, a recent report by Okamura et al. has demonstrated that synthetic particles surface-modified by “platelet aggregation” promoting H12 peptides and those surface-modified by “adhesion” promoting vWF-binding recombinant rGPIbα motifs, when introduced together in a thrombocytopenic bleeding model in rabbits, showed a higher hemostatic efficacy via cumulative (adhesion plus aggregation) effects, compared to either of these particles alone [56]. Building on this rationale, we envision that platelet-mimetic hemostatic efficacy of synthetic constructs can be further enhanced if the “aggregation”-promoting component and “adhesion”-promoting component can be combined on the same particle. Therefore, in subsequent studies, we will combine the adhesion-promoting VBP and CBP motifs
along with aggregation-promoting Fgmimetic RGD peptide motifs on the same liposome, and investigate whether these functionally integrated constructs can themselves adhere to vWF/collagen surfaces under flow and promote recruitment and aggregation of platelets at the sites of liposome adhesion.

It is to be noted that the model particle used in our studies were spherical unilamellar liposomes about 150 nm in diameter. Several recent mathematical modeling, as well as experimental, studies have demonstrated that there exist significant correlations between the shape and size of particles to their location in hemodynamically relevant flow patterns [57–59]. For natural platelets, their hemostatic functions at the vessel wall depend upon their ability of “margination” to the wall injury site through RBCs and other blood components. This hemodynamic migration is significantly influenced by platelet’s shape and size [60]. On the basis of such observations, we rationalize that an additional critical component of synthetic platelet design will be identification of the optimum particle geometry (size and shape) that can facilitate enhanced wall-margination of the particles. We envision that, by integration of margination-favoring optimal physical parameters (size and shape) with adhesion- and aggregation-promoting optimal biological parameters (chemistry and density of ligand modifications), an ideal biomimetic design of synthetic platelet can be achieved. Also, these design components and resultant insight can provide novel avenues to target such particles as efficient drug delivery vehicles to vascular disease sites with exposed vWF or collagen.

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Chapter 5 : Peptide-decorated Liposomes Promote Arrest and Aggregation of Activated Platelets under Flow on Vascular Injury Relevant Protein Surfaces in Vitro

Based on:

1. Introduction

Transfusion of natural platelet suspensions and platelet-derived products is routinely used clinically for the treatment of various hematologic and oncologic bleeding disorders as well as in trauma or surgery-related heavy bleeding complications [1–4]. However, these natural platelet products are highly expensive, have limited availability due to shortage of blood donors, have a very short shelf life (3–7 days), and can present a variety of contamination and biological risks [5], [6]. Hence, there is a significant clinical interest in synthetic constructs that exhibit hemostatically relevant platelet-mimetic functionalities while allowing easy scalable fabrication, long shelf life, and reduced biological risks. Research in developing such synthetic platelet mimics over the past two decades has resulted in several designs (Table 5.1) [7–18]. As evident from the Table, most of these designs have focused on creating particles that can amplify the aggregation of platelets (particles coated with fibrinogen or fibrinogen-derived RGD and H-12 peptides), whereas a few have focused on creating particles that exhibit platelet-mimetic adhesion under flow (e.g., particles coated with GPIbα or GPIa/IIa motifs). The designs that just amplify platelet aggregation can render only partial benefit in hemostasis at high particle doses, as demonstrated in several reports based on bleeding models in small animals [14–17]. Additionally, these reports are not mutually comparable because some
have utilized thrombocytopenic animals with subacute bleeding (e.g., tail-vein or ear-punch bleeding), whereas others have utilized normal platelet count animals with acute (traumatic) bleeding (e.g., femoral artery laceration). Also, some reports have used particle concentration in numbers per kilogram of animal body weight or per unit injection volume (n per kg or n per mL), whereas some others have used particle concentration in total mass per injection volume or per kilogram of animal body weight (mg per mL or mg per kg). Therefore, although some hemostatic effect of the various designs has been demonstrated in all of these reports, whether this effect is actually therapeutically efficacious remains to be a topic of further research. We rationalize that the hemostatic benefit from particles capable of just amplifying platelet aggregation will be therapeutically relevant and efficient only in heavy bleeding conditions with normal platelet counts because, in such cases, a large number of activated platelets will already be available, adhering (and being recruited) at the injury site, whose aggregation can then be kinetically enhanced by particle-mediated clustering. However, in bleeding

Table 5.1: Various design approaches carried out in past research regarding the development of platelet-mimetic synthetic hemostat particles.
complications that are subacute or involve reduced platelet numbers (e.g., thrombocytopenic or thrombostenic conditions), particles that just amplify platelet aggregation may not be therapeutically effective in maintaining hemostasis because they may not find sufficient number of activated platelets already adhering or being recruited to the bleeding site. Instead, these particles may end up clustering the low number of available active platelets in circulation to form free-floating aggregates that may increase the risk of embolism. Similarly, for particles having only the functionalities that exhibit platelet-mimetic adhesion [10–14], there may be partial benefit if the particles attaching at the injury site can themselves staunch bleeding. However, this may not be therapeutically sufficient because the particles cannot further amplify active platelet aggregation at the site. Interestingly, in a recent publication by Okamura et al [19], it was demonstrated that synthetic particles surface-modified by “platelet aggregation” promoting motifs (fibrinogen γ-chain derived H-12 peptides) and those surface-modified by “vWF-adhesion” promoting motifs (recombinant rGPIbα fragments), when introduced together in a thrombocytopenic bleeding model in rabbits, showed significantly higher hemostatic efficacy compared with either of these particles injected alone. These results suggest that the simultaneous presence of platelet-mimetic “adhesion-promoting” and “aggregation-promoting” functionalities on a single particle may result in a more efficacious design of a synthetic hemostat.

On the basis of the above rationale, here we describe the development and experimental results from integrating platelet-mimetic adhesion- and aggregation-promoting functionalities on a single particle, by decorating the surface of 150 nm diameter liposomes simultaneously with three peptides: a vWF-binding peptide (VBP), a
collagen-binding peptide (CBP), and an active platelet GPIb-IIIa-binding peptide (cRGD). We have previously demonstrated that liposomes bearing VBP and CBP motifs undergo platelet-mimetic adhesion under flow on vWF and collagen-coated surfaces in vitro at low-to-high shear in parallel plate flow chamber (PPFC) experiments [20]. Here we first demonstrate that cRGD-modified liposomes pre-adhered to a surface can enhance the aggregation of ADP-activated platelets onto themselves, even at a low platelet concentration (LPC). Subsequently, we demonstrate that liposomes bearing all three peptides (VBP, CBP, and cRGD), when introduced in a PPFC flow system along with low concentration of ADP-activated platelets over a vWF/collagen mixed coated surface, are able to adhere to the surface under high shear and promote arrest and aggregation of active platelets onto sites of liposome adhesion.

2. Materials and Methods

Materials.

Phosphate-buffered saline (PBS), 3.8% w/v sodium citrate, paraformaldehyde (PFA), avidin, bovine serum albumin (BSA), and ethanol were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Cholesterol, dimethyl sulfoxide (DMSO), and collagen were purchased from Sigma Aldrich (Saint Louis, MO). Fluorescently labeled monoclonal antibody, Alexa Fluor 647-anti- CD62P (staining activated platelet P-selectin), was purchased from BioLegend (San Diego, CA). The lipids distearyl phosphatidyl choline (DSPC), distearyl phosphatidyl ethanolamine (DSPE), poly(ethylene glycol)-modified DSPE (DSPE-PEG2000), carboxy-poly(ethylene glycol)-modified DSPE (DSPE-PEG2000-COOH), and biotinylated poly-(ethylene glycol)-modified DSPE (DSPE-
PEG2000-Biotin) were purchased from Avanti Polar Lipids (Alabaster, AL). ClearOx and N-hydroxysuccinimide-modified fluorescein (NHS-fluorescein) were purchased from Invitrogen (Carlsbad, CA). Human vWF (FXIII free) was purchased from Hematologic Technologies (Essex Junction, VT). The PPFC system for dynamic flow studies was purchased from Glycotech (Gaithersburg, MD). The dimensions of the chamber were flow width = 1 cm and distance between plates = 0.00254 cm. The peptide sequences used were TRYLRIHPQSWVHQI (VBP), [GPO]7 (CBP), and cyclo-CNPRGDY(OEt)RC (cRGD). The VBP, CBP, and the linear precursor of the cRGD peptide were synthesized using fluorenlymethoxycarbonyl chloride (FMoc)-based solid-phase chemistry on a Knorr resin and characterized using mass spectroscopy (see mass spec results for VBP and CBP in Figure 4.3). The linear precursor of cRGD was subjected to sulfhydryl oxidation of cysteine termini using ClearOx reagent to achieve disulfide-based cyclization, and this cyclization was confirmed using mass spectroscopy (Figure 5.1). The rationale behind using these peptides has been explained in previous

![Figure 5.1: MALDI-TOF mass spectrometric characterization of the cyclic RGD peptide (cRGD) and synthesized via FMoc-based solid phase chemistry on Knorr resin using Applied Biosystems solid phase synthesizer. Confirmation of cyclization of the cRGD peptide via sulfhydryl oxidation of cysteine termini using ClearOx reagent.](image)
publications [20], [21].

Preparation of Platelet Suspensions.

Venous blood from healthy, medication-free, adult donors was drawn into 3.8% w/v sodium citrate anticoagulant at a 9:1 ratio (by volume), in compliance with CWRU IRB-approved protocols. Platelet-rich plasma (PRP) was obtained by centrifuging human whole blood at 150 g for 15 min, and platelet count was monitored using a Coulter Counter. To prepare LPCs mimicking thrombocytopenic conditions, we further centrifuged a portion of PRP at 2500g for 25 min to obtain platelet-poor plasma (PPP). This PPP was then added volumetrically to PRP such that final platelet concentration was adjusted to ∼20 000/μL, as monitored by a Coulter Counter. These LPC suspensions were used immediately after preparation.

Fabrication of Surface-Modified Liposomes.

The VBP, CBP, and cRGD peptides were conjugated via their N-termini to the carboxyl terminus of DSPE-PEG2000-COOH via standard carbodiimide chemistry adapted from previously reported methods [21], [22], resulting in the various DSPE-PEG-peptide molecules. NHS-fluorescein was reacted with DSPE-PEG2000-COOH to form DSPE-PEG-fluorescein for fluorescence labeling of liposomes. DSPE-PEG-peptides were mixed at specific mol % with DSPC, cholesterol, DSPE-PEG, DSPEPEG-biotin, and DSPE-PEG-fluorescein. These mixed lipid formulations were used toward fabricating liposomes using standard reverse-phase evaporation and extrusion techniques, as previously described [23]. Extrusions were carried out near the glass-transition
temperature of DSPC (∼60 °C) through nanoporous (200 nm pore-size) polycarbonate membranes, resulting in unilamellar liposomal constructs (∼150 nm average diameter). A general schematic of fabricating the “functionally integrated” liposomes (simultaneously bearing all three peptides VBP, CBP and cRGD) is shown in Figure 5.2.

In Vitro Platelet Aggregation Studies.

For studying whether the cRGD-modified liposomal constructs pre-adhered to a surface can induce aggregation of activated platelets even from LPCs, DSPC (49 mol %), cholesterol (45 mol %), DSPE-PEG (2.5 or 5 mol %), and DSPE-PEG-Biotin (1 mol %) was combined with or without DSPEPEG-cRGD (2.5 mol %) to form cRGD-modified or unmodified biotinylated liposomal constructs. These non-fluorescent cRGD-modified or unmodified biotinylated liposomal constructs.
unmodified biotinylated liposomes were incubated with avidin-coated glass coverslips for 1 h and subsequently washed with PBS to remove any loosely bound liposomes. This produced coverslips with a stable coating of cRGD-modified or unmodified liposomal constructs, as shown in the schematic of coverslips in the left columns of Figure 5.3.

**Figure 5.3:** Schematic representation and representative fluorescence images from studies of platelet aggregation in the absence or in the presence of ADP on unmodified versus cRGD-modified biotinylated liposomes, pre-adhered as a monolayer on avidin-coated coverslips. Only cRGD-modified liposomes show enhanced arrest and aggregation of platelets (red fluorescence) on them in the presence of ADP-induced activation. For imaging, platelets were stained with P-selectin specific Alexa Fluor 647-anti-CD62P antibody and imaged using a Zeiss Axio Observer.D1 inverted fluorescence microscope fitted with a photometrics chilled CCD camera and a 63× objective. These studies were carried out in a gyratory shaker under gentle agitation (100 rpm), which results in very low (almost static) wall shear stress between 0.5 and 1.25 dyn/cm².

LPC was obtained as previously described and incubated with the construct-coated coverslips for 1 h in the absence or in the presence of platelet agonist ADP, under gentle agitation (100 rpm in gyratory shaker). Previous studies have shown that speeds of
50–100 rpm in gyratory shakers result in very low wall shear stress between 0.5 and 1.25 dyn/cm² [24]. Post-incubation, the coverslips were gently washed with PBS to remove loosely bound platelets from the construct-coated surface. Subsequently, the coverslips were stained with mouse anti-human Alexa Fluor 647-anti-CD62P (red fluorescence, λmax ≈ 570 nm), which labels P-selectin on activated platelets. These stained coverslips were mounted onto glass slides, and the fluorescence of active platelets aggregated onto the coverslips was imaged using inverted fluorescence microscopy. The working hypothesis behind these experiments was that coverslips coated with cRGD-modified liposomal constructs would induce enhanced GPIIb-IIIa-binding mediated aggregation of ADP-activated platelets compared with the controls. In the absence of cRGD-modification on liposomes (unmodified liposome coating), a percentage of ADP-activated platelets may still undergo some clustering mediated by the fibrinogen present in the plasma of LPC suspension, but these platelet clusters will aggregate only minimally on the unmodified liposome surface because bare or PEG-ylated phospholipids (liposome membrane component) are known to prevent platelet adhesion and arrest [25]. Platelet aggregation was quantified as the percentage of coverslip surface area covered by platelet fluorescence. All statistical analysis was performed using ANOVA, and significance was considered to be p < 0.05.

**In Vitro Evaluation of Functionally Integrated Liposomal Constructs.**

For developing functionally integrated liposomal constructs where the platelet-mimetic “matrix-adhesion” and “aggregation” properties are combined on a single-particle platform, DSPEPEG-VBP (1.25 mol %), DSPE-PEG-CBP (1.25 mol %), and DSPEPEG-
cRGD (2.5 mol %) were combined with DSPC (49 mol %), cholesterol (45 mol %), and DSPE-PEG-fluorescein (1 mol %). Negative control liposomal constructs did not contain any lipid–peptide conjugate in their formulations but instead contained 5 mol % DSPE-PEG. Comparison liposomal formulations contained only “adhesion” functionality (1.25% DSPE-PEG-VBP and 1.25 mol % DSPE-PEG-CMP together with 2.5 mol % DSPE-PEG, 49 mol % DSPC, 45 mol % cholesterol and 1 mol % DSPE-PEG-fluorescein) or only “aggregatory” functionality (2.5 mol % DSPE-PEG-cRGD together with 2.5 mol % DSPE-PEG, 49 mol % DSPC, 45 mol % cholesterol, and 1 mol % DSPE-PEG-fluorescein). For these experiments, glass slides were coated with adjacent circular regions of albumin (control surface with no specific adhesive interaction with any liposome formulation) and 50:50 vWF/collagen (vascular injury site mimicking protein surface with adhesive interaction with VBP- and CBP-decorated liposomes). The coated glass slides were vacuum sealed within the PPFC chamber, with the coated sides exposed to the flow (schematic shown in Figure 5.4). Platelets in LPC were pre-incubated with ADP and pre-stained with red fluorescent Alexa Fluor anti-CD62P. These LPC suspensions were allowed to flow through the PPFC along with various formulations of green fluorescent liposomes (unmodified, only adhesive peptide-modified, only aggregatory peptide-modified, or functionally integrated ones modified by all three peptides), over the coated glass slides. The flow was maintained to produce wall shear stresses of 5–55 dyn/cm2 for 30 min in a closed loop circulation. In brief, the wall shear stress \((\tau_w)\) of the PPFC can be modulated as a function of flow rate \((Q)\) by: \(\tau_w = 6 \mu Q/bh^2\), where \(\mu = \) fluid viscosity (0.015 dyn/cm2), \(b = \) width of the chamber (1.0 cm), and \(h = \) distance between plates (0.00254 cm) [26]. After 30 min, flow of just PBS was
maintained for an additional 15 min in an open loop to remove any loosely bound constructs and platelets. The working hypothesis for this experimental design was that liposomal constructs bearing all three peptides (VBP, CBP, and cRGD) will be able to adhere stably to the vWF/collagen surface under low-to-high shear flow, recruit activated platelets in flow, and promote aggregation of the activated platelets onto themselves at sites of liposome adhesion. Liposomal constructs bearing only “adhesive” peptides (only VBP and CBP) or only “aggregatory” peptide (only cRGD) will have much reduced capability of demonstrating platelet-mimetic dual functions of promoting adhesion and arrest/aggregation of active platelets from flowing LPC suspensions. The slides were imaged at various time points (5, 15, 30, and 45 min) of flow using an inverted fluorescence microscope. For each image, liposome fluorescence (green) and activated platelet fluorescence (red) intensity were quantified using Adobe Photoshop CS4 software. The colocalization of these two fluorescence colors was considered to be a
quantitative measure of liposomes adhering to the vWF/collagen surface under flow and then promoting arrest and aggregation of activated platelets onto themselves. This colocalization is qualitatively shown in pseudo-colored yellow overlay in the results. The colocalization was quantified using Axiovision software by acquiring the percentage of green pixels that also had red pixels superposed on them (at fixed pixel size) for every image and multiplying this percentage by the pixel-averaged green fluorescence intensity for that specific image. All statistical analyses were performed using ANOVA, and significance was considered at p < 0.05.

3. Results and Discussion

Platelet Aggregation on Biotinylated cRGD-Modified Liposomes Coated on Avidin Surfaces.

The last column in Figure 5.3 shows representative fluorescence images for platelet interaction with the coverslip-coated liposome formulations in the absence or in the presence of ADP. Figure 5.5 shows the corresponding quantitative data from these studies. The images and the data indicate that in the absence of ADP-induced activation, quiescent platelets hardly undergo any interaction with the liposome layer, irrespective of whether the liposomes were unmodified or cRGD-modified. This also suggests that the liposomes themselves, whether unmodified or cRGD-modified, do not activate (and hence aggregate) quiescent platelets. In contrast, upon ADP-induced activation, platelets undergo significantly enhanced interaction with the coverslip-adhered cRGD-modified liposomes, resulting in high extent of platelet aggregation. In comparison, the unmodified liposomes show only minimal aggregation of activated platelets onto themselves. This
establishes that the cRGD-modified liposomal constructs adhered onto a surface are capable of promoting recruitment and aggregation of activated platelets onto themselves.

**Figure 5.5:** Quantitative analysis of platelet aggregation studies described in Figure 2. Results show percent of surface area covered in red fluorescence (aggregation of platelets to cRGD-modified or unmodified liposome surfaces in the presence and in the absence of ADP under gentle agitation at 100 rpm in a gyratory shaker). cRGD-modified liposomes promote significant aggregation of ADP-activated platelets compared with the other conditions ($p < 0.05$).

**Evaluation of Functionally Integrated Liposomal Constructs in Promoting Arrest and Aggregation of Platelets on vWF/Collagen Surface under Flow.**

**Figure 5.6** shows a representative set of fluorescence images, and **Figure 5.7** shows the quantitative data from the PPFC studies evaluating the various liposomal constructs interacting with activated platelets while flowing over the vWF/collagen-coated or the albumin-coated surface. In the images, the green fluorescence represents adhered liposomal constructs, the red fluorescence represents arrested and aggregated active platelets, and the yellow pseudo-color represents colocalization of the green liposomes and red fluorescent platelets in the same field of view. As evident from the images in the
fifth row of Figure 5.6, the albumin surface showed hardly any adhesion of liposomes or arrest of platelets, and consequently, the quantitative values of liposome or platelet fluorescence (and colocalization) from the albumin surfaces are not included in the quantitative data in Figure 5.7. Although images were taken at six shear values between 5 and 55 dyn/cm² and at four time points between (5, 15, 30, and 45 min), representative fluorescence images are shown at only one shear value (35 dyn/cm²) and one final time point.

**Figure 5.6:** Experimental setup and resulting representative fluorescence images from studies involving unmodified and various peptide-modified liposomal constructs under flow with low concentrations of activated platelets through the PPFC over albumin-coated and vWF/collagen-coated surfaces. Green fluorescence (from DSPE-PEG-Fluorescein) represents liposomal constructs adhered onto vWF/collagen and albumin surfaces. Red fluorescence (from Alexa Fluor 647-anti-CD62P) represents activated platelets aggregated onto vWF/collagen and albumin surfaces in the same field of view as the liposome adhesion images. Yellow overlay represents colocalization of the green and red fluorescence signifying adhered liposome-promoted aggregation of activated platelets. Only images at the 45 min time point for shear stress value of 35 dyn/cm² are shown here for convenience.
point (45 min) for convenience. The quantitative data in Figure 5.7 are shown for this 45 min time point across all shear stress values studied.

From the images in Figure 5.6, it is evident that liposomes bearing only aggregation-promoting cRGD peptides are unable to undergo significant adhesion to the vWF/collagen surface and promote aggregation of activated platelets from the LPC condition onto themselves, even if they may cluster some activated platelets in flow. This is suggested by the minimal green fluorescence and minimal yellow colocalization shown in the second row of images. The red fluorescence shown in this row indicates a certain extent of platelet arrest and aggregation, which is possibly due to the direct interaction of activated platelets with the vWF/collagen surface rather than being mediated by liposomes. Constructs bearing only adhesion-promoting peptides (VBP and CBP) but no cRGD can adhere stably to vWF/collagen surface under flow but are unable to promote significant arrest and aggregation of activated platelets onto themselves from the flowing LPC suspension. This is suggested by the presence of considerable green fluorescence but minimal yellow colocalization in the first row of images. As before, the red fluorescence shown in this row indicates a certain extent of platelet arrest and aggregation due to the direct interaction of activated platelets with the vWF/collagen surface rather than being mediated by liposomes. Unmodified liposomes (no peptide modification) show insignificant adhesion to vWF/collagen surface (minimal green fluorescence) and consequently do not promote any arrest and aggregation of active platelets (minimal yellow fluorescence), as shown in the fourth row of images in Figure 5.6. As before, some platelet fluorescence (red) is seen here on the vWF/collagen surface due to direct interaction and arrest of activated platelets on this surface. In contrast with
all of these, functionally integrated liposomal constructs bearing all three peptides (VBP, CBP, and cRGD) show high extent of green fluorescence as well as red fluorescence with significant yellow overlay, suggesting colocalization of the green fluorescent liposomes and the red fluorescent platelets on the vWF/collagen surface (third row of images in Figure 5.6). This indicates the enhanced ability of these functionally integrated constructs to undergo stable adhesion to the vWF/collagen surface under flow and promote arrest and aggregation of activated platelets onto themselves, mimicking the primary hemostatic action of natural platelets. The qualitative results indicated by the fluorescence images are further validated by the quantitative data analysis for liposome fluorescence, platelet fluorescence, and colocalized fluorescence intensity shown in separate graphs in Figure 5.7. From the graphs, it is evident that the functionally integrated liposomes have significantly enhanced ability to adhere to the vWF/collagen surface and promote arrest and aggregation of activated platelets onto them (blue bars in the colocalization graph) compared with unmodified, pro-aggregatory, or matrix-adhesive liposomes. The pro-aggregatory liposomes (modified by cRGD only) seemed to cause statistically higher aggregation compared with the unmodified and the matrix-adhesive liposomes (red bars compared with the brown and green bars in the colocalization graph), but this is probably an effect of the cRGD-modified liposomes causing clustering of active platelets in free flow and some of the heavier clusters migrating down and sticking to the vWF/collagen surface. However, this effect of pro-aggregatory liposomes is still statistically lower than the action of the functionally integrated liposomal constructs. These results establish that combining the platelet-mimetic key hemostatic functionalities
adhesion-promotion and aggregation-promotion on a single particle platform can lead

Figure 5.7: Quantitative analysis of the fluorescence data showing (A) liposome adhesion (quantification of green fluorescence intensity), (B) platelet aggregation (quantification of red fluorescence intensity), and (C) colocalization of green and red fluorescence for PPFC experiments with unmodified and various peptide-modified liposomal constructs under flow with low concentrations of activated platelets through the PPFC over albumin-coated and vWF/collagen-coated surfaces. The data are shown for wall shear stress values of 5–55 dyn/cm² at the 45 min time point. The results demonstrate that the functionally integrated liposomes (surface-modified simultaneously with VBP, CBP, and cRGD) have significantly enhanced capacity to adhere to the vWF/collagen surface under flow at the various shear stress values and promote significant arrest and aggregation of active platelets onto themselves (p < 0.05).
to a more refined design of a synthetic hemostat.

In the native mechanism of platelet-mediated primary hemostasis in vascular injury, platelets initially adhere to vWF at the injury site via interaction between GPIbα of platelet surface receptor complex GPIb-IX-V. This adhesion is enhanced with increasing shear as vWF multimerizes with increasing shear, thereby allowing larger extent of GPIbα interaction. The GPIbα-vWF interaction is supplemented by additional binding interaction of platelet surface receptors GPVI and GPIa/IIa to fibrillar collagen, which secures the “rolling” vWF-adhered platelets and arrests them at the injury site. In our design, these two mechanisms of platelet adhesion are mimicked by decoration of multiple VBP and CBP copies on the liposome surface. Furthermore, in natural hemostasis, the arrested, adhered platelets get activated and act as nucleation points for recruitment and aggregation of more activated platelets via interaction between native ligand fibrinogen with the surface integrin GPIIb-IIIa on active platelets. In our design, to mimic and amplify this process, we decorated the liposome surface by multiple copies of fibrinogen-mimetic cRGD peptides, which have high affinity and selectivity to active platelet GPIIb-IIIa. The results from in vitro PPFC studies with “functionally integrated” liposomal constructs establish successful platelet-mimicry of our design. Although in our experiments the various peptides were presented on a liposome surface, they can be potentially conjugated to any other particle platform if needed. Also, in the experiments reported here, the absolute mol % of the different peptides in the various liposome formulations was kept constant. (VBP and CBP were always at 1.25 mol % each, and cRGD was always at 2.5 mol %.) For “adhesion only” liposomes (VBP and CBP modification only) and “aggregation only” liposomes (cRGD modification only), 2.5 mol
% of just DSPE-PEG was added along with the DSPE-PEG-peptide conjugates to keep the total PEG-ylation content at 5 mol%. In the “functionally integrated” liposomes, this was achieved by combining VBP and CBP at 1.25 mol % each and cRGD at 2.5 mol %, and hence no additional DSPE-PEG was incorporated in these formulations. This initial metric of peptide composition was chosen to investigate the feasibility of our platelet-mimetic design approach. Our results successfully demonstrate the platelet-mimetic bioactivity of our design as a function of the “type” of peptide incorporation. It can be expected that along with the type, the relative densities of peptide incorporation will also influence the platelet-mimetic bioactivities of such constructs. Our ongoing and future studies are focused on modulating the peptide incorporation percentages to optimize their surface decoration density for the best hemostatic performance, and this will be an area covered in future publications.

Besides transfusion of natural platelet-based products, there are a few other synthetic or recombinant hemostatic products in current clinical use for treatment of bleeding complications such as QuikClot (a zeolite that induces platelet aggregation) [27], [28] and NovoSeven (coagulation factor VII that triggers the activation of the coagulation cascade) [29], [30]. QuikClot is relevant only in topical application on open wounds and not in intravenous transfusion medicine. However many bleeding complications are internal, where hemostasis will require intravenous therapy. NovoSeven can be used intravenously but has been reported to present risks of immunogenic and thromboembolic complications [31], [32]. In addition, being a coagulation factor, it requires the presence of other factors and activated platelet membranes for effective action. Therefore, the application and efficacy of these products
in hemostasis are limited. In contrast, a synthetic particle construct that avoids biologic contamination risks and side effects, has convenient preparation methodology, and has a long storage life can be potentially administered intravenously, can efficiently mimic both adhesive and aggregatory actions of natural platelets toward primary hemostasis, and can have significant potential in transfusion medicine. Our biomimetic approach of combining the key hemostatic functions of platelets on a single particle addresses the above criteria and can therefore lead to a highly efficacious design of synthetic hemostats.

4. Conclusions

We have successfully demonstrated that liposomes heteromultivalently surface-modified with peptides mimicking platelet’s adhesion and aggregation properties can adhere to vascular-injury-relevant protein-coated surfaces and promote arrest and aggregation of active platelets onto themselves under a dynamic shear flow environment in vitro. The platelet-mimetic activity of these liposomal constructs was demonstrated with LPCs, suggesting that in normal physiological platelet concentrations these constructs can potentially have even higher activity. Optimization of this approach can lead to a highly efficient design of synthetic hemostat with application in transfusion medicine.

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6. References


Chapter 6: In Vitro and In Vivo Hemostatic Capabilities of a Functionally Integrated Platelet-mimetic Liposomal Nanoconstruct


1. Introduction

Platelet transfusions are routinely needed in the clinic to prevent bleeding-associated morbidities and mortalities in patients with hematologic and oncologic platelet disorders, chemo/radio-therapy induced myelosuppression, and trauma- or surgery-associated heavy bleeding [1–9]. These transfusions primarily use allogeneic natural platelet concentrates (PCs) suspended in autologous plasma. These PCs have a very short shelf-life (~3-5 days) due to high risk of contamination [10–16]. In recent years, several pathogen reduction technologies (PRT) have been developed (e.g. psoralen-based UV treatment) to reduce the contamination risks, but these have extended the platelet shelf-life only up to ~7 days [17–19]. Other platelet-based hemostats under investigation, e.g., frozen (-80°C) platelets, cold-stored (4°C) platelets, lyophilized platelets, platelet-derived microparticles, and infusible platelet membranes (IPM), suffer from similar issues [10], [11]. Also, since all of these products are prepared from pooled blood of multiple donors, they present high risk of blood-borne infection that can only partly be prevented by serological testing and expensive leukoreduction [14], [16], [20]. Even then, these products present risks of alloimmunization-induced refractoriness, graft-versus-host disease, and transfusion-associated immunosuppression [21–23]. To add to these issues, limited availability of blood donors causes substantial shortage in supply of platelet products [10]. Altogether these factors severely limit the therapeutic benefit of natural
platelet-based hemostats. Consequently, there is a significant clinical interest in *intravenous synthetic hemostats* that can mimic platelet’s hemostatic action while allowing large-scale preparation, minimum contamination, sterilization and long storage life, and absence of biologic side effects [10], [11], [13], [24].

Driven by this interest, several intravenous synthetic hemostat designs have been investigated, by decorating synthetic particle surfaces with molecular motifs that mimic platelet’s hemostatic *biological* mechanisms of either undergoing vascular matrix protein *adhesion* or fibrinogen (Fg)-mediated *aggregation* [25–32]. We have rationalized that platelet-mimetic synthetic designs that exhibit *only adhesion* or *only aggregation* functionality may have sub-optimal hemostatic capabilities since *both* of these functions in tandem are required for effective hemostasis. Our rationale was also supported by recent findings from one research group that co-administration of particles that render platelet-mimetic adhesion and particles that render platelet-mimetic aggregation exhibited higher hemostatic potential than either of these particles alone [33]. However, combination of both adhesion and aggregation functionalities on a single particle platform was not reported by this group. This is possibly due to the fact that for adhesion they had used large recombinant GPIbα or GPla/IIa protein fragments, while for aggregation they had used a much smaller Fg-mimetic dodecapeptide (H-12), that when combined together on a single particle, can lead to mutual steric masking because of size disparities. Our research was the first to report on a functionally integrated platelet-mimetic design where mechanistic mimicry of platelet adhesion to VWF and collagen, and that for promoting platelet aggregation were combined on a single model nanoparticle platform (~150 nm diameter liposomes) using heteromultivalent small
peptide decorations that avoided mutual steric interference (Figure 6.1). The liposomes were simultaneously surface-decorated with a von Willebrand Factor (VWF)-binding peptide (VBP), a collagen-binding peptide (CBP) and an active platelet GPIIb-IIIa-binding cyclic RGD-based fibrinogen-mimetic peptide (cRGD). The VBP and CBP decorations are to promote platelet-mimetic adhesion of the liposomes at a vascular injury site, while the cRGD decorations are to promote enhanced aggregation of available active platelets at this site for primary hemostasis [34], [35]. In these initial studies, we have demonstrated in vitro that this platelet-mimetic design undergoes enhanced adhesion and promotes aggregation of active platelets selectively at a vascular injury-relevant surface (VWF/collagen).
Building on our previous work, here we have investigated *in vitro* whether our platelet-mimetic design allows compositional modulation of the heteromultivalent ligand decorations on the construct surface, to enable optimization of platelet-mimetic adhesion and aggregation capabilities under shear flow. Furthermore, using a mouse tail transection model, we have studied *in vivo* whether the functionally integrated platelet-mimetic constructs promote enhanced hemostasis compared to constructs that bear only adhesive or only aggregatory functionality.

2. Materials and Methods

*Materials*

Phosphate-buffered saline (PBS), 3.8% w/v sodium citrate, paraformaldehyde (PFA), and avidin were purchased from Thermo Fisher Scientific (Pittsburgh, PA). Cholesterol, and collagen were purchased from Sigma Aldrich (Saint Louis, MO). Fluorescently labeled monoclonal antibody, Alexa Fluor® 647-anti-CD62P (staining active platelet P-selectin), was purchased from BioLegend (San Diego, CA). The lipids distearyl phosphatidyl choline (DSPC), distearyl phosphatidyl ethanolamine (DSPE), poly(ethylene glycol)-modified DSPE (DSPE-PEG2000), carboxy-poly(ethylene glycol)-modified DSPE (DSPE-PEG2000-COOH), biotinylated poly-(ethylene glycol)-modified DSPE (DSPE-PEG2000-Biotin), and Rhodamine B-labeled dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE-Rhodamine) were purchased from Avanti Polar Lipids (Alabaster, AL). Adenosine Diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA, USA). ClearOx and was purchased from Invitrogen (Carlsbad, CA). Human VWF (FXIII-free) was purchased from Hematologic
Technologies (Essex Junction, VT). The PPFC system (flow width = 1 cm and distance between plates = 0.00254 cm) for dynamic flow studies was purchased from Glycotech (Gaithersburg, MD). The peptide sequences used were TRYLRIHPQSWVHQI (VBP), [GPO]_{7} (CBP), and cyclo-CNPRGDY(OEt)RC (cRGD). The synthesis and characterization of these peptides have been reported previously [34], [35]. Female wildtype black C57 mice (~20-25g) were obtained from Jackson Labs (Bar Harbor, ME), and experiments were performed at ~10 weeks of age.

**VBP and CBP-decorated liposome fabrication and PPFC studies for platelet-mimetic adhesion**

The VBP and CBP peptides were conjugated via their N-termini to the carboxyl terminus of DSPE-PEG_{2000}-COOH via standard carbodiimide chemistry adapted from previously reported methods, resulting in DSPE-PEG-peptide molecules [36], [37]. These DSPE-PEG-peptide molecules were combined with DSPC, cholesterol, DSPE-PEG, and DHPE-Rhodamine, to fabricate peptide-decorated ~150 nm diameter liposomes via reverse phase evaporation and extrusion (RPEE) technique [38]. For liposomes bearing only VBP or only CBP peptides, the corresponding DSPEG-PEG-peptide concentrations were maintained at 2.5, 5 or 10 mol% of total lipid. For liposomes bearing both VBP and CBP in combination, liposomes contained 2.5 mole % each of DSPE-PEG-CBP and -VBP (1:1 ratio). For PPFC-based adhesion experiments, glass slides were coated with 50:50 VWF:collagen or albumin, vacuum sealed within the PPFC chamber and the coated surfaces exposed to the flow of liposome suspensions, producing wall shear stresses of 5–55 dyn/cm². Our experimental set-up has been described previously [34]. The
unmodified or peptide-modified liposomes were allowed to flow through the system in a recirculating loop for 30 min to allow adhesion, followed by flow of PBS for an additional 15 min in open loop to remove any loosely bound constructs and study retention of adhered constructs. The slides were imaged at various time points (5, 15, 30 and 45 min of flow) using an inverted fluorescence microscope and adhered liposome fluorescence intensity (red from Rhodamine) was recorded. For image analysis, the fluorescence intensity was analyzed using a MATLAB script and statistical comparisons were carried out between adhesion of liposomes bearing only VBP or CBP (at increasing mole%) and those bearing both VBP and CBP (2.5 mol% each). All statistical analyses were performed using ANOVA, and significance was considered at p < 0.05. To further determine the effect of varying relative peptide ratios when VBP and CBP were combined on the same construct, liposomes were fabricated with varying ratios of DSPE-PEG-CBP to DSPE-PEG-VBP (80:20, 60:40, 50:50, 40:60, and 20:80) at a fixed total peptide decoration of 5 mole%. Adhesion of these liposomes from flow on a VWF/collagen or albumin surface, was studied using a PPFC set-up similar to the previous adhesion studies. The imaging of adhered liposome fluorescence and statistical analysis of fluorescence intensity was carried out as described previously. As before, significance was considered at p < 0.05.

cRGD-decorated liposome fabrication and studies of liposome-promoted platelet aggregation

The cRGD peptide was conjugated via the N-termini to the carboxyl terminus of DSPE-PEG2000-COOH via standard carbodiimide chemistry, resulting in DSPE-PEG-cRGD
molecules. Liposomal constructs with varying mole % of DSPE-PEG-cRGD (2.5, 5, 10 mole %) were fabricated using the RPEE technique as before, but incorporating 1 mole% DSPE-PEG-Biotin. These biotinylated cRGD-decorated liposomes were pre-adhered to an avidin-coated coverslips to form liposome-covered surfaces, as described in our recent report [35]. These liposome-covered coverslips, bearing the various mole% of cRGD decorations, were incubated with ADP-activated human platelets. We have previously demonstrated that the cRGD-decoration on liposomes have only minimal interaction with inactive resting platelets and do not promote platelet aggregation [35]. Hence in the current studies, only activated platelets were used. To isolate platelets, venous blood from healthy, medication-free, adult donors was drawn in compliance with CWRU IRB-approved protocol, centrifuged (150g for 15 min) to obtain platelet-rich plasma (PRP), which was further centrifuged (2500g for 25 min) to obtain platelet-poor plasma (PPP). This PPP was then added volumetrically to PRP such that final platelet concentration was adjusted to ~20 000/μL, as monitored by a Coulter Counter. This low platelet concentration (LPC) was incubated with the liposome-covered coverslips for 1 h in the presence of ADP, under gentle agitation (100 rpm in gyratory shaker). Post-incubation, the coverslips were gently washed with PBS to remove loosely bound platelets and subsequently, aggregated active platelets were fixed with 4% PFA, and stained with mouse anti-human Alexa Fluor® 647-anti-CD62P (orange-red fluorescence, λmax ≈ 570 nm), which labels P-selectin. These stained coverslips were mounted onto glass slides, and the fluorescence of the platelets aggregated onto the coverslips was imaged using inverted fluorescence microscopy. The working principle behind these experiments was that coverslips coated with cRGD-decorated liposomes would induce aggregation of
ADP-activated platelets via binding to active platelet GPIIb-IIIa. Platelet aggregation as an effect of varying mole % of cRGD was quantified as the surface-averaged platelet fluorescence intensity. All statistical analysis was performed using ANOVA, and significance was considered to be $p < 0.05$.

*Tail vein bleeding studies in mice in vivo*

The mouse model experiments were carried out in accordance to CWRU IACUC-approved protocols. The tail transection and bleeding time measurement assay is a simple and well-established model to determine efficacy of hemostatic therapies [39], [40]. For this, mice were injected via the tail vein with 10mL/kg saline only or saline containing 30mg/kg of unmodified, ‘aggregation only’ (2.5 mol% cRGD-decorated), ‘adhesion only’ (2.5 mol% CBP- plus 2.5 mol% VBP-decorated), or functionally integrated (2.5 mole% cRGD-, 1.25 mole% VBP-, and 1.25 mole% CBP-decorated) liposome constructs. In these pilot studies, 5 mice were used per group of injection. Thirty minutes after injection of the saline or liposomal suspension, the end of the tail was completely transected at a point with tail diameter of 1.5 mm. The injured tail was immersed in 37°C saline and the time for bleeding to stop was measured.

3. Results

*Platelet-mimetic construct adhesion with varying extent of VBP decoration*

Figure 6.2 shows the representative fluorescence microscopy images at the 30 min time-point, of VBP-decorated liposomes adhering to a VWF-collagen (50:50) coated mixed surface under flow at 5, 30 and 55 dyn/cm$^2$ wall shear stress conditions in a PPFC set-up.
The first three rows of images are of liposomal constructs bearing VBP decoration at 2.5, 5 and 10 mole % of total lipid concentration, while the last row shows adherent liposomal constructs that were heteromultivalently surface-modified with 2.5 mole % VBP and 2.5 mole % CBP (hence total 5 mole % of peptide). Figure 6.2 shows the quantitative analysis of fluorescence intensity of adhered liposomes at the 30 min time-point (adhesion from flow) and the 45 min time-point (retention), for the three shear stress values. As evident from the results, VBP-decoration on the liposome surface resulted in high extent of liposome adhesion on the VWF-collagen substrate compared to unmodified liposomes, but this adhesion was not found to significantly increase with the
increasing mole % of VBP decoration at each shear value tested. Interestingly, the extent of adhesion (fluorescence intensity) was found to become almost double for all VBP-decorated liposomes at 30 dyn/cm² compared to that at 5 dyn/cm², indicating platelet-mimetic higher VWF binding at higher shear. However, further increase in VBP-decorated liposome adhesion was not observed when the shear was further increased to 55 dyn/cm². This is possibly because the amount of VWF domains available for binding of the VBP, gets already saturated by liposome binding at 30 dyn/cm² and hence significantly more binding domains do not become available at the next higher shear condition. The fluorescence intensities at the 45 min time points (retention) are similar to

**Figure 6.3:** Platelet-mimetic construct adhesion with varying extent of VBP decoration: Quantitative analyses of fluorescence intensity of adhered liposomes at the 30 min time point at 5, 30, and 55 dyn/cm² show enhanced adhesion of VBP-decorated liposomes with increasing shear stress, compared to unmodified liposomes (*p<0.001). Increasing the total mol % of ‘VBP only’ decoration did not further enhance adhesion; however, heteromultivalent liposomes (VBP plus CBP) showed significantly higher adhesion and retention to the VWF-collagen surface compared to any ‘VBP only’ decorations (**p<0.001).
The intensities at the 30 min time point (adhesion) for each of the three shear values tested, indicating that once the liposomes adhere via VBP decorations binding to the VWF, they are significantly retained and not dislodged under the flow environment. It is interesting to note that for all shear values, heteromultivalent modification of liposomes with VBP and CBP (2.5 mole % each) resulted in a significantly higher adhesion, as well as, retention of liposomes on the VWF-collagen mixed surface compared to any of the ‘VBP only’ decorations. This suggests that for such heteromultivalently decorated constructs, cumulative effects of binding to VWF and to collagen can provide significantly enhanced adhesion and retention capabilities, even with a lower amount of total mole % peptide decoration.

**Figure 6.4:** Platelet-mimetic construct adhesion with varying extent of CBP decoration: Representative fluorescence microscopy images of varying extent of unmodified, CBP-decorated (2.5, 5, and 10 mole %), and heteromultivalent (VBP plus CBP at 2.5 mol % each) liposomes adhering to a VWF-collagen substrate after 30 mins of flow at 5, 30, and 55 dyn/cm².
Figure 6.4 shows the representative fluorescence microscopy images at the 30 min time-point, of CBP-decorated liposomes adhering to a VWF/collagen coated surface under flow at 5, 30 and 55 dyn/cm² wall shear stress conditions in the PPFC set-up. Similar to the VBP-liposome data, the first three rows of images are of liposomal constructs bearing CBP decoration at 2.5, 5 and 10 mole % of total lipid concentration, while the last row shows the same image as Figure 6.3 for liposomes heteromultivalently surface-modified with VBP plus CBP (2.5 mole % each) for comparison. Figure 6.5 shows the quantitative fluorescence intensity data of adhered liposomes at the 30 min (adhesion) and the 45 min time-point (retention) for the three shear stress values. As evident from the results, CBP-decoration on the liposome surface resulted in high extent of liposome adhesion on the VWF-collagen surface compared to unmodified liposomes, but this adhesion was not found to significantly increase with the increasing mole % of CBP decoration at each of the three shear values tested. Also, the extent of adhesion (fluorescence intensity) was found to be comparable for all shear values, which is different from the VBP-decorated liposome adhesion described in the previous section. This is possibly because, for natural platelets increased adhesion to collagen under increasing shear is actually mediated by VWF multimerization on collagen and subsequent platelet GPIbα binding to VWF. Without the VWF-binding component, natural platelet-binding to collagen does not get much influenced by shear. This is further validated by the fact that, when VWF-binding capability is combined with the collagen-binding capability by heteromultivalent decoration with 2.5 mole % of each peptide, the overall adhesion of these liposomes significantly increases compared to liposomes bearing CBP alone for all shear values. In addition, from Figure 6.5 it is evident that the fluorescence intensities at the 45 min time
Figure 6.5: Platelet-mimetic construct adhesion with varying extent of CBP decoration: Quantitative analyses of fluorescence intensity of adhered liposomes at the 30 min time point at 5, 30, and 55 dyn/cm$^2$ show enhanced adhesion of CBP-decorated liposomes compared to unmodified liposomes (*p<0.001). Increasing the total mole % of ‘CBP only’ decoration did not further enhance adhesion; however, increasing CBP from 2.5 to 5 and 10 mole % resulted in enhancement of adhered construct retention (45 min time point) at high shear (55 dyn/cm$^2$) (***p<0.05). In addition, heteromultivalent liposomes (VBP plus CBP) showed significantly higher adhesion and retention to the VWF-collagen surface compared to any ‘CBP only’ decorations (**p<0.001).

Points (retention) are similar to the intensities at the 30 min time point (adhesion) for the low (5 dyn/cm$^2$) and medium (30 dyn/cm$^2$), for all mole % CBP decorations, but at higher shear (55 dyn/cm$^2$) the retention of the liposomal constructs with 2.5 mole % CBP is significantly lower than liposomes bearing higher (5 or 10 mole %) amount of CBP. In comparison, at this high shear value, the adhesion of liposomes bearing 2.5 mole % VBP was much higher. This is possibly because at high shear, the binding of VBP to VWF is reasonably high even for low mole % VBP decorations but a higher amount of CBP is needed to stay retained and avoid dislodgement. Once again, when the VWF-binding and
the collagen-binding capabilities were combined on the liposomal construct by simultaneous decoration with VBP plus CBP, the retention of the liposomes at all shear values significantly increased compared to liposomes bearing CBP only. As before, this strengthens the rationale that cumulative effects of VBP binding to VWF and CBP binding to collagen can provide significantly enhanced adhesion and retention even with a lower total mole % peptide decoration.

Construct adhesion for varying VBP: CBP ratios at fixed total peptide decoration

As the results in the previous sections indicated that liposomes decorated simultaneously with VBP and CBP have higher adhesion and retention capabilities compared to liposomes bearing VBP only or CBP only, further PPFC experiments were carried out where the VBP:CBP ratio was varied keeping the total peptide decoration at 5 mole %, to determine whether modulation of VBP:CBP ratio results in variation of liposome adhesion and retention at the various shear values. Figure 6.6 shows representative fluorescent images for adhesion (30 min time point) and retention (45 min time point) of liposomes to VWF/collagen surface, for the varying ratios of VBP:CBP at total peptide decoration of 5 mole %. Figure 6.7 shows the corresponding quantitative data at the three shear values. As evident from the results, after 30 minutes of flow under 5 dyn/cm\(^2\) shear, liposomes bearing more CBP (80:20 and 60:40 CBP:VBP) showed significantly more adhesion compared to liposomes that have equal ratio (50:50) or more VBP (40:60, and 20:80 CBP:VBP). After an additional 15 minutes of PBS flow through the system (45 minute time point), the liposomes modified with more CBP showed enhanced retention compared to the liposomes decorated with equal ratio or more VBP. These results suggest
that at low shear conditions, collagen-binding via CBP provides a more predominant mechanism of adhesion and retention of our platelet-mimetic constructs, compared to VWF-binding via VBP. When the shear was increased to 30 dyne/cm$^2$, liposomes with close to equal ratios of CBP:VBP (40:60, 50:50, 60:40) showed significantly more adhesion and retention compared to liposomes with 80% of either the CBP or VBP. When the shear was further increased to 55 dyn/cm$^2$, liposomes bearing higher VBP decorations (20:80, 40:60 CBP:VBP) or equal ratio (50:50) showed significantly higher adhesion and retention compared liposomes with low amounts of VBP (80:20 CBP:VBP).

These results indicate that our heteromultivalent constructs provide a way to fine-tune the mimicry of platelet’s adhesion mechanism at low-to-high shear values, by modulating the synergistic binding mechanisms to VWF and collagen.

**Figure 6.6:** Construct adhesion for varying VBP: CBP ratios at fixed total peptide decoration: (A) Representative fluorescent images for adhesion (30 min time point) and retention (45 min time point) of liposomes to VWF-collagen surface, for the varying ratios of VBP:CBP at total peptide decoration of 5 mol %.
Figure 6.7: Construct adhesion for varying VBP: CBP ratios at fixed total peptide decoration: Quantitative analysis of fluorescence intensity of adhered liposomes show that under 5 dyn/cm² shear, liposomes bearing more CBP (80:20 and 60:40 CBP:VBP) showed significantly more adhesion and shear retention compared to liposomes that have equal ratio (50:50) or more VBP (40:60, and 20:80 CBP:VBP) (*p<0.05); Quantitative analysis of fluorescence intensity of adhered liposomes show that under 30 dyn/cm² shear, liposomes with close to equal ratios of CBP:VBP (40:60, 50:50, 60:40) showed significantly more adhesion and retention compared to liposomes with predominantly higher (80%) amount of either CBP or VBP (*p<0.05); Quantitative analysis of fluorescence intensity of adhered liposomes show that under 55 dyn/cm² shear, liposomes bearing higher VBP decorations (20:80, 40:60 CBP:VBP) or equal ratio (50:50) showed significantly higher adhesion and retention compared liposomes with low amounts of VBP (80:20 CBP:VBP) (*p<0.05).

Construct-promoted platelet aggregation with varying extent of cRGD decoration

As mentioned previously, we have recently demonstrated the ability of 2.5 mole% cRGD decoration on liposomes to promote significant aggregation of active platelets, with minimal effect on resting platelets [35]. Building on this, in the current study we aimed to determine whether increasing the cRGD decoration mole % had an increasing effect on platelet aggregation. Figure 6.8 A shows representative fluorescent images of platelets aggregated onto the liposome-coated coverslips, while Figure 6.8 B shows the
quantitative analysis of the aggregated platelet fluorescence. As evident from the results, without cRGD modification there was only minimal aggregation of active platelets on the liposomes. With cRGD modification there was a significant increase in aggregation of active platelets (*p<0.0001) onto the liposome-coated coverslip surface. When the cRGD decoration was increased from 2.5 mole % to 5 mole % or from 5 mole % to 10 mole %, there was no statistical difference in extent of liposome-promoted platelet aggregation. Interestingly, a statistical increase was observed between samples with 2.5 mole % cRGD versus those with 10 mole % cRGD (**p<0.05).

Hemostatic effect of constructs in mouse tail bleeding model
In our previously reported *in vitro* studies, liposomal constructs decorated simultaneously with VBP, CBP and cRGD, were found to be capable of promoting active platelet aggregation selectively at the sites of liposome adhesion [35]. In the current studies, we have investigated the capability of this functionally integrated platelet-mimetic design to promote hemostasis *in vivo* in a tail transection bleeding model in mice (Figure 6.9 A).

**Figure 6.9:** Hemostatic effect of constructs in mouse tail transection bleeding model: (A) Experimental schematic of the tail transection bleeding time assay. (B) Mice tail bleeding time post-transection show that pre-injection of ‘only adhesive’ or ‘only aggregation-promoting’ liposomes resulted in approximately ~50% decrease in the time for bleeding to stop compared to injection of saline or unmodified liposomes, indicating enhancement of hemostasis (*p<0.002*). Pre-injection with the functionally integrated liposomal constructs resulted in ~70% reduction of bleeding time compared to saline or unmodified liposome injection, which was significant improvement over liposomes bearing adhesion function or aggregation function only (**p<0.05**).
Figure 6.9 B shows the results from our experiments where mouse tail bleeding time post-transection was monitored with pre-injection (30 min earlier) of saline, unmodified liposome suspension, ‘only adhesive’ liposomes, ‘only aggregation-promoting’ liposomes or functionally integrated liposomes (n=5 for each group). Without any injection, the normal time for the transected tail bleeding to stop (hemostasis) in the mice was found to be ~100-120 seconds, which is in accordance with other groups [40]. Pre-injection of saline or of unmodified liposome suspension slightly increased bleeding time to ~160 sec due to dilution of the blood (hence of platelets) by the injection volume. Pre-injection of ‘only adhesive’ or ‘only aggregation-promoting’ liposomes resulted in approximately ~50% decrease in the time for bleeding to stop compared to injection of saline or unmodified liposomes, indicating enhancement of hemostasis. These results are comparable to other platelet-mimetic synthetic construct designs reported by various researchers where only one functionality (mostly aggregation) was mimicked [30], [41]. Pre-injection with the functionally integrated liposomal constructs resulted in further significant decrease of bleeding time to ~50 sec, which is almost a 70% reduction from bleeding time compared to saline or unmodified liposome injection. This indicates that integrating the platelet-mimetic adhesion functionalities and the aggregation-promoting functionalities on a single nanoconstruct platform may lead to a synthetic intravenous hemostat design with significantly higher therapeutic efficacy in vivo, compared to approaches that use ‘adhesion only’ or ‘aggregation only’ designs.

4. Discussion
The clinical interest in intravenous synthetic hemostats is significant because of potential applications in treatment of trauma-related bleeding, pre-surgical settings and congenital
or induced bleeding disorders. To this end, our functionally integrated platelet-mimetic design has shown higher hemostatic capabilities in vitro and in vivo, compared to designs that only mimic adhesion or only amplify active platelet aggregation.

The VBP that we have used is derived from the C2 domain (residues 2303–2332) of the coagulation factor FVIII, which forms a complex with VWF prior to thrombin- or factor Xa-catalyzed activation in the coagulation cascade [42]. The mature monomeric VWF protein is a ~250 kDa molecule containing domains arranged in the order D’-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK, and the FVIII binding domain is in the D’-D3 region [43]. Each VWF monomer can therefore bind only one FVIII, and self-association and multimerization of VWF can lead to multiple binding sites in large molecules. However, in physiological circulation, the multimeric VWF is mostly in globular conformation with many of its binding domains unexposed [44]. This is possibly why approximately one FVIII is bound per 60–130 VWF monomers [45]. Upon binding to collagen via its A3 domain (e.g. at a vascular injury site) or under high shear flow (e.g. vascular injury or thrombosis), the globular multimeric VWF molecules can undergo rapid conformational changes to unravel and expose various binding domains [44], [46]. In fact, this is a primary mechanism by which the A1 domain is exposed for platelet GPIbα binding and the A2 domain is exposed for ADAMTS13-induced cleavage. We postulated that such unraveling of VWF will also expose multiple unused D’-D3 domains that can then be amenable to the binding of the FVIII-derived VBPs decorated on synthetic particles. This would effectively allow platelet-mimetic enhanced binding of VBP-decorated particles with increasing shear and at injury site. Our previous and current results validate our design rationale [34].
The CBP that we have used is based on previous reports of the Glycine-Proline-Hydroxyproline (GPO) tripeptide sequence that has helicogenic affinity to fibrillar collagen. It has been shown previously that short GPO-trimer repeats as high as a 30-mer (10 repeats) are unable to effectively activate platelets and only partially interact with platelet GPIa/IIa and GPVI integrins; yet they can effectively bind to fibrillar collagen [47], [48]. Several studies have shown that to actually activate platelets, long multimeric triple-helical forms of collagen is necessary, the chances of which is minimal for short GPO repeats [49]. Hence, we postulated that a short GPO tripeptide chain can be utilized to decorate a liposome surface for promoting adhesion to VWF/collagen surface. For our studies we selected a 7-repeat unit, i.e. \((GPO)_7\), that has a melting temperature \((T_m)\) of ~37°C, and hence has enhanced degree of freedom at physiological temperature for helicogenic interaction with fibrillar collagen [47], [48]. Our previous and current results validate our design rationale. Our studies further established that combining VWF-binding (via VBP decoration) and collagen-binding (via CBP decoration) properties on the liposomal platform significantly enhances the adhesion and retention capabilities on a VWF/collagen surface, with a lower amount of total ligand decoration. In addition, it also allows modulation of CBP:VBP ratios to fine-tune adhesion capabilities at low-to-medium-to-high shear conditions. These findings can help customize the design for therapeutic applications in high flow (e.g. acute bleeding injury) and low-to-moderate flow (e.g. chronic thrombocytopenic bleeding disorders) conditions.

Integrating the platelet-mimetic adhesion functionalities with an active platelet aggregation promoting function (via cRGD decoration) on the liposomal platform enabled a synthetic hemostat design that can render enhanced aggregation of active
platelets selectively at the sites of liposome adhesion. In this context, varying the extent of cRGD decoration may provide a way to modulate the extent of platelet aggregation, as demonstrated in our results. It is also important to note that the cRGD used in our design has a much higher selectivity to active platelet GPIIb-IIIa compared to other ubiquitous RGD motifs previously reported for synthetic platelet applications [30], [50]. This can lead to increased functional selectivity and efficacy in vivo.

In the feasibility studies carried out in mouse tail transection bleeding model, the functionally integrated liposomal constructs showed higher hemostatic efficacy compared to constructs bearing only the adhesion functionality or the aggregation functionality. It is interesting to note that in our previous in vitro studies, liposomes bearing only adhesion functionality (VBP plus CBP- decorated) were found to undergo high adhesion to a VWF-collagen surface but did not promote much aggregation of platelets onto themselves [35]. However, in the in vivo bleeding model, these liposomes seemed to promote hemostasis (hence platelet aggregation). We think this may be because of the possibility that the VBP-CBP-decorated liposomal constructs can adhere to the vascular injury site and can then promote further binding and multimerization of soluble mouse VWF to the available exofacial VBP decorations on the liposome, which in turn can then promote binding of mouse platelets at the site (Figure 6.10 A). Our rationale is based on reports that human FVIII has comparable affinity to both human and mouse VWF and the VBP is derived from human FVIII [51]. On the other hand, the enhanced hemostasis caused by the cRGD-decorated liposomal constructs is possibly more due to the clustering of active platelets onto the liposomes via cRGD-to-GPIIb-IIIa interactions and attachment of these clusters at the injury site via direct adhesion of the platelets (Figure
Compared to these two possible mechanisms, the functionally integrated constructs show significantly higher hemostatic efficacy possibly because of their capability of undergoing enhanced adhesion at the injury site and then promoting enhanced platelet recruitment and aggregation at that site by both cRGD and VBP (Figure 6.10 C). Further in vivo studies in appropriate bleeding models (e.g. VWF-knockout or GPIIb-IIIa-knockout models) are needed to corroborate these mechanistic possibilities.

Successful use of liposomes as a model platform in our functionally integrated platelet-mimetic design opens up additional possibilities of incorporating site-selective drug delivery parameters along with the engineered hemostatic functionalities. Such possibilities may provide unique strategies to not only promote, but also regulate, hemostatic activities. For example, controlled release of platelet agonists or coagulation factors, like ADP or thrombin, may be possible from such synthetic platelet-mimetic constructs, as recently demonstrated by Okamura et al [33], [52]. Additionally, the
membrane composition of the liposome itself can be engineered to regulate hemostasis. For example, on activated platelets, negatively-charged phosphatidylserine (PS) components expressed on the plasma-exposed exofacial membrane surface is known to enhance FXa-mediated conversion of prothrombin to thrombin [53]. Following this mechanistic rationale, PS-rich liposomes can be engineered to modulate coagulatory capabilities, as demonstrated recently [54]. Furthermore, surface decoration with VBP, CBP and cRGD can be adapted to other particle platforms that may allow modulation of drug release kinetics or promotion of platelet-mimetic hemodynamic transport and margination properties, as discussed in our recent review article [24]. These approaches can lead to more efficient designs of platelet substitutes.

5. Conclusion

We have demonstrated that by heteromultivalent decoration of a liposomal surface with VWF-binding, collagen-binding and active platelet GPIIb-IIIa-binding peptide ligands, a functionally integrated platelet-mimetic construct can be engineered. Our design allowed modulation of relative peptide decoration ratios to control platelet-mimetic adhesion and aggregation promotion properties. Furthermore, the integration of the adhesive and the aggregatory properties on a single liposomal platform resulted in an intravenous construct that showed higher hemostatic efficacy in vivo compared to constructs bearing adhesive property only or aggregatory property only. Further optimization and in vivo evaluation of our constructs in appropriate bleeding models can lead to a superior synthetic intravenous hemostat.
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7. References


animal models of antibody-induced hemophilia and von Willebrand disease.,”


Chapter 7: An FVIII-derived Peptide Enables VWF-binding of an Artificial Platelet Nanoconstruct without Interfering with Natural Platelet Adhesion to VWF

Based on:

1. Introduction

Von Willebrand Factor (VWF) is a major protein that mediates physiological (hemostasis) as well as pathological (thrombosis) adhesion of platelets in vascular injury [1–3]. VWF is secreted from the Weibel-Palade bodies of injured endothelial cells and alpha(α)-granules of activated platelets [4]. Each monomeric subunit of VWF consists of several domains with specific bioactivity, e.g. the A1 domain mediates binding to platelet glycoprotein GPIbα component, the A3 domain mediates binding to sub-endothelial collagen, the C1-C2 domain mediates binding to fibrinogen (Fg) and to integrin GPIIb-IIIa on activated platelets, the D’-D3 domain acts as a carrier for coagulation factor FVIII before thrombin-induced activation, and the A2 domain undergoes cleavage via metalloprotease ADAMTS-13 enzyme for regulation of VWF multimer size [5], [6]. The multi-domain VWF monomeric subunits can multimerize via disulphide bonds, and this multimeric VWF circulates as a globular protein [7], [8]. However, at a vascular injury site due to increasing hemodynamic shear, the globular VWF multimers can unravel and further self-associate to enhance the VWF availability for bioactive functions (Figure 7.1) [9].
The primary mechanism of platelet adhesion at a vascular injury site is the tethering of platelet GPIbα to VWF’s exposed A1 domains. Therefore, simulating this functional aspect is a critical component of our research to design an artificial platelet analog. The clinical interest for artificial platelet analogs stems from the issues that natural platelet-based products pose, e.g. shortage in supply, very short shelf-life (3-5 days) due to high

**Figure 7.1:** (A) Schematic of normal vascular endothelium and subsequent endothelial injury leading to VWF secretion, its shear-induced conformational change and multimerization on subendothelial collagen, and platelet adhesion, activation and aggregation on VWF/collagen matrix; (B) Schematic showing shear-induced conformational unraveling of VWF multimers leading to self-association along with atomic force microscopy (AFM) images of (i) globular and (ii) stretched VWF (adapted with permission from Marchant RE et al., *Current Protein and Peptide Science* 2002; 3: 249-74); (C) A closer schematic look at the various domains of VWF with specific bioactive functions.
risk of pathologic contamination, storage lesions and a variety of biological side effects [10]. An effective approach to design artificial platelet analogs is to decorate the surface of biocompatible intravenously-administrable particles with motifs that render platelet-mimetic hemostatic functions. To this end, we have focused on mimicking platelet’s key primary hemostatic actions of injury site-selective adhesion and site-selective amplification of platelet aggregation and have combined them on a single synthetic platform. For this, we have utilized self-assembly of lipid-peptide bioconjugates to form unilamellar liposomal constructs (~150nm in diameter) that are heteromultivalently decorated with VWF-binding peptides (VBP), collagen-binding peptides (CBP) and active platelet glycoprotein GPIIb-IIIa binding fibrinogen-mimetic peptides (cRGD). Our design rationale is that the VBP and CBP will promote injury site-selective adhesion of the constructs via VWF- and collagen-binding, while the cRGD will promote site-directed aggregation of active platelets onto the adhered constructs to amplify primary hemostasis. We have recently demonstrated the platelet-mimetic abilities of our constructs at the cellular scale [11–13].

Building on these studies, we now focus on establishing a molecular scale mechanistic model of the platelet-mimetic functions exhibited by the nanoconstructs. As the first component of this mechanistic investigation, here we report on our analysis of how surface decoration with VBP enables the binding of the constructs on VWF. In this context, we rationalized that an artificial platelet design for VWF-binding should not interfere with the binding of available natural platelets to the same VWF. Hence, the mechanisms of nanoparticle binding to VWF should be different from that of natural platelets binding to VWF. To achieve this exclusivity at the molecular scale, we have
focused on the FVIII-binding D’-D3 domain of VWF and have utilized a FVIII-derived VBP that has moderately high affinity (IC\textsubscript{50} ~ 9 μM) to this domain [14], [15]. It is also reported that although each VWF monomeric subunit contains one D’-D3 domain that can theoretically bind one FVIII molecule, physiologically VWF contains ‘bound FVIII’ in ~ 1:50 (FVIII:VWF) ratio [16]. Hence we rationalized that physiological VWF will have sufficient ‘unoccupied’ D’-D3 domains that can enable adhesion of VBP-decorated nanoparticles under flow, while available natural platelets can still, in parallel, bind to the A1 domain of VWF via GPIbα without mutual interference. Also, since the VBP we used does not include the thrombin-binding Arg\textsuperscript{1689} region but rather residues 2303–2332 of the C2 domain of FVIII [14], [15], we further rationalized that the binding of VBP to VWF will not be thrombin-cleavable. Furthermore, we hypothesized that co-decoration of the VBP-decorated nanoconstructs with cRGD motifs will potentially enable the constructs to recruit and amplify the aggregation of locally activated platelets and thereby...

**Figure 7.2:** A schematic of the envisioned mechanism of action of the VBP-cRGD-co-decorated liposomal constructs interacting with VWF and platelets to enhance the primary hemostatic processes of platelet recruitment and aggregation. In the schematic, ‘Fg’ stands for Fibrinogen. The VWF-binding peptide (VBP) peptide is the sequence TRYLRHIPQSWHVQI and the Fibrinogen-mimetic peptide (cRGD) containing the Arginine-Glycine-Aspartic Acid (RGD) sequence is cyclo-CNPRGDY(OEt)RC.
cumulatively enhance primary hemostasis, as envisioned in Figure 7.2. Here we report on our experimental studies of our rationale and hypotheses at cellular and molecular scale in vitro.

2. Experimental Procedure

Materials

Phosphate Buffered Saline (PBS), glass coverslips and microscope slides, 3.8% w/v sodium citrate, paraformaldehyde (PFA), and bovine serum albumin (BSA) were obtained from Thermo Fisher Scientific (Waltham, MA). FVIII free and physiologic human Von Willebrand Factor (VWF) and alpha(α) thrombin were obtained from Hematologic Technologies (Essex Junction, VT). Glycocalicin was purchased from USCN Life Science (Wuhan, China). Calcein was purchased from Invitrogen (Carlsbad, CA) and Ristocetin from Helena Laboratories (Beaumont, TX). For liposomal construct fabrication, cholesterol was purchased from Sigma Aldrich (Saint Louis, MO). The lipids diestearyl phosphatidyl choline (DSPC), poly(ethylene glycol)-modified DSPE (DSPE-PEG2000), carboxypoly(ethylene glycol)-modified DSPE (DSPE-PEG2000-COOH), and Rhodamine B-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE-Rhodamine) were purchased from Avanti Polar Lipids (Alabaster, AL). Adenosine Diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA). The peptides TRYLRIHPQSWVHQI (VBP) and cyclo-CNPRGDY(OEt)RC (eRGD) were synthesized and characterized as reported in our previous publications [11], [12].

Preparation of surfaces, VBP-decorated constructs and platelets for binding experiments
The platelet-adhesive role of VWF is physiologically and pathologically facilitated by shear-induced ‘conformational unraveling’ of globular VWF under blood flow environment to expose the GPIbα-binding A1 domain (Figure 7.1). It has been reported that such platelet-adhesive conformational changes in VWF can also be achieved in static incubation conditions by treating VWF with the antibiotic ristocetin [17], [18]. Hence in our experiments, we have used ristocetin-treated VWF adsorbed on a glass coverslip surface as our ‘test’ substrate, versus untreated VWF as control. Additionally, we have used BSA-coated coverslips as a second control surface, since albumin is known to have no specific adhesive activity towards platelets or VBP [19–21]. For blocking ristocetin-treated VWF’s exposed A1 domain, we used glycocalicin, which is the carbohydrate-rich extramembranous portion of platelet GPIbα [22], [23]. Since it is the GPIbα that undergoes binding to VWF’s A1 domain, we rationalized that pre-treatment with soluble excess glycocalicin will effectively inhibit this specific binding to the A1 domain while still allowing bioactivity of the other domains of VWF. Our rationale is strengthened by reports of using glycocalicin as a viable substitute for recombinant GPIbα in the VWF:Ristocetin cofactor assay [24]. Exposure of platelets and VBP-decorated constructs to VWF without glycocalicin pre-treatment were used as control conditions. For all platelet-based experiments, platelets were isolated using serial centrifugation from human whole blood collected via venipuncture from Aspirin-refraining healthy donors using institution-approved protocols. The platelets were stained with calcein (λ_{ex}=495nm, λ_{em}=515nm, green fluorescence) to enable imaging of their binding to experimental surfaces. For all construct-based experiments, VBP-decorated as well as VBP-cRGD-co-decorated lipid-peptide nanoconstructs were formed using reverse-phase evaporation and
extrusion, resulting in ~150 nm diameter (confirmed by dynamic light scattering) vesicles bearing 5 mol% VBP (for single decoration) or 2.5 mol% of VBP and cRGD each (for co-decoration). To enable imaging of constructs, DHPE-Rhodamine ($\lambda_{ex}=540\text{nm}, \lambda_{em}=625\text{nm}$, red fluorescence) was incorporated in the construct membrane at 1 mol% during fabrication.

**Binding studies with platelets on VWF surface**

FVIII-free VWF (10μg/ml in 1x PBS pH 7.4) was adsorbed on glass coverslips by incubating overnight at 4°C. The VWF-adsorbed surfaces were exposed to incubation with calcein-stained (green) platelet suspension ($2*10^6$ platelets/μl in 1%BSA/1xPBS, pH 7.4) in the presence of ristocetin (1mg/ml in 1x PBS, pH 7.4) for 1 hr at room temperature. In control experiments, similar VWF-adsorbed surfaces were first exposed to incubation with soluble glycocalicin (1μg/ml in 1x PBS, pH 7.4) in the presence of ristocetin (1mg/ml in 1x PBS, pH 7.4) for 1 hr at room temperature, the surfaces were then gently washed with PBS and subsequently incubated with platelet suspension. In additional control experiments, the VWF-adsorbed surfaces were not subjected to ristocetin treatment but exposed to incubation with platelet suspension with or without prior exposure to glycocalicin. In another control experiment, BSA-coated coverslip surfaces were exposed to ristocetin followed by incubation with the platelet suspension. In all these experiments, the platelets were not deliberately activated with ADP, since the tethering interactions between platelet GPIbα and VWF A1 domain can occur for ‘resting’ platelets marginating to a vascular injury site [25], [26]. The coverslips were mounted onto glass microscope slides, imaged by a Zeiss Axio Observer.D1 inverted fluorescence microscope fitted with a photometrics chilled CCD camera and the degree
of platelet binding was quantified by surface averaged intensity analysis for calcein (green) fluorescence.

**Binding studies with VBP-decorated constructs on VWF surface**

The VBP-decorated constructs, at a concentration of $2 \times 10^6$ particles/μl in 1x PBS, pH 7.4, were incubated with FVIII-free VWF-adsorbed surfaces with and without ristocetin treatment. Also, in comparison studies, the construct incubation with the ristocetin-treated VWF surface was carried out after exposing the surface to glycocalicin pre-incubation. In control experiments, VBP-decorated constructs were incubated with BSA-coated surfaces and undecorated constructs were incubated with VWF-adsorbed surfaces in presence of ristocetin. The VBP we used is derived from the C2 domain (residues 2303−2332) of FVIII [14], [15]. Physiologically, VWF-bound FVIII is cleaved by thrombin at the Arg$^{372}$ and Arg$^{740}$ in the heavy chain A1-A2 domains and at the Arg$^{1689}$ position of light chain (C2-containing) domain of FVIII, to release the A3-C1-C2 based activated FVIIIa fragment [27–30]. Based on these reports, we rationalized that the VBP-mediated binding of constructs to VWF should remain unaffected by thrombin. To test this, VBP-decorated Rhodamine-labeled (red) constructs were allowed to adhere to ristocetin-treated VWF-adsorbed surfaces, and then the surfaces were exposed to incubation with thrombin (0.067μg/ml in 1x PBS, pH 7.4) for 1 hr at room temperature. Fluorescence from VWF-bound constructs was imaged prior to and after thrombin exposure and quantified by intensity analysis. In a separate group of experiments, all the above studies with VBP-decorated constructs were carried out using surfaces adsorbed with physiological VWF instead of FVIII-free VWF to test our hypothesis component that physiological VWF is capable of binding the VBP-decorated constructs. As before,
the construct-binding was imaged by fluorescence microscopy and quantified by intensity analysis.

*Studies involving simultaneous binding of constructs and platelets on VWF*

For these experiments, the VWF-adsorbed surfaces were treated with ristocetin and then exposed to incubation with calcein-stained (green) platelets and rhodamine-labeled (red) VBP-decorated constructs, simultaneously. For control conditions, the VWF-adsorbed ristocetin-treated surfaces were incubated with platelets and unmodified (no VBP decoration) constructs, simultaneously. A further component of our hypothesis was to study whether co-decoration of VBP-decorated constructs with fibrinogen-mimetic peptides (cRGDs) enables the VWF-adhered constructs to promote amplified aggregation of active platelets via platelet GPIIb-IIIa interaction with the cRGDs (envisioned in Figure 7.2). We have previously demonstrated that cRGD-decorated biotinylated constructs pre-adhered on avidin-coated surfaces can promote enhanced aggregation of active platelets, while without cRGD-decoration or without platelet activation such aggregation was minimal [12]. Building on these prior studies, constructs co-decorated with VBP and cRGD peptides (VBP-cRGD-liposomes) were incubated simultaneously with platelets on VWF-adsorbed ristocetin-treated surfaces. We rationalized that if the constructs undergo VBP-mediated adhesion on the VWF surfaces and, in effect, promote cRGD-mediated enhanced aggregation of active platelets onto them, then this will be exhibited by the significant overlap between the construct fluorescence (red) and platelet fluorescence (green). Comparison with experimental results from incubation of platelets with constructs that bear VBP-decoration only but not cRGD co-decoration was regarded as a control. Also for comparison purposes, in one group of these experiments the
platelets were not activated by any external addition of agonists, while in another group the platelets were pre-activated by agonist (ADP) treatment. The rationale for such experimental design was that, for the ‘predominantly unactivated platelet’ group some of the platelets will effectively bind naturally to VWF’s A1 domain, and this binding will activate these platelets resulting in secretion of agonists (e.g. ADP) to activate more platelets locally. These locally activated platelets can then undergo direct binding to VWF’s C1-C2 domain as well as undergo cRGD-mediated enhanced aggregation onto the VWF-adhered constructs. In comparison, for the ‘significantly pre-activated platelet’ group the pre-existing population of activated platelets can possibly undergo amplified aggregation promoted by the VBP-cRGD-constructs. Aggregation of pre-activated platelets on VWF in presence of ristocetin but with unmodified constructs (no VBP and cRGD decoration) was also included for comparison in this group. As before, the binding of constructs and platelets were imaged and the overall platelet recruitment/aggregation (green fluorescence) was quantified by fluorescence intensity analysis.

Statistical Analysis

All fluorescence data of construct binding and platelet adhesion were quantified as surface-averaged fluorescence intensity. Student’s t-test was used to analyze the difference between two means. All other statistical analyses between multiple groups were performed using one-way ANOVA with Tukey method. In all analyses, significance was considered to be $p < 0.05$.

3. Results

Binding of platelets on VWF surfaces
**Figure 7.3** shows representative fluorescence images as well as the quantitative data for binding of calcein-stained (green) platelets to FVIII-free VWF in presence or absence of ristocetin treatment and additional presence or absence of glycocalicin pre-incubation. As evident from the results, ristocetin treatment led to a significant increase in platelet adhesion to VWF compared to conditions without ristocetin. However, this adhesion was significantly reduced when glycocalicin pre-incubation was used on the ristocetin-treated VWF-surface prior to platelet incubation. In fact, this reduced adhesion was found to be statistically similar as that of platelet adhesion to VWF without ristocetin or platelet
adhesion to the control BSA surface. These results suggest that incubation with glycocalicin was effective in specifically blocking the A1-domain of VWF to cause significant reduction of platelet adhesion.

**Binding of VBP-decorated liposomal constructs on VWF surfaces**

**Figure 7.4** shows representative fluorescence micrographs as well as the quantitative data for the binding of VBP-decorated constructs to FVIII-free VWF in presence or absence of ristocetin treatment and additional presence or absence of glycocalicin pre-incubation.

![Figure 7.4: Representative fluorescence microscopy images (scale bar 100 μm) and quantitative fluorescence intensity data of interaction of calcein-stained (green) platelets to glass coverslip-adsorbed FVIII-free VWF in presence or absence of ristocetin (Risto) treatment with additional presence or absence of glycocalicin (Glyco) pre-incubation. Platelets were found to significantly bind to Risto treated VWF compared to binding in absence of Risto; the platelet-binding to Risto-treated VWF was significantly reduced by pre-incubation with Glyco (p < 0.002) and this reduction was comparable to low platelet-binding on negative control BSA surface.](image)
As evident from the results, the presence or absence of glycocalicin pre-incubation had no drastic effects on the binding of VBP-decorated constructs (red) on VWF. The binding of the constructs was only slightly lowered (statistically not significant) if the VWF-adsorbed surfaces were not first treated with ristocetin. In contrast, the binding of the VBP-decorated constructs was significantly reduced on the negative control BSA surface, and this reduced level was similar to that of undecorated constructs on VWF-adsorbed surface. Comparison of results in Figure 7.4 and Figure 7.3 demonstrate that glycocalicin can significantly reduce platelet-adhesion to ristocetin-treated VWF but does not affect the binding of VBP-decorated constructs to VWF under the same conditions. This suggests that the VBP possibly interacts with a VWF domain that is different from the platelet GPIbα-binding A1 domain.

From additional experiments, Figure 7.5A compares fluorescence intensity results of VWF surface-adhered VBP-decorated constructs prior to and after exposure to thrombin.

Figure 7.5: Representative fluorescence microscopy images (scale bar 100 μm) and quantitative fluorescence intensity data of interaction of calcein-stained (green) platelets to glass coverslip-adsorbed FVIII-free VWF in presence or absence of ristocetin (Risto) treatment with additional presence or absence of glycocalicin (Glyco) pre-incubation. Platelets were found to significantly bind to Risto treated VWF compared to binding in absence of Risto; the platelet-binding to Risto-treated VWF was significantly reduced by pre-incubation with Glyco (p < 0.002) and this reduction was comparable to low platelet-binding on negative control BSA surface.
As evident from the results, the fluorescence intensity before and after thrombin-exposure remains statistically unchanged, suggesting that VBP binding (hence construct binding) to the VWF was not cleaved by thrombin. This is important to ensure that the construct adhesion is stable under the locally relevant presence of thrombin. From further experiments, Figure 7.5B shows the fluorescence intensity results for VBP-decorated constructs binding to FVIII-free VWF compared to physiologic VWF adsorbed on glass coverslips in presence of ristocetin. As evident from the results, there was no statistical difference in construct binding between the two VWF scenarios, suggesting that VBP-decorated constructs will remain capable of effectively binding to physiological VWF in vivo.

**Simultaneous binding of peptide-decorated constructs and platelets on VWF surfaces**

Figure 7.6 A1-to-D3 show representative fluorescent images from these studies along with schematic depictions of envisioned interactive mechanisms of the red liposomal constructs and green platelets on VWF. Figure 7.6E shows quantitative fluorescence intensity data of platelet fluorescence (calcein green fluorescence) as a measure of overall platelet recruitment and aggregation on the VWF surface when co-incubated with the various test and control liposomal constructs. The co-localization of red constructs and green platelets are shown in pseudocolor yellow (in Figure 7.6 A3, B3, C3 and D3). The control condition included constructs without any peptide decoration (unmodified). As evident from the results, without VBP-decoration, the liposomal constructs could not substantially bind to the VWF surface (minimum red fluorescence in A1), but the platelets could themselves naturally bind to the VWF surface (green fluorescence in A2). This resulted in minimum co-localization of red and green fluorescence (yellow in A3).
Figure 7.6: (A1-D3) Representative fluorescence microscopy images (along with envisioned mechanistic schema) of peptide-decorated rhodamine-labeled (red) constructs and calcein-stained (green) platelets incubated simultaneously on Risto-treated VWF adsorbed on glass coverslips. (E) Quantitative overall fluorescence intensity data of platelets (green) adhered and aggregated on the VWF-adsorbed coverslips. A1, B1, C1 and D1 represent construct binding; A2, B2, C2 and D2 represent platelet binding; A3, B3, C3 and D3 represent merged results to exhibit co-localization in yellow. The conditions tested were undecorated (Unmod-Lipo), VBP-decorated (VBP-Lipo) and VBP-cRGD-co-decorated (VBP-cRGD-Lipo) liposomal nanoconstructs incubated with predominantly inactive platelets (Platelet) and ADP-activated platelets (Act Platelet). Undecorated constructs showed minimal VWF-binding and platelet co-localization, VBP-decorated constructs showed concomitant VWF-binding with platelets but minimal platelet co-localization and VBP-cRGD-co-decorated constructs showed substantial VWF-binding as well as platelet co-localization, especially if platelets were already in a pre-activated state.

Incubation of the VBP-decorated constructs (red) and platelets (green) on ristocetin-
treated VWF-adsorbed surfaces resulted in their concomitant binding on the surface without mutual interference (B1 and B2) with minimal co-localization (yellow in B3). When the constructs were co-decorated with VBP and cRGD motifs, their incubation with platelets on the VWF-adsorbed surface demonstrated a slight increase of the overall platelet recruitment and aggregation on the surfaces (increased platelet fluorescence shown by the third bar in 6E), and the corresponding images showed slight enhancement of yellow overlap (C3) indicating increased co-localization of red and green fluorescence (C1 and C2). These results were obtained with platelets isolated from freshly drawn whole blood via serial centrifugation but without deliberate pre-activation by ADP. We have previously shown by flow cytometry analyses that such freshly prepared platelet suspensions still have ~20-25% of the platelets activated, possibly due to blood draw and storage [31]. Therefore, we rationalize that the slight enhancement in platelet recruitment/aggregation is a cumulative result of these low percentage of pre-active platelets binding directly to the C-domain of VWF as well as to the cRGD ligands co-decorated on the surface of the VWF-adhered VBP-decorated constructs (schematic shown in image panel), plus, the binding of small number of platelets that may get locally activated due to action of agonists secreted by the VWF-adherent platelets themselves. In comparison, when the platelets were deliberately pre-activated by ADP treatment prior to incubation with peptide-modified constructs on ristocetin-treated VWF-adsorbed surfaces, the overall platelet fluorescence (fifth bar in 6E) and corresponding co-localization (yellow in D3) of constructs (red, D1) and platelets (green, D2) were found to be significantly enhanced. This enhancement was also statistically higher than when such ADP-activated platelets were incubated with unmodified (no VBP and cRGD
decoration) constructs (fourth bar in 6E). These results suggest that in presence of pre-activated platelets, the VBP-decorated constructs do not interfere with platelets binding to VWF but rather amplify recruitment/aggregation of the active platelets as a cumulative effect of the platelets directly binding to VWF’s C-domains as well as significantly binding to the cRGD ligands co-decorated on the construct surface (schematic shown in fluorescence image panels of Figure 7.6). Therefore, the ‘primary hemostasis’ processes of platelet adhesion and aggregation can be efficiently mimicked and amplified by our platelet-inspired nanoconstruct design, possibly by the mechanism depicted previously in Figure 7.2.

4. Discussion

Hemostasis is a complex multi-step process involving platelet margination, adhesion, activation and aggregation (primary hemostasis), coagulation processes on adhered active platelet membrane (secondary hemostasis), and subsequent spatio-temporal regulation of clot retraction. Design of platelet-inspired synthetic hemostats should aim at adapting various functional components of these natural phenomena. To this end, several nano- and microscale design approaches are investigating (i) surface-modification of synthetic particles with platelet aggregation-promoting fibrinogen and fibrinogen-derived peptides, (ii) collagen or VWF-adhesion promoting recombinant glycoprotein moieties, (iii) encapsulation of platelet agonists and coagulation promoters within particles, and (iv) fabrication of particles with platelet-mimetic physico-mechanical properties that allow platelet-mimetic margination [10]. While these approaches have been mutually independent, for an optimized design of platelet-inspired synthetic hemostat, several of these components may potentially need to be integrated. In this aspect, a crucial
component is the integration of the ‘adhesion-promoting’ and ‘aggregation-promoting’ components on a single particle. Past strategies to achieve this by co-decorating a particle surface with adhesion- and aggregation-promoting recombinant protein moieties have indicated difficulties stemming from mutual steric interference between the moieties due to their large size [11], [32]. In our research, we have been able to resolve this issue by co-decorating a particle surface heteromultivalently with adhesion- and aggregation-promoting small molecular weight peptides that do not have mutual steric interference [11–13]. Compared to particles that bear only adhesion-promoting or only aggregation-promoting moieties, our ‘functionally integrated’ design that combines adhesion- and aggregation-promoting functionalities have indicated a statistically enhanced capability of hemostasis in a mouse tail transection model [13]. Building on these studies, we are presently focused on establishing a molecular scale mechanistic model for the hemostatic action of our platelet-inspired constructs. As a first step towards this in the current study, we have investigated whether our VWF-binding peptide (VBP) is capable of promoting construct adhesion on VWF without interfering with the natural platelet interaction to VWF’s A1 domain via platelet GPIbα. Our results indicate that even when VWF’s A1 domain is significantly ‘blocked’ by treatment with glycocalicin (resulting in significant reduction of natural platelet adhesion), the VBP-decorated constructs remain capable of binding to VWF, possibly via a different VWF domain. Furthermore, in the absence of glycocalicin-based ‘blocking’ of the A1 domain, the VBP-decorated constructs and platelets remain capable of simultaneously binding to VWF without mutual interference. In addition, when the VBP-decorated constructs were co-decorated with active platelet
GPIIb-IIIa-binding cRGDs, the constructs were capable of cumulatively increasing the recruitment and aggregation of active platelets on the VWF-adsorbed surfaces.

At the molecular level, the VBP itself has only a moderately high affinity to VWF, as indicated by its IC_{50} value of ~ 9 μM for inhibiting FVIII binding to VWF [14], [15]. However, decoration of multiple copies of this peptide on a nano- or microparticle surface is expected to significantly enhance the overall affinity of the particles to VWF. Such overall affinity enhancement via multi-copy decoration of ligands on nanoparticles has been reported for a variety of surface-engineered nanoparticle design, we rationalize that optimization of VBP decoration density on our nanoconstructs will render similar enhancement of VWF-binding of our constructs [33–35]. In the current studies we have utilized only one fixed molar composition of VBP in surface-decorating the nanoconstructs, since the focus of this study was to investigate whether VBP-decoration allows construct binding to VWF without interfering with natural platelets. In future studies, the VBP-decoration density will be varied and correlated to overall construct binding affinity to VWF utilizing established surface plasmon resonance (SPR) techniques [36]. It is also interesting to note that the binding of the VBP-decorated constructs to VWF adsorbed on glass coverslips showed no statistical difference between ristocetin-treated versus ristocetin-untreated conditions, whereas significant difference in platelet-binding was noted between those two conditions. This is indicative of the possibility that even without ristocetin treatment, the incubation and adsorption of VWF onto glass slides renders some conformational exposure of VWF that may allow interaction with VBP-binding regions but not sufficient conformational changes to allow substantial exposure of platelet GPIbα binding A1 domain. Such possibility can be
further rationalized from the fact that when VBP-liposomes or unmodified liposomes are exposed to soluble VWF without ristocetin and allowed to flow over collagen-coated surfaces at low shear ($< 10$ dynes/cm$^2$), only minimal binding of the VBP-liposomes on the collagen-coated surface is observed (c.f. supplemental data). Also, the adhesion of the VBP-decorated constructs on VWF surfaces were comparable between FVIII-free VWF and physiologic VWF and was unaffected by thrombin. Since the VBP is derived from residues 2303–2332 of the C2 domain of FVIII that does not contain the thrombin-binding Arg$^{1689}$ site and since physiologically FVIII binds to VWF’s D’-D3 domain, we rationalize that the VBP-decorated constructs bind to VWF’s D’-D3 domain without interfering with the platelet-binding A1 domain. Future studies will be directed at validating this molecular model rationale by utilizing D’-D3-domain specific and A1-domain specific antibodies to VWF. Altogether, our results indicate substantial promise of utilizing the VBP-peptide to promote VWF adhesion of platelet-inspired nanoconstructs towards efficient design of synthetic platelet analogs. The VBP-decorated design may also be potentially used to develop vehicles that can actively target vascular pathology sites rich in endothelium- and platelet-secreted VWF for drug delivery [37].

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6. References


Chapter 8: Platelet-inspired Nanomedicine Targeted to Thrombotic Disease Sites

Based on:

1. Platelet involvement in pathology of atherosclerosis, thrombosis and restenosis

The hemostasis mechanisms of platelets discussed in Chapter 3, when dysregulated, lead to hyperactivity of platelet-mediated clot formation, which is the main characteristic of thrombosis. Anatomically, thrombosis can be venous (RBC-rich red clot) or arterial (platelet-rich white clot), but irrespective of this distinction, platelets play a pivotal role in thrombosis [1], [2]. In related context, restenosis is a phenomenon where after a thrombosed artery is cleared via interventional procedures (e.g. angioplasty, stenting etc.), thrombosis and inflammatory processes can re-occur to occlude the vessel. Atherosclerosis, on the other hand, is an even more complex multi-factorial disease of blood vessels which is initiated by inflammatory events partly mediated by platelets and can then propagate into sub-endothelial plaque formation, erosion and rupture, leading to acute occlusive thrombosis. Platelets play a major role in mediating these inflammatory and thrombotic events both in atherosclerosis and restenosis [3–5]. Platelet’s role in arterial thrombosis stems from its previously described ability to rapidly adhere to the site of luminal endothelial injury, get activated by action of multiple agonists, undergo aggregation to form a primary platelet plug and concomitantly propagate coagulation mechanisms on active platelet membrane surface. As described previously, the initial injury site adhesion is mediated by VWF-to-platelet GPIbα and collagen-to-platelet
GPIa-IIa and GPVI interactions, and the aggregation is mediated principally by Fg interaction with active platelet GPIIb-IIIa. The GPIIb-IIIa integrins can also bind to VWF domains and fibronectin, which provide additional mechanisms of thrombus stabilization. Besides stimulated GPIIb-IIIa-integrin, another major surface marker for activated platelets is P-selectin, which is present within cytoplasmic α-granules in quiescent platelets but becomes significantly upregulated on the platelet surface as the granules fuse with the cell membrane upon platelet activation. P-selectin mediates recruitment of inflammatory cells by interaction with P-selectin Glycoprotein Ligand-1 (PSGL-1) on leukocytes, and also interacts with other platelets via sulfatides, as well as, with endothelial cells via GlyCAM-1, CD34 and MadCAM-1 [6], [7]. Activated platelets aggregated via these mechanisms can then aid in further propagation of coagulatory processes. In normal hemostasis, these clot-forming mechanisms are precisely balanced by innate mechanisms of clot retraction, dissolution and healing. For example, fibrinolytic pathways mediated by plasminogen and tissue-type plasminogen activator (tPA) interact to generate plasmin, which in turn digests fibrin to dissolve the clot. In pathological thrombosis, this fine balance is disrupted, resulting in excessive clotting. In venous thrombosis, the same platelet-mediated mechanisms come into play as blood hypercoagulability, flow stasis and damaged endothelium act in concert (Virchow’s triad) to result in thrombosis [8].

The thrombotic events described above also have a significant spatio-temporal involvement of inflammatory processes at the site. As mentioned previously, P-selectin expressed on active platelets can directly interact with leukocytes via PSGL-1, as well as
via other receptor-ligand pairs like CD40-CD40L (CD154), CD11b/CD18-to-β2 integrins etc. [9]. Activated platelets also secrete pro-inflammatory biomolecules like interleukin-1β (IL-1β), Regulated Upon Activation Normal T-cell Expressed and Secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1) and macrophage colony stimulating factor (M-CSF) which promote inflammatory cell recruitment and transformations that facilitate atherosclerosis [5]. Activated platelets also secrete platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) that stimulate smooth muscle cell (SMC) migration, proliferation and collagen synthesis. In addition, activated platelets, along with macrophages, produce matrix-metalloproteases (MMPs) that degrade extracellular matrix to aid infiltration of SMCs and inflammatory cells. These processes are highly characteristic of restenosis where SMCs migrate from

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**Figure 8.1:** Illustration of cell-cell and cell-matrix interactions of platelets in thrombosis and possible restenotic and inflammatory processes; platelet hyperactivity and aggregation is mediated by GPIIbIIIa-to-Fg interaction and P-selectin-to-PSGL-1 interaction; P-selectin based interactions also lead to recruitment and adhesion of leukocytes.
the medial layer of the vascular wall towards the intimal layer, while monocytes and macrophages translocate from the luminal blood flow into the intimal matrix [4]. Figure 8.1 shows a mechanistic scheme of cell-cell and cell-matrix interactions mediated by platelets in thrombosis. Utilizing these can provide unique ways of targeted nanomedicine to these diseases.

2. Platelet-inspired nanomedicine targeted to vascular disease site

Rationalizing from the mechanisms described above, several research strategies have been directed towards engineering of nanomedicine vehicles that actively target vascular disease sites. For example, echogenic liposomes that actively target fibrinogen, fibrin or intercellular adhesion molecule-1 (ICAM-1) via anti-fibrinogen, anti-fibrin or anti-ICAM-1 antibodies conjugated onto the liposome surface, demonstrated the ability to enhance ultrasound imaging of atheroma in a Yucatan miniswine model [10], [11]. Such echogenic liposomes have also provided a way to utilize ultrasound-induced cavitation to deliver thrombolytic/fibrinolytic agents (e.g. tPA and streptokinase) [12], [13]. A PEG-lipid based micelle system, surface-modified with a fibrin-targeted pentapeptide, has been reported for targeted delivery of a fluorescent probe (for imaging) and a therapeutic agent (hirulog, an anticoagulant) to atherosclerotic lesion sites in ApoE-/- mice [14]. Fibrin-targeted nanovehicles have also been reported for the clot-targeted delivery of imaging contrast agents, and atherosclerosis-targeted delivery of anti-proliferative agents (e.g. paclitaxel and fumagilin) [15–17]. Regarding direct targeting of disease-associated active platelets, gas-filled microbubbles surface-modified with platelet integrin GPIIb-IIIa-specific antibody abciximab (marketed as ReoPro® by Eli Lilly) were
found to enhance targeted molecular imaging of thrombus in vitro and in vivo [18]. In previous research by Sen Gupta et al. regarding platelet-targeting, liposomes were developed that were surface-modified with peptide ligands that have selectivity and high affinity to activated integrin GPIIb-IIIa on the platelet surface [19–21]. These liposomes demonstrated effective platelet binding in vitro as well as localization to a carotid artery
injury site in vivo in a rat model. Following the rationale of platelet adhesion to VWF at a diseased endothelium site, efficient thrombus-targeting has also been suggested for the previously mentioned albumin-based particles surface-decorated with GPIbα fragments [22]. Similar VWF-binding was also reported recently for nanoparticles made of gelatin (denatured collagen), since VWF and collagen have mutual binding interactions [23]. In another recent study, a lipid-polymer nanoparticle surface-decorated with peptides that target basement membrane collagen IV, was able to undergo enhanced binding to vascular injury sites in vivo and deliver the anti-proliferative drug Paclitaxel to reduce SMC activity [24]. In yet another study, the nanomedicine design utilized the presence of high shear environment near thrombotic sites to allow shear-activated polymeric nanoparticle microaggregates to disintegrate and release thrombolytic drugs [25]. Figure 8.2 shows selected research results in the area of nanomedicine-based targeted treatment of vascular diseases. Besides targeting active platelets or coagulation proteins, a large volume of nanomedicine research has also been directed towards targeting of other cell types involved in atherosclerosis, thrombosis and restenosis, e.g. macrophages, neutrophils and migratory SMCs. A comprehensive description of these approaches is available in a recent review [26].

3. References


Chapter 9: Heteromultivalent Liposomal Nanoconstructs for Enhanced Targeting and Shear-Stable Binding to Active Platelets for Site-selective Vascular Drug Delivery

Based on:


1. Introduction

Vascular diseases, leading to thrombo-occlusive and ischemic end points, are the leading cause of tissue morbidity and mortality in the United States and globally [1]. In vascular disease manifestations, such as atherosclerotic plaque progression and rupture as well as events leading to thrombosis and restenosis, disease site-selective delivery of therapeutic agents (e.g. thrombolytic or anti-proliferative drugs) and diagnostic probes (e.g. MRI or CT contrast agents) can provide significantly enhanced treatment efficacy compared to systemic administration of the same agents [2–4]. This is because, in direct systemic administration, a significant fraction of the agents may get cleared rapidly or may get deactivated by plasma action, thereby reducing their therapeutic concentration at the target disease site. Moreover, systemic distribution of the drug can cause unwanted side-effects in un-involved tissues, such as systemic coagulopathic and hemorrhagic effects as seen in direct intra-vascular delivery of thrombolytic agents in stroke therapy [5–7]. In these scenarios, disease site-selective or local delivery may have significant clinical benefit. Indication of such benefit is seen, for example, in the application of drug-eluting stents (DES), where local sustained release of anti-proliferative agents (e.g. Sirolimus) from the
stent polymer coating at the treated vascular site prevents in-stent restenotic events [8–10]. Although DES approaches provide considerable clinical benefit, they also pose risks of late-stage thrombosis when the drug reservoir runs out without allowing the stented site to have healed effectively [11–13]. Additionally, a significant number of patients are not stented but undergo angioplasty, bypass grafting, and other interventional procedures to restore blood flow and then are prescribed prolonged oral or systemic pharmacotherapy to prevent post-procedural thrombotic and restenotic events. In these scenarios, local or targeted delivery can provide improved strategies for detection and treatment.

Based upon the above rationale, our research is aimed at developing delivery systems that can specifically target and bind to the site of vascular disease and can allow site-selective delivery of various bioactive and therapeutic agents. Besides having

![Figure 9.1: Design of liposomal constructs surface-functionalized with two types of ligands for simultaneous targeting of GPIIb-IIIa integrin and P-selectin expressed at high quantity on the membrane surface of activated platelets involved in thrombotic and inflammatory events in vascular disease.](image-url)
selectivity to the vascular disease site, an additional requirement for such a delivery system is to bind the target site with sufficient strength so that it remains stably attached under the wall shear of hemodynamic flow. To meet these design criteria, we have developed liposomal nanoconstructs that are simultaneously surface-modified with two types of peptide ligands having specificity and high affinity to two different cell-surface receptors on activated platelets, namely integrin αIIbβ3 (also known as glycoprotein GPIIb-IIIa) and P-selectin (Figure 9.1). Natural ligands binding to these two receptors are responsible for stabilizing active platelet interactions at vascular disease sites under a hemodynamic shear environment. Integrin αIIbβ3 on activated platelets, in its stimulated ligand-binding conformation, binds to the bi-dentate ligand fibrinogen (Fg) to promote interlinking of active platelets in primary thrombus formation [14], [15]. This integrin also allows interaction of activated platelets with diseased endothelial cells (ECs) via Fg-mediated crosstalk with integrin αvβ3 [16], [17]. P-selectins, expressed specifically on activated platelets and stimulated endothelial cells via membrane fusion of cytoplasmic granules, are responsible for allowing interaction of platelets and ECs with monocytes via binding to P-selectin Glycoprotein Ligand-1 (PSGL-1) present on the surface of monocytes [18], [19]. P-selectin also mediates inter-platelet interactions via sulfatides and platelet interaction with endothelial cells via GlyCAM-1, CD34, and MadCAM-1, providing supplementary mechanisms of platelet-monocyte-EC interaction stabilization at vascular disease sites. Hence, we rationalized that exploiting simultaneous binding to these two receptors by our liposomal nanoconstructs will not only enhance vascular disease site-selectivity, but also provide sufficient binding stability under hemodynamic flow at the target site.
Our choice of activated platelets as an ideal target for vascular diseases stems from their multiple roles in promoting, regulating, and actively participating in major vascular disease manifestations, for example in atherosclerosis, thrombosis, and restenosis [20–27]. In these disease situations, platelets mediate monocyte-endothelial interactions, partly regulate transcytotic and matrix remodeling events via secretion of various cytokines and enzymes, and actively participate in clot formation via Fg-mediated inter-platelet bridging and induction of the coagulation cascade via activation of coagulation factors on activated platelet membrane [28], [29]. Hence, platelets have an enhanced spatio-temporal distribution in various types and stages of vascular diseases. For specific targeting to activated platelets, we have chosen integrin α₁β₃ and P-selectin as our molecular target epitopes since they are present at high levels, specifically on the surface of activated platelets. This will enable high selectivity of our liposomal nanoconstructs towards activated platelets and not quiescent platelets and, therefore, will enhance selectivity to vascular disease sites. We have previously demonstrated enhanced targeting of α₁β₃ on activated platelets by decorating liposome surface with fibrinogen-mimetic RGD peptides [16], [17], [27]. In current research, we combined α₁β₃-targeting by a fibrinogen mimetic GSSGRGDSPA peptide (FMP) with P-selectin targeting by a sialoprotein mimetic DAEWVDVS peptide (SMP) that was recently identified by Molenaar et. al. as having higher affinity to P-selectin compared to the traditional sialyl Lewis acid (sLeᵃ and sLe⁵) ligands [30], [31]. We carried out heteromultivalent surface-modification of the liposome membrane with these peptides and studied the interaction (specific binding and retention) of the resultant nanoconstructs with collagen-adhered activated platelets in a parallel plate flow chamber (PPFC) at various wall shear values.
The binding and retention of dual targeted constructs were compared to non-targeted or singly targeted constructs.

2. Materials and Methods

Materials

Phosphate Buffered Saline (PBS), 3.8% w/v sodium citrate, parformaldehyde (PFA), Bovine Serum Albumin (BSA), Trifluoroacetic acid (TFA), chloroform, methanol, and ethanol were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA). Cholesterol, Ninhydrin, Phenol, Potassium cyanide, Pyridine, 1,2-Ethanedithiol (EDT), Thiolanisole, and collagen were obtained from Sigma Aldrich (St. Louis, MO, USA). All amino acids were purchased from Advanced ChemTech (Louisville, KY, USA). Polycarbonate filters with 200nm pores were obtained from Whatman (Kent, UK). Fluorescently labeled monoclonal antibodies, namely, FITC-anti-CD41a (staining platelet GPIIb-IIIa) and AlexaFluor® 647-anti-CD62P (staining activated platelet P-selectin), were obtained from BD Biosciences (San Jose, CA, USA) and BioLegend (San Diego, CA, USA), respectively. Adenosine Diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA, USA). The lipids Distearyl Phosphatidyl Choline (DSPC), Distearyl Phosphatidyl Ethanolamine (DSPE), Polyethylene Glycol-modified DSPE (DSPE-PEG_{2000}), DSPE-NHS ester, and Carboxy-Polyethylene Glycol-modified DSPE (DSPE-PEG_{2000}-NHS ester) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). N-Hydroxysuccinimide (NHS)-modified fluorophores, namely, NHS-Fluorescein and NHS-Rhodamine were obtained from Invitrogen Corporation (Carlsbad, CA, USA).
The Parallel Plate Flow Chamber (PPFC) system was purchased from Glycotech (Gaithersburg, MD, USA).

**Peptide synthesis and fabrication of peptide-modified liposomal nanoconstructs**

A linear RGD peptide, GSSSGRGDSPA, and a P-selectin targeting peptide, DAEWVDVS, were synthesized using Fmoc based solid phase chemistry and characterized using MALDI-TOF mass spectroscopy (Figure 9.2). Negative control peptide sequences, namely GSSSGRGESA and DAEWVEVS, were synthesized and characterized in the same manner. All peptides were conjugated ‘on resin’ to NHS-ester modified-lipid (DSPE-PEG-NHS ester) by reductive amidation to form DSPE-PEG-peptide (schematic in Figure 9.3). Conjugation yield was determined to be 95.8 ± 4.9% using a Ninhydrin assay [32]. To synthesize DSPE-Fluorescein (green fluorescence,
λmax ~ 530 nm) or DSPE-Rhodamine (red fluorescence, λmax ~ 570 nm), the free NH2 termini of DSPE was reacted to the amine reactive NHS-Fluorescein or NHS-Rhodamine at a basic pH. The liposomes were fabricated by homogenizing DSPC (49 mol%), DSPE-PEG-peptide (5 mol%), cholesterol (45 mol%) and DSPE-PEG-Rhodamine or Fluorescein (1 mol%) in 1:1 chloroform:methanol and subjecting the mixture to reverse phase evaporation through several cycles of freeze-thaw, followed by extrusion through a 200nm polycarbonate membrane to achieve unilamellar vesicles. The formulations of the liposomes used in the experiments are shown in Table 9.1. For singly targeted liposomes, the DSPE-PEG-GSSSGRGDSPA or DSPE-PEG-DAEWVDVS concentration was kept at 5 mol% total lipid, while for the dual targeted liposomes the DSPE-PEG-peptide composition was kept at 2.5 mol% total lipid for each type of targeting peptide, such that the cumulative DSPE-PEG-peptide composition was still at 5 mol% total lipid. The size distribution of the liposomal constructs was characterized using dynamic light

![Diagram](image-url)

**Figure 9.3:** Process for fabricating the heteromultivalent liposomal constructs for dual targeting of activated platelets; bottom panel shows representative DLS characterization data for the constructs before and after extrusion; after extrusion the constructs are largely monodisperse with an average diameter of 150 nm.
scattering (DLS) and the constructs were found to have an average diameter of ~150nm following extrusion (Figure 9.3).

**In vitro studies to confirm targeting specificity and enhanced binding stability of constructs**

**Receptor-specific binding studies in a static environment.**

Human whole blood was obtained from healthy, aspirin-refraining donors by venipuncture following IRB-approved protocol and collected in citrated tubes. Platelet-rich-plasma (PRP) was obtained from the whole blood by centrifugation (150 x g, 15 min, room temperature), and platelet concentration was verified at 200×10^6 platelets/ml. The platelets in PRP were activated using ADP at a concentration of 2×10^{-5} M, and were allowed to adhere to glass coverslips pre-coated with collagen. The adhered platelets were subsequently fixed with 4% paraformaldehyde, and their presence on the coverslip was confirmed by immunostaining with AlexaFluor® 647-anti-CD62P (staining P-
selectin) and FITC-anti-CD41a (staining integrin $\alpha_{IIb}\beta_3$) followed by fluorescence microscopy imaging. For establishing the receptor-specificity of liposome binding, collagen coated coverslip-adhered platelets were incubated with fluorescently-labeled, non-targeted (unmodified) or $\alpha_{IIb}\beta_3$-targeted (FMP-modified) or P-selectin-targeted (SMP-modified) liposomes (schematics in Figure 9.4 and Figure 9.5). After one hour of incubation, the coverslips were gently washed with PBS to remove loosely bound liposomal constructs and imaged using fluorescence microscopy. All images were captured using a Zeiss Axio Observer.D1 inverted fluorescence microscope fitted with a

**Figure 9.4:** Fluorescence microscopy experiment to establish the specific binding of FMP-modified liposomes to activated platelets; top panel shows the experiment procedure; middle panel shows representative microscope images of control and test conditions (A through E); the quantitative analysis of fluorescence intensity from multiple images (n=10 per test or control condition) at these conditions are shown in the graph at the bottom, confirming the enhanced binding of activated platelets by FMP-modified liposomes compared to unmodified liposomes.
photometrics chilled CCD camera and a 63X objective using exposure time of 800ms. To confirm that nonspecific binding of the liposomes was not occurring, fluorescently-labeled peptide-modified liposomal constructs were incubated with coverslips coated with collagen only but no activated platelets, followed by gentle washing and imaged using fluorescence microscopy keeping same exposure times (800ms).

Receptor-specific blocking studies in a static environment
As additional verification of the peptide-modified liposomal constructs binding specificity to the respective activated platelet surface target receptors, blocking studies were carried out. First, activated platelets were adhered onto collagen-coated coverslips as previously described. Next, nonfluorescent FMP-modified or SMP-modified liposomal constructs were incubated with the coverslip-adhered platelets for 1 hour. After 1 hour, the coverslips were gently washed with PBS and further incubated with fluorescent FITC-anti-CD41a or AlexaFluor® 647-anti-CD62P for 30 min. Similar activated platelets adhered onto collagen-coated coverslips were incubated with FITC-anti-CD41a or AlexaFluor® 647-anti-CD62P without prior incubation with the non-

Figure 9.6: Specific receptor blocking studies analyzed by fluorescence microscopy to establish that FMP-modified liposomes bind to GPIIb-IIIa on activated platelets; top panel shows the experiment procedure; middle panel shows representative microscope images of control and test conditions (A through E); the quantitative analysis of fluorescence intensity from multiple images (n=10 per test or control condition) at these conditions are shown in the graph at the bottom, confirming that pre-incubation of activated platelets with FMP-modified liposomes results in significant blocking of fluorescent antibody specifically binding to activated platelet GPIIb-IIIa; this result in combination with results shown in Figure 3 confirm that the FMP-modified liposomes can specifically target and bind to GPIIb-IIIa on activated platelets.
fluorescent peptide-modified liposomes. The rationale was that if the peptide-modified liposomal constructs bound their specific target receptors, then the pre-incubation with the liposomes would possibly occupy many of these receptors and block them from binding the fluorescent antibodies in the subsequent step (schematics in Figure 9.6 and Figure 9.7). On the other hand, without liposome pre-incubation, the fluorescent antibodies would successfully stain the respective receptors. All coverslips were imaged as previously described.

*Flow cytometry analysis of platelet-targeting by peptide-modified constructs*
Liposomal construct binding to activated platelets was also studied using an LSRII (Becton Dickinson) flow cytometer (Blue 488nm and Red 640nm lasers). Whole blood aliquots were incubated with ADP for 30 minutes to activate the platelet population. Samples were then fixed with 4% paraformaldehyde for 1 hour. The level of platelet activation was assessed by co-staining the aliquot with FITC-conjugated anti-CD41a antibody and AlexaFluor® 647-conjugated anti-CD62P antibody and running the sample through the flow cytometer to assess the fluorescence associated with the gated platelet population. Following confirmation of platelet activation, duplicate whole blood aliquots were incubated with Rhodamine B-labeled non-targeted (unmodified), singly targeted (FMP-modified OR SMP-modified), or dual targeted (both FMP and SMP modified) liposomes at a final concentration of 500µM total lipid for 30 min and run through the flow cytometer to analyze platelet-associated fluorescence. For control studies, the aliquots were subjected to liposome incubation without pre-incubation with ADP (no platelet activation). For all analyses, gated platelet population fluorescence was analyzed at 20,000 counts per aliquot.

Binding stability and retention studies in Parallel Plate Flow Chamber (PPFC)

A standard PPFC system (10mm width x 0.01in height) fitted to a peristaltic pump and placed under an inverted epifluorescence microscope was used for studying interaction of liposomal constructs with surface-adhered platelets under a flow environment (schematic in Figure 9.8). The PPFC system allows variation of wall shear stress ($\tau_w$) at the plate surface by modulating the flow rate ($Q$) of the fluid through the chamber, according to the equation $\tau_w = 6\mu Qw^4h^{-2}$, where ‘$\mu$’ is fluid viscosity, ‘$w$’ is chamber width and ‘$h$’ is
chamber height [33], [34]. Similar flow chamber set-ups have been reported for analysis of cell-material interactions in a dynamic flow environment [35–37]. For PPFC studies, glass microscope slides were coated in two equal circular area regions with albumin and collagen. The slides were allowed to sit in contact with PRP in presence of ADP or BSA for 1 hour to render adhesion of activated platelets to the collagen-coated region; the albumin-coated region does not adhere activate platelets and hence act as the control area. After fixing with 4% paraformaldehyde and immunostaining with FITC-anti-CD41a and AlexaFluor® 647-anti-CD62P, the collagen-coated region revealed dense adhesion and aggregation of platelets (simulating a thrombotic region), while the albumin-coated

![Figure 9.8: Schematic representation of experimental set-up and procedure for the PPFC experiments to establish the enhanced binding and retention of peptide-modified liposomes to activated platelets under hemodynamic flow relevant shear stress ranges over time; the bottom left also shows representative SEM images of albumin-coated surface area and collagen-coated surface area after incubation with activated platelets, confirming that the collagen-coated surface has a significantly high density of active platelets; allowing the test and control liposomes to interact with platelet-rich (collagen-coated) and platelet-deficient (albumin-coated) surface regions on the same slide under various shear stress ranges in the PPFC set-up for effective analysis of liposome binding and retention.](image-url)
region had almost no adhered platelets. This fluorescence microscopy information was further complimented by Scanning Electron Microscopy (SEM) images (representative images shown in Figure 9.8). After confirming the activated platelet-adhered and platelet-deficient regions on the glass slides, similar PRP-incubated slides but without platelet immunostaining were placed in the PPFC, and Rhodamine-labeled (red fluorescent) peptide-modified (singly-targeted or dual-targeted) or unmodified liposomes were allowed to flow through the chamber in a PBS suspension (total lipid concentration of 1mM) for 30 minutes. The flow was maintained at various flow rates for different batches of experiments to allow varying wall shear stress values in the range between 5-60 dynes/cm². After 30 minutes, the liposome solution was replaced with PBS and the flow was maintained for another 15 min to determine the stability of binding of the liposomes in the dynamic flow environment. Throughout this procedure, at various time points and for various flow rates (hence shear stresses), the glass slides were imaged under an epifluorescence microscope as previously described.

Data Analysis

For fluorescence images from the binding/blocking studies under both static and dynamic conditions, adhesion and retention was quantified using raw image analysis of surface averaged intensity values in Adobe Photoshop® CS4 software. Statistical analysis of the static binding/blocking results was done using Paired Student’s t-tests, and statistical analysis of dynamic binding results was done using ANOVA. For all statistical analysis, significance was considered at p<0.05.

3. Results
Receptor-specific binding studies and blocking studies in a static environment

Figure 9.4 and Figure 9.5 show representative fluorescence microscopy images for the receptor-specific binding studies with FMP-modified and SMP-modified liposomal constructs, respectively, along with corresponding quantitative data of surface-averaged fluorescence intensity analysis. As evident from the data, both FMP-modified and SMP-modified liposomal constructs were able to significantly bind activated platelets by specific interaction with their respective target receptors, but had minimal non-specific binding with collagen itself in the absence of adhered active platelets. Constructs without any peptide modification or bearing negative control (RGE or EWVEV) peptides had negligible binding to activated platelets. The target receptor specificity of the binding was further confirmed by the results of the receptor blocking studies as shown in Figure 9.6 and Figure 9.7. As evident from the figures, pre-incubation with the FMP-modified nonfluorescent constructs was able to block subsequent binding of FITC-anti-CD41a to integrin α_{IIb}β_{3}, while pre-incubation with the SMP-modified nonfluorescent constructs was able to block subsequent binding of AlexaFluor® 647-anti-CD62P to P-selectin on activated platelets.

Flow cytometry analysis of binding:

Figure 9.9 shows representative flow cytometry scatter plot of the whole blood aliquot, the gated activated platelet population analyzed for fluorescence, and representative fluorescence histograms of ADP-activated aliquots incubated with non-targeted, singly targeted (FMP-modified or SMP-modified) and dual targeted (simultaneously FMP- and SMP-modified) liposomal constructs. As evident from the data, the FMP-modified and
the SMP-modified constructs were individually able to bind activated platelets significantly higher than non-targeted constructs. Furthermore, when the two peptide modifications were combined on a single construct, the resultant dual-targeted constructs showed much enhanced binding to activated platelets compared to the singly targeted constructs. This validates our rationale that the dual targeting approach will render

Figure 9.9: Representative flow cytometry results showing (A) the gated activated platelet population in whole blood aliquots under analysis and (B) the fluorescence histograms from platelet population interacting with unmodified (non-targeted), FMP- or SMP-modified (singly targeted) and simultaneous FMP- and SMP-modified (dual-targeted) fluorescently labeled liposomes; it is evident that though the singly-targeted liposomes are capable of binding activated platelets significantly more than the non-targeted liposomes, the dual-targeted liposomes have even higher extent of binding activated platelets when compared to the singly-targeted liposomes.
enhanced targeting efficacy towards activated platelets, which in essence, would enhance the binding selectivity of these constructs at sites of vascular disease where large numbers of activated platelets are involved.

*Binding stability and retention studies in Parallel Plate Flow Chamber (PPFC)*

**Figure 9.10** shows representative fluorescent images of construct interaction with test (platelet-covered) and control (albumin without platelet) surface regions on the glass slide in PPFC at three shear values (5, 25 and 45 dyn/cm²) for the 30 min time point. The bottom panel of **Figure 9.10** also shows quantitative analysis of surface-averaged fluorescence from images at three shear stress values over a period of 45 min (30 min of liposomal construct flow in recirculating loop + 15 min of plain PBS flow in open loop) for the various test and control samples. As evident from the qualitative images, the FMP-modified liposomal constructs, the SMP-modified liposomal constructs, and the dual-targeted constructs were all able to significantly bind the activated platelet covered surface under flow compared to unmodified (non-targeted) liposomal constructs or platelet-deficient (albumin) surfaces. The quantitative analysis shows that the dual-targeted liposomal constructs have significantly enhanced binding and retention on activated platelets under flow compared to the singly targeted constructs. This validates our rationale that the dual targeting not only enhances the selectivity of activated platelet targeting (complementary results to flow cytometry data), but also enhances the strength of binding to ensure higher retention at the target site under a dynamic flow environment.
Figure 9.10: Representative fluorescence microscopy images and quantitative data from PPFC experiments to study binding and retention of test (single or dual-targeted) and control (non-targeted) liposomes to activated platelet-coated surface versus platelet-deficient surface under flow at three different shear stress values (low-to-high shear) over a period of 45 min (30 min liposomal suspension flow + 15 min 1X PBS flow); it is evident that in a dynamic flow environment, dual-targeted liposomes are capable of binding and staying retained on target cells (activated platelets) at significantly enhanced levels over time at all shear stress values compared to non-targeted and even singly-targeted liposomes.
4. Discussion

With the advent of the concepts of ‘targeted drug delivery’ and ‘nanomedicine’, significant research has been focused on enhancing the therapeutic distribution and efficacy at disease sites via direct local administration devices or drug-loaded micro- and nano-particulate delivery vehicles. The delivery vehicle-mediated approaches have shown a higher success in targeted therapeutics for cancer pathologies compared to vascular pathologies. This is because in cancer, the vehicles are designed to extravasate within the tumor tissue through tumor-associated leaky vasculature, and remain in a pseudo-static reservoir environment due to compromised lymphatic drainage in tumors; this is known as the ‘enhanced permeation and retention’ (EPR) mechanism. In contrast for vascular diseases, the vehicles are expected to marginate to the vascular wall and bind the disease site under a hemodynamic environment, and should stay retained at that target site under flow. Hence, for targeted drug delivery in vascular diseases beyond ‘targeting specificity’, the ‘binding stability’ also is critical to ensure site-selective drug release [2]. Based on this requirement, we rationalized that mimicking the multi-receptor binding mechanisms of natural platelets in a vascular disease environment on a synthetic delivery vehicle may provide enhanced ‘targeting specificity’ as well as ‘binding stability’ of the constructs under flow. In fact, a number of recent reports have demonstrated that targeting specificity and efficacy of drug delivery vehicles can be enhanced by utilizing a multi-receptor targeting approach [38], [39].

Our experimental results validate our design rationale and approach. Liposomal constructs surface-modified by GPIIb-IIIa-binding, fibrinogen mimetic (FMP) peptides or P-selectin-binding, sialoprotein mimetic (SMP) peptides were both able to specifically
bind activated platelets significantly more than unmodified constructs in a static environment (incubation in a well plate). This enhanced binding was also observed in a dynamic environment (i.e. in PPFC); but for the singly targeted constructs (FMP-modified or SMP-modified), the extent of stable retention on activated platelet layer over time under low-to-high shear stress values seemed to decrease significantly after about 30 min under flow, as evident from the representative data shown in Figure 9.10. In contrast, for constructs bearing dual targeting functionalities (both FMP and SMP peptides), not only did the specific binding to activated platelets occur at a significantly enhanced level, but the retention of the constructs on the target cells over time under flow remained significantly higher at low-to-high shear values. This can be of considerable benefit in ensuring the prolonged residence of these constructs at the vascular disease sites under a hemodynamic flow environment to guarantee site-selective release of therapeutic payload.

In our current study, we have kept the total mol% of peptide decorations on the liposomal constructs constant, and also for the dual targeted constructs we have maintained the ratio of GPIIb-IIIa-targeted peptides and P-selectin targeted peptides to be 50:50. These specific formulations have already exhibited results that validate our design rationale and approach. Future research will include varying the total mol% as well as the peptide decoration ratio for particle surface modification to determine whether there is an optimum surface-density and ratio that guarantees maximum enhanced binding and retention to activated platelets under flow. Also, we have used liposomes as our model drug delivery system to demonstrate the effect of hetero-multivalent surface-modification in vascular targeting. However, the scientific approach remains flexible enough to be
applied to any particulate delivery system for targeted vascular therapy. In fact, interesting theoretical and experimental research in recent years have indicated that there might be a significant correlation between the dimensional parameters (shape and size) of particulate drug delivery systems and their preferential transport to vascular wall through blood components under hemodynamic flow [40], [41]. Future work will also be focused on integrating such dimensional parameters to surface-modification parameters (i.e. ligand type and surface-density) towards optimizing the construct design for maximum efficacy in vascularly targeted drug delivery systems.

5. Conclusion

We rationalized that vehicles surface-modified with multiple types of ligands that simultaneously target multiple types of receptors at a vascular disease site will have higher selectivity as well as higher binding stability under flow, ensuring enhanced targeted delivery of therapeutics and diagnostics in a hemodynamic environment. We tested our rationale by using liposomes as the delivery vehicles and modifying their surface with two different types of ligands, namely a FMP-peptide and a SMP-peptide, that simultaneously target two different receptors on vascular disease-relevant active platelets, integrin GPIIb-IIIa and P-selectin, respectively. Our results indicate that liposomes surface-modified with both peptides (dual targeting mechanism) have higher binding selectivity and retention to activated platelets under flow compared to liposomes bearing any one type of peptide (single targeting mechanism). The results establish the feasibility and benefit of using heteromultivalent surface modification of delivery
vehicles to enhance their site-selectivity and stability of binding under dynamic flow to ensure effective drug delivery in the vascular compartment.

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7. References


Chapter 10: Active Platelet Binding and Clot Lysis Evaluation of a Thrombus-targeted Enzyme-triggered Vascular Nanomedicine System

1. Introduction

Vascular diseases continue to be the number one cause of tissue morbidities and mortalities in the United States and globally [1], [2]. Consequently, significant research and clinical efforts are directed in prevention and treatment of these diseases. Although vascular diseases fall into many categories, many of them have common spatio-temporal cellular and molecular mechanisms, the most prominent and worrisome of which is the formation of intravascular occlusive clots (thombi) that reduce or even completely occlude blood flow to vital tissues and organs such as the heart and the brain. Therefore, many clinical strategies are focused on prophylactic, emergent and sustained prevention of thrombo-occlusive events to maintain normal blood flow to tissues and organs. The prophylactic strategies mainly involve oral or systemic administration of anti-coagulant (e.g. heparin) and anti-platelet (e.g. Aspirin and Clopidogrel) agents, the emergent strategies mainly involve mechanical (e.g. thrombectomy, balloon angioplasty etc.), surgical (e.g. bypass grafting etc.) and fibrinolytic pharmacotherapy (e.g. IV bolus or infusion of plasminogen activators like streptokinase and tPA) procedures, while the sustained strategies mostly involve post-procedural prolonged oral administration of anti-coagulant and anti-platelet drugs. As evident from these descriptions, systemic (oral and IV) administration of drug molecules that prevent platelet activation and aggregation (anti-platelet agents), block coagulation pathways (anti-coagulant agents), degrade clot proteins (fibrinolytic agents) or down-regulate unwanted cellular proliferation (anti-
proliferative agents) remain a major component of clinical regimen in treating occlusive vascular disease conditions. Such systemic direct administration of drugs show only partial clinical benefit, because they present a number of problems [3–6]:

a) Rapid drug washout and clearance from the target site due to dynamic blood flow
b) Plasma-induced inactivation of the drugs and reduced circulation half-life
c) Systemic non-specific distribution of the drugs resulting in sub-optimal availability at the target site
d) Systemic non-specific action of the drugs leading to harmful side effects like coagulaopathy, neuro- and nephrotoxicity and hemorrhage

These issues can be potentially resolved by localizing the delivery (and action) of the drugs at the target clot sites.

Localizing delivery of drugs at a vascular target site has been shown to improve therapeutic efficacy, as in the case of trans-catheter site-proximal release of drugs [7], [8], as well as deployment of Drug Eluting Stents (DES) [9], [10]. However, catheterization and stenting are still invasive interventional procedures that are expensive and require specially trained clinical personnel. Such expertise and expenses may not be available or applicable in many locations. In this framework, a safer and more applicable strategy can be achieved by creating delivery systems that can be administered into the patient’s circulation with minimal invasiveness (e.g. intravenous (IV) injection), yet can actively anchor onto the thrombo-occlusive site, stay retained under hemodynamic flow conditions, and allow controlled release of encapsulated payload (e.g. anti-platelet or thrombolytic agents) for site-specific action. To this end, packaging therapeutic agents within thrombus site-targeted nanoparticles provides a promising avenue for such
localized therapy. Previous approaches have included nanoparticles surface-decorated with fibrin-specific ligands (e.g. perfluorocarbon particles bearing fibrin-specific antibodies and micelles bearing fibrin-binding peptides) or sub-endothelial matrix-binding ligands (e.g. lipoplexes bearing collagen-binding peptides) [11–15]. Fibrin is produced in the final stage of the coagulation cascade; hence to ensure a high amount of vehicle binding, extensive coagulation must have already occurred which may offset the therapeutic benefits of the delivered drug. As for particles targeting sub-endothelial matrix proteins (e.g. collagen), such proteins may not have a sufficient extent of exposure since rapid thrombus formation on the exposed protein may outcompete the vehicle binding. A more ideal target would be an entity that has sufficiently accessible spatio-temporal presence during thrombus formation and clotting propagation.

Based upon the above rationale, we have identified thrombus-associated active platelets as an ideal target. Active platelets play multiple roles in promoting and regulating thrombus formation (as discussed in Chapter 8). Specifically, active platelets mediate monocyte-endothelial interactions via platelet P-selectin binding to PSGL-1 on monocytes and CAMs on endothelial cells, partly regulate matrix remodeling events via secretion of various cytokines and enzymes, actively participate in clot formation via inter-platelet bridging through blood protein fibrinogen (Fg) binding to platelet integrin GPIIb-IIIa, and participate in the activation of coagulation factors on their membrane [16], [17]. Consequently, active platelets demonstrate excellent spatio-temporal accessibility during various types and stages of vascular diseases. In order to target active platelets, we have chosen integrin GPIIb-IIIa and P-selectin as our molecular target epitopes because they are expressed at high levels on the active platelet membrane. Since
platelets render stable disease site binding via multiple simultaneous ligand-receptor interactions, we rationalize that platelet-inspired heteromultivalent binding of our drug delivery vehicles to these receptors will render thrombus-specific targeting as well as retention under hemodynamic flow. Furthermore, once retained at the thrombus site, internal or external trigger mechanisms can be utilized to render site-specific drug release from the delivery vehicle. To test this, we have used liposomes as a model vehicle and a fibrinogen-mimetic GSSGGRGDSPA peptide (FMP) and a sialoprotein-mimetic DAEWVDVS peptide (SMP) as ligands to facilitate binding to active platelet integrin GPIIb-IIIa and P-selectin, respectively. We have recently published on our initial work in design and characterization of platelet-targeted heteromultivalently surface-modified liposome system [18]. Our results showed that liposomes surface-modified with both peptides (dual targeting mechanism) have higher binding selectivity and retention to activated platelets under flow, *in vitro*, compared to liposomes bearing any one type of peptide (single targeting mechanism). These results established the feasibility and benefit of using heteromultivalent surface modification of delivery vehicles to enhance their site-selectivity and stability of binding under dynamic flow to ensure effective drug delivery in the vascular compartment. Building on this, here we demonstrate that our

**Figure 10.1:** Design components of a delivery system that can specifically target and bind the site of vascular disease via simultaneously binding to activated platelet GPIIb-IIIa and P-selectin can allow site-selective delivery of various bioactive and therapeutic agents via PLA₂-triggered payload release.
heteromultivalent design allows compositional modulation of the ligand decorations on
the construct surface to optimize their binding to activated platelets in low, moderate, and
high shear stress environments, in vitro. Once localized to the thrombus site via
heteromultivalent binding to active platelets, these constructs can be further refined to
release their therapeutic cargo via vascular disease site abundant enzymes. For example,
phospholipase A2 (PLA₂) has been shown to be secreted by activated platelets, leading to
elevated levels of the enzyme at sites of vascular diseases [19–25]. Additionally, recent
studies have demonstrated the ability of PLA₂ to degrade phosphatidylcholine-containing
lipid vesicles, resulting in rapid content release [26], [27]. With this rationale, here we
demonstrate release of a model payload, carboxyfluorescein, from our liposomal
nanoconstructs triggered by PLA₂. Combining the platelet targeting and PLA₂-triggered
payload release functionalities on liposomes containing a model thrombolytic drug
(streptokinase, SK) (Figure 10.1), we demonstrate enhanced clot lysis under flow, in
vitro. Lastly, using a mouse carotid artery thrombosis model, we have carried out initial
studies of thrombus site localization of our vascular nanomedicine system, in vivo.

2. Materials and Methods

2.1 Materials
Phosphate Buffered Saline (PBS), 3.8% w/v sodium citrate, parformaldehyde (PFA),
Bovine Serum Albumin (BSA), Trifluoroacetic acid (TFA), chloroform, methanol,
ethanol, 5(6)-Carboxyfluorescein, Tris HCl, Whatman #1 filter paper, and calcium
chloride (CaCl₂) were obtained from Thermo Fisher Scientific (Pittsburgh, PA, USA).
Cholesterol, Ninhydrin, Phenol, Potassium cyanide, Pyridine, 1,2-Ethanedithiol (EDT), Thiolanisole, collagen, Streptokinase from β-hemolytic *Streptococcus*, Phospholipase A2 (PLA₂) from *Naja mossambica mossambica*, TritonX-100, and ferric chloride (FeCl₃) were obtained from Sigma Aldrich (St. Louis, MO, USA). All amino acids were purchased from Advanced ChemTech (Louisville, KY, USA). Polycarbonate filters with 200nm pores were obtained from Whatman (Kent, UK). Adenosine Diphosphate (ADP) was purchased from Bio/Data Corporation (Horsham, PA, USA). The lipids Distearyl Phosphatidyl Choline (DSPC), Distearyl Phosphatidyl Ethanolamine (DSPE), Polyethylene Glycol-modified DSPE (DSPE-PEG₂₀₀₀), DSPE-NHS ester, and Carboxy-Polyethylene Glycol-modified DSPE (DSPE-PEG₂₀₀₀-NHS ester) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). N-Hydroxysuccinimide (NHS)-modified fluorophores, namely, NHS-Fluorescein and NHS-Rhodamine were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Calcein AM was purchased from Life Technologies (Grand Island, NY). Human alpha-thrombin was obtained from Hematologic Technologies Inc. (Essex Junction, VT). Glass microscope slides containing a concave well were purchased from Celestron (Torrance, CA). The Parallel Plate Flow Chamber (PPFC) system was purchased from Glycotech (Gaithersburg, MD, USA). Ketamine was purchased from Fort Dodge Animal Health (Fort Dodge, IA) and xylazine was purchased from Hospira (Lake Forest, IL).

2.2 *Heteromultivalently-decorated liposome fabrication and PPFC studies for platelet binding in vitro*
Heteromultivalently-decorated, platelet-targeted liposomes were fabricated as previously described [18]. Briefly, a fibrinogen mimetic GPIIb-IIIa-binding peptide (FMP), GSSSGRGDSPA, and a sialoprotein-mimetic P-selectin-binding peptide (SMP), DAEWVDVS, were synthesized using Fmoc-based solid-phase peptide chemistry on a Knorr resin and characterized using mass spectrometry. The peptides were then conjugated via their N-termini to NHS-ester modified-lipid (DSPE-PEG-NHS ester) by reductive amidation to form DSPE-PEG-peptide conjugates. Fluorescently-labeled lipids were fabricated by reacting the free amine NH\textsubscript{2} termini of DSPE with NHS-Fluorescein (green fluorescence, λ\textsubscript{max}~530nm) or NHS-Rhodamine (red fluorescence, λ\textsubscript{max}~570nm) to allow for microscopy detection. These DSPE-PEG-peptide molecules were combined with DSPC, cholesterol, DSPE-PEG, and DSPE-Rhodamine to fabricate peptide-decorated ~150nm diameter (characterized by DLS) liposomes via the reverse phase evaporation and extrusion (RPEE) technique. For liposomes bearing only the FMP or SMP peptides, the corresponding DSPE-PEG-peptide concentrations were maintained at 2.5, 5, or 10 mol % of total lipid. These liposomes were used to evaluate the effect of varying total peptide concentration on the extent of platelet binding. For liposomes bearing both FMP and SMP in combination, liposomes were fabricated with varying ratios of DSPE-PEG-FMP to DSPE-PEG-SMP (80:20, 60:40, 50:50, 40:60, and 20:80) at a fixed total peptide decoration of 5 mol %. These liposomes were used to further determine the effect of varying relative peptide ratios when combined on the same construct. Particle lipid composition is summarized in Table 10.1.
For parallel plate flow chamber (PPFC)-based platelet binding experiments, acid washed glass microscope slides were coated with two equal circular regions of albumin.

<table>
<thead>
<tr>
<th>Liposome name</th>
<th>DSPC (mol %)</th>
<th>DSPE-PE (mol %)</th>
<th>Cholesterol (mol %)</th>
<th>DSPE-PE-FMP (mol %)</th>
<th>DSPE-PE-SMP (mol %)</th>
<th>DSPE-Rhodamine (or Fluorescein) (mol %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>49</td>
<td>5</td>
<td>45</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2.5% SMP</td>
<td>49</td>
<td>2.5</td>
<td>45</td>
<td>0</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>2.5% FMP</td>
<td>49</td>
<td>2.5</td>
<td>45</td>
<td>2.5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5% SMP</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>5% FMP</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10% SMP</td>
<td>44</td>
<td>0</td>
<td>45</td>
<td>0</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>10% FMP</td>
<td>44</td>
<td>0</td>
<td>45</td>
<td>10</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5% heteromultivalent (20 FMP:80 FMP)</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5% heteromultivalent (40 FMP:60 FMP)</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>5% heteromultivalent (50 FMP:50 FMP)</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>2.5</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>5% heteromultivalent (60 FMP:40 FMP)</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5% heteromultivalent (80 FMP:20 FMP)</td>
<td>49</td>
<td>0</td>
<td>45</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 10.1**: Summary of liposome compositions.

and collagen and incubated with activated platelets as previously described [18]. Briefly, human whole blood was drawn into citrated tubes from healthy donors, and platelet rich plasma (PRP) was obtained by centrifugation (150 x g, 15 min, room temperature). Platelets were activated using ADP (2 x 10^{-5} M) and were allowed to incubate on glass microscope slides pre-coated with collagen or albumin. The platelets adhered to the
collagen-coated region but did not adhere to the control albumin region. After fixing with 4% paraformaldehyde, the microscope slides were vacuum sealed within a standard PPFC system (10mm width x 0.01 in height) and were exposed to the flow of liposome suspensions, producing wall shear stresses of 5-55 dyn/cm$^2$. The unmodified or peptide-modified liposomes were allowed to flow through the system in a recirculating loop for 30 min to allow binding to the surfaces, followed by flow of PBS for an additional 15 min in an open loop to remove any loosely bound constructs and study the retention capabilities of the bound constructs. The slides were imaged for fluorescent liposome binding at various time points (5, 15, 30, 45 min of flow) using a Zeiss Axio Observer.D1 inverted fluorescence microscope with a 10x objective. Liposome binding was evaluated by quantifying the surface averaged fluorescence intensity values of the raw images using AdobePhotoshop® CS4 software.

2.3 Carboxyfluorescein encapsulation in liposomes and PLA$_2$-triggered release study

Carboxyfluorescein (CF) was chosen as a model payload to evaluate PLA$_2$-triggered release because of its inherent property of self-quenching at high concentrations (e.g. when encapsulated in nanoparticles) and increasing fluorescence when diluted (e.g. when released from nanoparticles). CF-loaded liposomes were fabricated using the RFEE technique described above, but by reconstituting the lipid film with 100mM CF in Tris-HCl buffer. Separation of free CF from CF associated with the liposomes was achieved by ultracentrifugation at 100,000 rpm for 20 min at room temperature using an Optima TLX Ultracentrifuge (Beckman Coulter). The supernatant containing the unencapsulated CF was aspirated and placed into a separate falcon tube. To determine encapsulation...
efficiency, the liposome pellet was resuspended in fresh buffer containing 10% TritonX-100 to disrupt the liposome membrane and release the encapsulated CF. Absorbance (at 492 nm) and fluorescence (λex~492 nm, λem~517 nm) of the CF in both the supernatant and contained in the liposome pellet were measured using a plate reader, CF concentrations in the two solutions were calculated using standard curves (Figure 10.4A-B), and CF encapsulation efficiency was determined using the following equation:

\[
EE (\%) = \frac{CF_{liposome \ pellet}}{CF_{liposome \ pellet} + CF_{supernatant}} \times 100
\]

To evaluate PLA₂-triggered payload release from our constructs, a standard release study was performed (Figure 10.5A) [26]. CF-loaded liposomes were fabricated as described above, unencapsulated CF was removed by ultracentrifugation, and the CF-loaded liposomes were resuspended in 5mM Tris-HCl + 1mM CaCl₂ buffer. Liposome suspensions were added to microcentrifuge tubes in the presence or absence of 2.5ng/ml PLA₂, which is within the elevated PLA₂ levels of cardiovascular patients [19]. The tubes were incubated on a gyratory shaker (~60 rpm) at 37°C for various time points between 0-5 hours. To quantify the amount of CF released at each time point, 100ul samples of the solutions were taken and CF fluorescence was measured using a plate reader. To determine percent release, the remaining liposome-contained CF was released by adding 10% TritonX-100 to the suspensions, and again fluorescence measurements were taken. Percent release was calculated as follows:

\[
Release (\%) = \frac{F_T - F_0}{F_{TritonX} - F_0} \times 100
\]
where $F_T$ is the fluorescence of CF in the solution at each time point, $F_0$ is the fluorescence of CF in the solution at $t=0$, and $F_{\text{TritonX}}$ is the fluorescence of the remaining CF disrupted from the liposomes.

2.4 *Streptokinase encapsulation, PLA$_2$-triggered release, and clot lysis under flow in vitro*

Once platelet binding of our constructs and PLA$_2$-triggered payload release were characterized, we evaluated the combination of these functionalities on clot lysis using streptokinase (SK) as a model thrombolytic drug under flow *in vitro* (Figure 10.6A). For this, SK was loaded within Rhodamine-labeled platelet-targeted liposomes using the RFEE technique with 25kU/ml SK in PBS to reconstitute the lipid film. Free SK was removed using ultracentrifugation, and encapsulation efficiency was evaluated as described in the previous section. To form fluorescein-labeled platelet-rich clots *in vitro*, PRP was obtained by centrifugation of human whole blood (150 x g, 15 min, room temperature), and the cytoplasm of the platelets was stained with Calcein AM at 2uM in PBS ($\lambda$ex~495 nm, $\lambda$em~515 nm) for 30 min at 37°C. The calcein-stained PRP was then centrifuged at 2500 x g for 25 min at room temperature to obtain platelet poor plasma (PPP) and a platelet pellet. The PPP was removed to get rid of excess calcein, and the platelet pellet was resuspended in fresh PPP. Clots were formed by adding 0.5nM thrombin and 0.5M CaCl$_2$ to the calcein-stained PRP, and subsequently incubating the activated PRP in a collagen-coated well of an acid washed microscope slide. These clot-containing slides were then assembled onto the PPFC system, and a solution of either control buffer (5mM Tris-HCl + 1mM CaCl$_2$) or Rhodamine-labeled SK-loaded platelet-
targeted liposomes in the presence of 2.5ng/ml PLA₂ was allowed to flow through the chamber and interact with the clot for 30 min at a wall shear stress value of 5 dyn/cm². The degree of clot lysis was examined by measuring the decrease in platelets’ calcein-associated fluorescence compared to the starting fluorescence in the well over time. The degree of Rhodamine-labeled liposome association with the clot was also measured.

2.5 Construct localization and binding to thrombus in vivo

The mouse model experiments were carried out in accordance to Cleveland Clinic Foundation IACUC-approved protocols. The ferric chloride (FeCl₃)-induced vascular injury is a simple and well-established model of occlusive thrombosis [28]. For this, 8-12 week old C57B16 mice were anesthetized with ketamine (100mg/kg)/xylazine (10mg/kg) via intraperitoneal injection, and the right common carotid artery (CA) is exposed. A 1x2 mm piece of Whatman #1 filter paper was saturated with 35% FeCl₃ solution and placed directly on the CA for ~5 min to produce a thrombus that occluded the vessel ~50% (Figure 10.7A). Fluorescein-labeled, non-targeted or platelet-targeted liposomes in saline were then injected at 30mg/kg in the right jugular vein. For real time intravital microscopy observation of particle localization and binding to the thrombus, a Leica DMLFS fluorescence microscope with an attached Gibraltar Platform and water immersion 10x objective was used. Video imaging was performed using a QImaging Retigo Exi 12-bit mono digital camera and Streampix version 3.17.2 software. To block background tissue fluorescence, a black plastic coffee stirrer was cut into a 3mm U-shaped piece and inserted behind the injured CA. Following the experiment, the mice were sacrificed and the CA was excised. Ex-vivo imaging of particle binding to the
thrombus site was performed using a Zeiss Axio Observer.D1 inverted fluorescence microscope with a 10x objective.

2.6 Statistical Analysis

Student’s t-test was used to analyze the difference between two means. All other statistical analyses between multiple groups were performed using one-way ANOVA with Tukey method. In all analyses, significance was considered to be $p < 0.05$.

3. Results

3.1 Platelet-binding of constructs with varying extent of FMP and SMP decoration

**Figure 10.2A** shows the fluorescence microscopy images at the 30 and 45 min time points of FMP- and SMP-decorated liposomes binding to an activated platelet surface under flow at 5, 25, and 55 dyn/cm$^2$ wall shear stress conditions in a PPFC setup. The first 2 rows are liposomes containing 2.5 mol % of SMP-only or FMP-only liposomes, respectively. The next 2 rows are liposomes containing 5 mol % of SMP-only or FMP-only liposomes, followed by 2 rows of liposomes containing 10 mol % of SMP-only or FMP-only. The last row shows platelet-bound liposomal constructs that were heteromultivalently surface-modified with 2.5 mol % FMP and 2.5 mol % SMP (hence total 5 mol % of peptide at 50:50 ratio). **Figure 10.2B-D** show the quantitative analysis of fluorescence intensity of platelet-bound liposomes at the 30 min time point (binding under flow) and the 45 min time point (retention under flow) for the three shear stress values. As evident from the results, increasing the mol % of the FMP- or SMP-only constructs from 2.5 to 5 mol % did not have a significant impact on the degree of particle
binding to the active platelets, but did show a significant increase in particle retention at high shear (55 dyn/cm²). Furthermore, increasing the ligand decoration from 5 to 10 mol% generally resulted in less platelet binding (except for SMP liposomes at 5 dyn/cm²), possibly due to steric interference or competition in receptor binding.

Additionally, heteromultivalently-decorated constructs showed the maximum binding and retention at all three shear stress values compared to constructs containing FMP only or SMP only, even when the total peptide content was less.
3.2 Platelet binding for varying FMP:SMP ratios at fixed total peptide decoration

As the results in the previous sections indicated that liposomes decorated simultaneously with FMP and SMP have higher platelet binding and retention capabilities compared to liposomes bearing FMP only or SMP only, further PPFC experiments were carried out where the FMP:SMP ratio was varied while keeping the total peptide decoration at 5 mol % to determine whether modulation of FMP:SMP ratio results in variation of liposome adhesion and retention at the various shear values. Figure 10.3 A shows representative fluorescence images of platelet binding (30 min time point) and retention (45 min time point) of liposomes for the varying ratios of FMP:SMP. Figure 10.3 B-D show the corresponding quantitative data at the three shear values. As evident from the data, all heteromultivalently-decorated constructs, regardless of FMP:SMP ratio, demonstrated enhanced platelet binding compared to FMP-only or SMP-only constructs at all three shear stress values. Under medium (25 dyn/cm$^2$) to high (55 dyn/cm$^2$) shear conditions, liposomes containing 60% FMP and 40% SMP demonstrate the highest degree of platelet binding and retention. This is probably due to the relative abundance of GPIIb-IIIa receptors (~80,000 per platelet) compared to P-selectin (~10,000 per platelet) on active platelets [29], [30]. Interestingly, at low shear (5 dyn/cm$^2$), constructs containing 40:60 and 80:20 FMP:SMP ratios demonstrated the highest platelet binding. However, since most vessels with thrombo-occlusions will have elevated shear stress values, we chose to continue the rest of the experiments using the optimally performing 60% FMP and 40% SMP containing constructs.
3.3 Carboxyfluorescein encapsulation and PLA$_2$-triggered release from liposomes

CF, a model payload, was loaded into the liposomes during the RFEE process. Figure 10.4 A and B show the CF absorbance and fluorescence calibration data, based on which the encapsulation efficiency (EE) was determined. Figure 10.4 C shows three batches of EE analysis. As evident from the data, the average EE was ~75%. These CF-loaded liposomes were then subjected to incubation in buffer at 37°C in the presence of absence of PLA$_2$ at pathological concentrations (Figure 10.5A). Figure 10.5B shows the CF release profile in these conditions. As evident from the data, the presence of PLA$_2$ leads
to significantly greater CF release, even at short time periods (e.g. 30 min), demonstrating the enzyme-triggered release.

### 3.4 Streptokinase-induced clot lysis under flow in vitro

Combining the platelet targeting and PLA2-triggered payload release functionalities demonstrated by the previous results on a single construct platform, we next evaluated the ability of streptokinase-loaded particles to facilitate clot lysis under a physiologically relevant flow environment in vitro using the PPFC setup (Figure 10.6A). SK was loaded into fluorescently-labeled, platelet-targeted liposomes during the RFEE process, and the encapsulation efficiency was determined to be 29.91±5.36%. These particles were then allowed to flow over and interact with a green fluorescent clot in the PPFC in the presence of PLA2. Buffer was used as a negative clot lysis control condition. Figure 10.6B shows representative fluorescence images of the clot (green) and particles bound to the clot surface (red) at various time points between 0-30 min during the assay. Percent clot lysis was determined by comparing the clot (green) fluorescence at each time point compared to the starting clot fluorescence, and the

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Figure 10.4: (A) and (B) show, respectively, the carboxyfluorescein fluorescence and absorbance calibration curves based on which the encapsulation efficiency (EE) of CF in the liposomes was determined. (C) is the EE analysis from three batches of liposomes, showing that the average EE was ~75%.

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quantitative results are shown in Figure 10.6C. As evident from the data, thrombus-targeted SK-loaded particles demonstrated significant clot lysis up to ~70% over the course of 30 min compared to the buffer control condition. Figure 10.6D shows a graph quantifying the particle fluorescence (red) intensity in the images, demonstrating increasing particle binding to the clot over the course of the 30 min experiment.

3.5 Platelet-targeted construct binding to thrombus in vivo
The results in our previous publication [18] as well as in the previous sections demonstrate the ability of our particles to bind to areas of activated platelets under physiologically relevant conditions \textit{in vitro}. In the current studies, we have investigated the capability of these platelet-targeted constructs to localize to the site of a thrombo-oclusion \textit{in vivo} in a ferric chloride (FeCl$_3$)-induced murine carotid arterial injury model (Figure 10.7A). Figure 10.7B shows representative snapshots of real time intravital

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure10_6.png}
\caption{(A) Schematic representation of PPFC clot lysis experiment. (B) Representative fluorescence images of the clot surface (green) and particles bound to the clot (red) at various time points. (C) Quantified analysis of clot lysis based on the change in clot (green) fluorescence over time shows significant clot lysis up to ~70% by the thrombus-targeted, SK-loaded particles in the presence of PLA$_2$ compared to the saline control condition. (D) Quantitative analysis of particle (red) fluorescence intensity shows an increase in amount of bound particles over the course of the experiment.}
\end{figure}
microscopy videos of particle localization to the CA thrombo-occlusion. Figure 10.7C shows representative ex-vivo fluorescence images of particles bound to the thrombus. As evident by the data, platelet-targeted constructs show enhanced localization and binding to the thrombus compared to unmodified control constructs. Although these were initial pilot studies (n=1 for each group), the results show the potential promise of these platelet-targeted constructs to localize to thrombus site in vivo.

![Diagram](image)

**Figure 10.7:** (A) Schematic representation of ferric chloride-induced carotid artery thrombo-occlusion formation in mice. (B) Representative grayscale snapshots of real time intravital microscopy video shows platelet-targeted enhanced particle accumulation at the site of thrombo-occlusion compared to non-targeted particles. (C) Representative ex-vivo fluorescence microscopy images of the excised carotid artery show enhanced targeted-particle binding (green fluorescence) to the thrombus both 1 and 2 hours post-occlusion compared to non-targeted control particles.

4. Discussion

The work presented here is driven by the need of a drug delivery strategy that can minimize the harmful issues associated with systemic delivery of vascular drugs (stated
in the introduction), while allowing sufficient drug availability and action at the target site. Specifically, our research is aimed at developing nanoparticle-mediated delivery systems that can specifically target and bind to the site of vascular disease via heteromultivalent interactions with active platelet surface receptors and can allow site-selective enzyme-triggered delivery of various bioactive and therapeutic agents. Our previous results have demonstrated that binding and retention of nanoparticles to active platelets under physiologically-relevant flow can be significantly increased utilizing a heteromultivalent targeting approach [18]. To this end, we modified the surface of liposomal nanoconstructs with two active platelet targeting peptides, a fibrinogen mimetic GPIIb-IIIa binding peptide (FMP) and a sialoprotein mimetic P-selectin-binding peptide (SMP), at equal ratios (50:50) and constant total mol%. In our current study, we have varied the total mol% ligand composition as well as the peptide decoration ratio for particle surface modification to determine an optimum surface-density and ratio that facilitates maximum enhanced binding and retention to active platelets under flow. Our results demonstrated that increasing the total amount of ligands on the particle surface from 2.5 to 5 mol% did not have a significant impact on modulating the degree of nanoparticle binding to platelets, but did show a significant increase in particle retention at high shear (55 dyn/cm²) (Figure 10.2). Furthermore, a more concentrated peptide decoration on the particle surface (10 mol%) demonstrated less platelet binding and retention, possibly due to steric interference or competition in receptor binding. This data is consistent with recent reports where too low or high ligand densities show decreased receptor binding properties and optimal nanoparticle binding is acheived at moderate ligand densities [31], [32]. In the case of our nanoparticle system, 5 mol% ligand
concentrations demonstrated consistent platelet binding and particle retention at low-to-high shear stress values, thus we can conclude that 5 mol% is our optimum ligand density. We also investigated the effect of varying heteromultivalent ligand ratios on platelet binding under flow in vitro (Figure 10.3). Particles containing 60% FMP and 40% SMP demonstrated superior platelet binding and retention at vascular disease-relevant medium-to-high shear stress values (25-55 dyn/cm²) compared to other ratios. These results suggest the benefit of having more GPIIb-IIIa binding motifs on the surface of the particles since this receptor is expressed at ~8x higher levels than P-selectin on active platelets. However, particles containing too few P-selectin binding motifs (0-20% SMP) show a decrease in platelet binding, demonstrating the importance of ligand cooperativity in heteromultivalent binding. Thus, we can conclude that optimized platelet targeting and binding retention under hemodynamic blood flow can be achieved with liposomal constructs containing 3 mol% FMP and 2 mol% SMP ligand decoration.

Once the particles have stably localized to the site of thrombo-occlusion via active platelet binding, we further refined our nanoparticle system to site-specifically deliver therapeutic agents triggered by vascular disease site abundant enzyme, PLA₂. PLA₂ is secreted by activated platelets and is found at elevated levels in areas of vascular disease. By incorporating PLA₂-sensitive phosphotidylcholine into our liposomal membrane, we have demonstrated triggered release of a model payload (carboxyfluorescein) from our constructs in the presence of PLA₂ (Figure 10.5). When combining the platelet binding with PLA₂-triggered release functionalities onto a single construct containing a model thrombolytic drug (streptokinase), our constructs demonstrated efficient clot binding and SK-induced clot lysis (~70% in 30 minutes) under flow conditions in vitro (Figure 10.6).
Furthermore, we have demonstrated the ability of our platelet-targeted constructs to localize to the site of a thrombo-occlusion in vivo in a ferric chloride (FeCl$_3$)-induced murine carotid arterial injury model (Figure 10.7).

The combination of our in vitro and preliminary in vivo data suggest the promise of this nanoparticle system to site-specifically deliver bioactive and therapeutic agents to sites of thrombo-occlusions. It is to be noted that the current study only evaluated the loading, release, and resultant clot lysis of a single concentration of SK, and further refinement of drug loading and dosage as well as evaluation of other therapeutic compounds should be performed to evaluate the efficacy of this construct for prevention and treatment of thrombo-occlusions. Also, lipid composition of the construct could be tailored to optimize PLA$_2$-triggered payload release in clinically appropriate time frames. Lastly, the therapeutic efficacy of this nanoparticle system needs to be evaluated in appropriate animal models. These components will become part of our ongoing and future studies.

We have chosen liposomes as our nanovehicle because of their biocompatibility, established clinical utility, and sensitivity to the vascular disease-abundant enzyme, PLA$_2$. However, liposomes have demonstrated stability issues in plasma due to their interactions with high and low density lipoproteins (HDL and LDL, respectively), leading to release of encapsulated drug into the plasma [33]. The addition of cholesterol into the liposome membrane has shown to increase their stability in plasma [34], but this also decreases the ease at which drug is released at the target site. Additionally, the concentration of drug loaded into the liposome can affect the stability of the final formulation, limiting the drug payload capacity of these particles [35]. Therefore,
alternative systems, such as polymeric nanoparticles, may need to be investigated to address possible issues with stability and limited drug loading. Furthermore, the PLA\(_2\)-triggered drug release mechanism presented here may not facilitate rapid enough release of payload to adequately lyse the clot site in a timely manner, \textit{in vivo}. Consequently, the nanoparticle system may need to be further refined to allow for alternative triggered release mechanisms, such as exogenous stimuli (e.g. heat, light, ultrasound, etc) \cite{36}.

5. Conclusions

The current study builds upon our previous report of utilizing a heteromultivalent targeting approach to facilitate active platelet binding under physiologically-relevant flow. To this end, platelet-targeted liposomes were developed that can simultaneously bind to active platelet GPIIb-IIIa via a fibrinogen-mimetic peptide (FMP) and P-selectin via a sialoprotein-mimetic peptide (SMP). Here, we demonstrate that our heteromultivalent design allows compositional modulation of the ligand decorations on the construct surface to optimize their binding to activated platelets in low, moderate, and high shear stress environments, \textit{in vitro}. Furthermore, these liposomes have been further engineered to release their contents in the presence of vascular disease site-abundant enzyme, PLA\(_2\). We demonstrate that combining these functionalities allows for efficient clot lysis using a model thrombolytic drug (SK) under flow \textit{in vitro}. Additionally, our platelet-targeted constructs were able to localize and stably bind to the site of thrombocombination in vivo. These results suggest the promise of such an approach in drug delivery for vascular diseases.
6. Acknowledgements

All animal studies were performed by Dr. Wei Li at the Cleveland Clinic Foundation’s Lerner Research Institute.

7. References


Chapter 11 : Conclusion and Future Directions

1. Summary and Conclusions of Completed Work

Conventional systemic administration of drugs and other bioactive molecules presents several limitations including dilution and deactivation of drug in the blood, poor biodistribution and pharmacokinetics, insufficient drug concentrations at the target site, and indiscriminate drug action leading to harmful side effects [1–3]. These issues with current therapies can potentially be resolved by localizing the delivery (and action) of the drugs specifically to the target site. With this rationale, nanomedicine is a broad and interdisciplinary field that has seen an explosion in research activity over the past few decades [4], [5]. The ideal nanomedicine drug delivery system should be able to package drug at appropriate doses and protect it from plasma deactivation, circulate through the body without rapid clearance, home to and accumulate/stay retained at the target site, and release drug at the target site in appropriate quantities and time frames. Nanomedicine strategies have demonstrated substantial pre-clinical and some clinical success in the area of cancer therapy; however, the clinical use of nanoparticles in other disease areas has been limited [6–10]. One field that can benefit significantly from nanomedicine applications is that of cardiovascular diseases [11–15]. However, the complex hemodynamic environment of the vascular system has presented several challenges for nanoparticle-mediated drug delivery. The first challenge in vascular nanomedicine is designing delivery vehicles that are able to traverse across the bulk blood flow and marginate towards the vascular wall. Secondly, the vehicles must bind specifically to the vessel wall at the target vascular disease site via active targeting strategies in order to allow sufficient disease site accumulation and stable retention under hemodynamic flow.
conditions. Lastly, once stably anchored to the vascular disease site, the particles must be able to deliver drug in a controllable fashion.

To address the engineering challenges in vascular nanomedicine, we have used blood platelets as a unique design paradigm. Platelets are megakaryocyte-derived, anucleated blood cells that have the innate ability to marginate to the vascular wall, interact actively with multiple specific cellular antigens and matrix proteins simultaneously at the vascular wall to render stable anchorage under hemodynamic flow, and release a variety of bioactive molecules to modulate physiologic (e.g. in hemostasis) or pathologic (e.g. in thrombosis) mechanisms [12], [16–27]. To this end, the design of a vascular nanomedicine platform can be envisioned that incorporates surface-mediated biological interactions, margination influencing particle shape/size/mechanical properties, and site-specific triggered drug release mechanisms to ensure maximum performance efficacy. Of these three design attributes, the current dissertation has focused principally on platelet-inspired cell-cell and cell-matrix biointeractions, and the central hypothesis of this dissertation is that utilizing platelet-inspired heteromultivalent biointeractions for surface-engineering of synthetic drug delivery platform will result in enhanced specific binding and retention of the delivery system at a vascular disease site under hemodynamically relevant flow conditions. To evaluate this hypothesis, I have investigated the functional mimicry of several platelet properties to design two vascular nanomedicine systems, (i) a platelet-inspired synthetic hemostat for management of bleeding complications and (ii) a platelet-inspired vehicle for targeted delivery of thrombolytic agents to a clot site.
1.1 Platelet-inspired synthetic hemostat

Platelet transfusion plays a major role in the treatment of bleeding complications in patients with various hematologic or oncologic platelet disorders as well as in rapidly staunching heavy bleeding in surgical or traumatic injury situations [28–33]. However, natural platelet products have several limitations such as limited availability, short shelf life (only 3-7 days), risk of biological/pathological contamination, and multiple biologic side effects [34–45]. Therefore, there is significant clinical interest in a synthetic construct that can mimic or amplify platelet’s hemostatic functionalities while allowing large-scale production, long storage life, and absence of biologic side effects [46]. The majority of approaches on mimicking platelet’s hemostasis-relevant functions have focused on amplifying platelet’s “aggregation” functionality by decorating synthetic particle surfaces with aggregation-promoting biomolecules like fibrinogen (Fg) or Fg-derived peptide sequences [47–53]. Several approaches have focused on mimicking platelet’s injury site “adhesion” functionality by decorating synthetic particle surfaces with adhesion-promoting biomolecules like recombinant GPIbα (to promote vWF-binding) or GPIa-IIa (to promote collagen-binding) [54–57]. However, in natural primary hemostasis, both platelet “adhesion” and “aggregation” functionalities in tandem are critical for regulating injury site-specific clot formation. With this rationale, we have designed a platelet-inspired synthetic hemostatic construct that can adhere to an injury site via simultaneous binding to vWF and collagen and subsequently promote platelet aggregation via GPIIb-IIIa binding utilizing a heteromultivalent ligand modification approach.
In Chapter 4, the development of a liposomal nanoconstruct was reported which combines the mimicry of platelet’s adhesion to vWF and collagen under flow via heteromultivalent decoration with a vWF-binding peptide (VBP, TRYLRIHPQSWVHQI) and a collagen binding peptide (CBP, [GPO]7). These constructs demonstrated shear-dependent adhesion to vWF surfaces and shear-independent adhesion to collagen surfaces under flow in vitro using a parallel plate flow chamber (PPFC) setup. Moreover, heteromultivalent decoration of constructs with both vWF and collagen binding motifs showed significantly enhanced adhesion to vWF/collagen mixed surfaces compared to liposomes bearing vWF-binding or collagen-binding ligands only.

Building on these results, Chapter 5 outlines the introduction of a third peptide onto the particle surface, specifically a fibrinogen mimetic active platelet clustering cyclic-RGD peptide (cRGD, cyclo-CNPRGDY(OEt)RC) which promotes the “aggregation” functionality of platelets. Surface-immobilized cRGD-containing liposomes demonstrated the capability to aggregate active platelets onto themselves. Subsequently, we demonstrated that heteromultivalent liposomes bearing VBP, CBP, and cRGD, when introduced in flow with ~20,000 activated platelets per microliter, are capable of adhering to vWF/collagen surfaces and promoting the recruitment/aggregation of platelets onto themselves.

In Chapter 6, the capability of optimizing the platelet-mimetic hemostatic properties of our liposomal constructs in vitro was demonstrated by modulating the ligand-decoration densities and ligand ratios. Modulation of VBP and CBP densities and relative ratios enabled optimized construct adhesion under varying shear flow conditions. Modulation of cRGD density enabled enhancement of construct-promoted platelet
aggregation. Furthermore, we demonstrated the enhanced hemostatic efficacy of functionally-integrated platelet-mimetic constructs (containing VBP, CBP and cRGD) \textit{in vivo} in a mouse tail-transection model compared to “adhesion-only” or “aggregation-only” constructs.

Chapter 7 further evaluated the molecular mechanism of VBP binding to vWF. The VBP sequence is derived from the coagulation factor VIII, which binds to vWF’s D’-D3 domain, while natural platelet GPIbα binds to vWF’s A1 domain. Therefore, we hypothesized that the VBP-decorated constructs would adhere to vWF without mutual competition with natural platelets. To this end, we selectively blocked vWF’s A1 domain with glycocalcin and studied the binding of VBP-decorated nanoconstructs versus platelets to ristocetin-treated vWF. The results of this study demonstrated that such A1 domain-blocking resulted in significant reduction of platelet adhesion but did not affect VBP-decorated construct binding. Furthermore, when co-decorated with VBP and cRGD, the constructs enhanced active platelet aggregation to vWF surfaces. These results indicate the significant promise in utilizing the FVIII-derived VBP for our synthetic hemostat since it does not interfere with vWF-binding of natural platelets but allows site-directed enhancement of platelet aggregation when combined with cRGD.

Altogether, these results show the feasibility and promise of integrating platelet’s hemostatic functionalities of “adhesion” and “aggregation” simultaneously on synthetic constructs via a heteromultivalent ligand modification approach.

\textit{1.2 Platelet-inspired thrombus-targeted drug delivery}
Vascular diseases, leading to thrombo-occlusive and ischemic end points, are the leading cause of tissue morbidity and mortality in the United States and globally [58]. Current clinical approaches in cardiovascular diseases are focused on prophylactic, emergent and sustained prevention of thrombo-occlusive events to maintain normal blood flow to vital tissues and organs. Many of these approaches involve the systemic (oral or IV) administration of anti-platelet, anti-coagulant, fibrinolytic, or anti-proliferative agents. However, systemic administration of these drugs presents a number of harmful issues including rapid drug washout from the target site, plasma-induced inactivation of the drugs and reduced circulation half-life, and systemic non-specific distribution and action of the drugs leading to sub-optimal availability at the target site and harmful side effects like coagulaopathy, neuro- and nephrotoxicity and hemorrhage [59–62]. Therefore, disease site-selective delivery of therapeutic agents can provide significantly enhanced treatment efficacy compared to systemic administration of the same agents. Based upon the above rationale, we have developed a nanoparticle-based delivery system that can specifically target and bind to the site of vascular disease and can allow site-selective triggered delivery of various bioactive and therapeutic agents.

In Chapter 9, the development of a liposomal nanoconstruct was reported which simultaneously binds to integrin GPIIb-IIIa and P-selectin on activated platelets to provide synergistic mechanisms for enhanced selectivity to vascular disease sites. Specifically, these constructs were heteromultivalently decorated with a fibrinogen mimetic GPIIb-IIIa-binding linear RGD peptide (FMP, GSSSGRGDSPA) and a sialoprotein mimetic P-selectin binding peptide (SMP, DAEWVDVS). The liposomes were studied for their platelet-specific interactions inside of a PPFC at low-to-high shear
stresses, and the interaction specificity with the platelet receptors was further confirmed by flow cytometry. The results indicated that liposomes surface co-modified with both FMP and SMPs have higher selectivity as well as retention to activated platelets under flow compared to liposomes bearing any one peptide type.

Building on this, Chapter 10 demonstrates optimization of this heteromultivalent platelet targeting strategy by modulating the ligand-decoration densities and ligand ratios. Furthermore, an enzyme-triggered payload release functionality is introduced into the nanoparticle design. Specifically, liposomes rich in phosphatidylcholine lipid show sensitivity to thrombus site abundant enzyme, phospholipase A2 (PLA2), and our results demonstrate triggered payload release from the liposomal constructs in the presence of PLA2. Combining the platelet targeting and PLA2-triggered payload release functionalities on liposomes containing a model thrombolytic drug (streptokinase, SK), we demonstrated enhanced clot lysis under flow, in vitro. Lastly, using a mouse carotid artery thrombosis model, the nanomedicine system showed thrombus site localization and binding in vivo.

Overall, these results suggest the promise of a platelet-inspired nanoparticle-based delivery system that can specifically target and bind to the site of vascular disease via heteromultivalent binding to active platelets and can allow site-selective enzyme-triggered delivery of various bioactive and therapeutic agents.

2. Future Directions
The work detailed in this dissertation has begun to address some of the current challenges in vascular nanomedicine, but there are still many avenues of study that need to be investigated further.

2.1 Quantification of heteromultivalent nanoparticle binding strength

Heteromultivalent modification of nanoparticles has demonstrated enhanced binding and retention under flow in our studies as well as those reported by others [63]. It is theorized that this ligand modification approach increases the binding strength and decreases the rate of nanoparticle dissociation from the target cell under the shear stress of fluid flow due to the cumulative strength of the individual ligand-receptor interactions, a concept known as avidity. Hong et al. have evaluated the binding avidity of a nanoparticle platform containing dendrimeric presentation of folate molecules using Surface Plasmon Resonance (SPR) to quantify the dissociation rate constants ($K_D$) [64]. Nanoparticles containing multivalent presentation of the targeting ligand demonstrated dramatically enhanced $K_D$ (~2,500- to 170,000-fold) compared to free folate molecules. Silpe et al. have further demonstrated that the avidity displayed by multivalent nanoparticles varies in response to the target cell receptor density [65]. Based on these studies, we can hypothesize that our heteromultivalent targeting approach also demonstrates enhanced binding strength due to avidity and that targeting two different surface receptors increases the “density” of available target receptors. This concept has been briefly explored through the preliminary development of a mathematical model of nanoparticle binding under flow (Appendix I). Taking avidity, target receptor density, and the shear forces of fluid flow into consideration, the model predicts the enhanced binding characteristics of heteromultivalently-decorated constructs compared to
respective homomultivalent particle controls. Future work should test this hypothesis experimentally by evaluating the binding strengths of our heteromultivalent nanoparticle systems compared to their respective homomultivalent counterparts, possibly using this SPR method. Atomic force microscopy (AFM) could provide another avenue to evaluate nanoparticle binding strength. AFM has been used to quantify the binding strengths of individual ligand-receptor interactions by modifying the cantilever tip with ligands and measuring interaction forces with receptor-coated surfaces in the pico-micro Newton range [66]. Based on this, we envision that the cantilever tip could be modified with heteromultivalently ligand-decorated nanoparticles, and the strength of interaction with their respective target cells can be quantified and compared to homomultivalently decorated particles.

2.2 Integration of margination, active targeting, and triggered payload release

The current dissertation focused principally on platelet-inspired heteromultivalent biointeractions, and the model particles used in our most of our studies were spherical unilamellar liposomes about 150 nm in diameter. As outlined in Chapter 1, one of the main design challenges in vascular nanomedicine is designing of platelet-inspired vehicles such that they are able to traverse across bulk blood flow and marginate to the vascular wall. Several recent mathematical modeling as well as experimental studies have demonstrated that there exist significant correlations between the shape, size, and modulus of particles to their location in hemodynamically relevant flow patterns [67–69]. For natural platelets, their functions at the vessel wall depend upon their ability of “margination” through RBCs and other blood components, and this hemodynamic migration is significantly influenced by platelet’s physical and mechanical properties
On the basis of such observations, we rationalize that an additional critical component of both the synthetic hemostat and vascularly targeted drug carrier designs will be identification of the optimum particle physical (e.g. size and shape) and mechanical (e.g. modulus) properties that can facilitate enhanced wall-margination of the particles. We envision that, by integration of margination-favoring optimal physical and mechanical parameters (size, shape, and modulus) with optimal biological parameters (chemistry and density of ligand modifications), site-specific accumulation of vascular nanomedicine strategies can be significantly enhanced. With this rationale, we have conducted preliminary studies with albumin-based platelet-like nanoparticles (PLNs) that closely mimic natural platelet’s shape and flexibility (modulus) (Appendix II). These PLNs demonstrated enhanced margination and binding under physiologically-relevant flow conditions in vitro compared to spherical or rigid (non-flexible) disc-shaped nanoparticles, suggesting the benefit of platelet-mimetic shape and modulus. When heteromultivalently decorated with our adhesion (VBP and CBP) and aggregation (FMP) promoting ligands, these PLNs demonstrated enhanced hemostatic capabilities in vitro and in an in vivo mouse tail transection model. Future work should include testing a more comprehensive metric of construct size, shape, and modulus to determine the optimum parameters to achieve maximum margination followed by coupling of these optimized particles with the heteromultivalent ligand decoration strategies established in this dissertation.

Additionally, although we demonstrated one possible approach for site-specific delivery of payload in regards to thrombolytic therapy (Chapter 10), we envision that the synthetic hemostat could also be engineered to release bioactive molecules to help
regulate hemostasis. For example, controlled release of platelet agonists or coagulation factors, like ADP or thrombin, may be used to help promote secondary hemostasis while subsequent release of other factors such as tissue plasminogen activator (TPA) may be used to promote clot resection once the injury has healed. Altogether, future work should include combining heteromultivalent biological interactions, margination influencing particle shape/size/mechanical properties, and site-specific triggered drug release mechanisms to ensure maximum performance efficacy of the vascular nanomedicine strategies described in this dissertation.

2.3 Future in vivo studies – platelet-inspired synthetic hemostat

The results from the pilot studies completed in this work demonstrated the ability of our heteromultivalently-modified, multifunctional synthetic hemostatic nanoparticles to reduce bleeding times in an acute but non-severe injury in normal mice. In order to further investigate the hemostatic potential of these constructs, it would be beneficial to evaluate our nanoparticle system in a severe injury model, such as the acute liver resection model in rats [71], [72]. In this model, the median lobe of the liver is resected, and blood loss and survival rates up to 1 hour after injury are recorded. Furthermore, future work should include investigating these constructs for applications in chronic bleeding disorders using a tail transection bleeding time assay in thrombocytopenic mice. To induce transient thrombocytopenia, an intra-peritoneal injection of bulsulfan at a dose of 1mg/kg of animal will be administered, which has been established to reduce platelet counts to ≤50,000/ul within a few hours [73–75]. As shown in our pilot studies, the transected tail bleeding time in normal mice is ~100s, and inducing thrombocytopenia should increase this bleeding time 2-4 fold. In both of these models, our hemostatic
nanoparticles would be administered ~30 min before injury (prophylactic) or immediately following injury (emergent), and outcomes would be compared to saline, blank nanoparticle, and current clinical treatment (e.g. recombinant FVIIa) controls.

2.4 Future in vivo studies – platelet-inspired vehicles for targeted delivery of agents to a clot site

Our preliminary in vivo studies outlined in this dissertation suggest the promise of our platelet-targeted nanoparticles to localize and bind to the site of a ferric chloride-induced arterial thrombo-occlusion in mice. However, this pilot study only investigated the localization of targeted versus non-targeted nanoparticles in one mouse each. Therefore, these studies should be continued with multiple animals per condition in order to get quantifiable data. Furthermore, this animal model of thrombo-occlusion could be used to evaluate the drug delivery and resultant thrombolysis aspect of our nanoparticle design. Although we chose to use streptokinase (SK) as a model thrombolytic drug for our initial in vitro clot lysis studies, there are several clinically relevant thrombolytic drugs that could be employed in our nanoparticle system. SK facilitates the lysis of clots by indirectly activating plasminogen through a two-step process, and this process requires the partial consumption of plasminogen (and plasmin), which can result in decreased therapeutic efficacy compared to direct plasminogen activators such as urokinase (UK) and tissue plasminogen activator (tPA) [76]. Additionally, because it is derived from streptococci, SK may induce antibody activity causing systemic reactions such as fever and even anaphylaxis [77]. Although UK activates plasminogen directly and demonstrates effective thrombolysis, it is not fibrin specific, and therefore suffers from issues of systemic fibrinogenolysis [76]. tPA, on the other hand, is fibrin specific and has
been shown to be ~10-300 times more efficient than UK on a molecular basis [78], [79]. Therefore tPA is the most common clinically used thrombolytic agent and may be a better thrombolytic agent to package in our thrombus-targeted nanomedicine system. To evaluate in vivo clot lysis, circulating platelets would be pre-stained to allow visualization of the clot using intravital microscopy, arterial thrombo-occlusion would be induced using ferric chloride, our platelet-targeted, PLA2-sensitive, thrombolytic drug-loaded liposomes would be subsequently injected via the tail vein, and thrombolysis would be evaluated by measuring the size of the fluorescent clot over time using intravital microscopy. As an additional measure of thrombolysis, restoration of blood flow can be monitored using a sonographic flow probe downstream of the clot. Saline, non-targeted particles, platelet-targeted particles without drug, and injection of free drug should be used as controls. A second method that could be investigated for inducing thrombo-occlusion formation in mice is a minimally invasive laser injury model [80]. The protocol for this injury involves focusing an argon-ion laser through a compound microscope on the vasculature of a mouse ear that is thin enough to visualize blood flow via intravital microscopy. The severity of the occlusion can be varied by modulating the intensity and time of laser illumination. Subsequent evaluation of nanoparticle targeting and thrombolysis can be evaluated in a similar manner to the ferric chloride model.

In addition to site-specific delivery of thrombolytic agents, other potential payloads of interest include contrast agents to facilitate simultaneous diagnosis and therapy, image guided therapy, or post-therapy image-assisted evaluation of treatment efficacy. A spectrum of molecular imaging approaches using nanovehicle systems are currently being studied in the vascular arena, especially atherosclerotic plaque and
thrombus imaging using magnetic resonance (MR) [81–83]. To this end, future work could include simultaneously incorporating MR contrast agents, such as gadolinium, into our nanoparticle system for theranostic applications in cardiovascular diseases.

3. References


Appendix I: Mathematical Model of Heteromultivalent Binding of Nanoparticles

Introduction

The field of nanomedicine, or using nanoparticles to package and deliver diagnostic or therapeutic cargo specifically to target disease sites in the body, has been an active area of research over the past few decades. In the context of drug delivery, the majority of nanomedicine strategies involve the encapsulation of drug molecules into nanoparticles that, because of their size, can passively accumulate at disease sites in the body.

Additionally, the surface of these nanoparticles can be modified with ligands to facilitate active binding to disease site receptors, further enhancing their site-specific accumulation and drug action [1]. These ‘actively targeted’ nanoparticles have been widely investigated for applications in cancer and vascular medicine and are rapidly expanding to other disease areas [2–4]. Although most ‘actively targeted’ nanoparticles are surface modified with only one type of ligand (termed homomultivalent) that binds to one type of disease site-specific receptor, recent studies have shown that nanoparticles that are simultaneously modified with multiple types of ligands (heteromultivalent) that bind to multiple disease site-specific receptors render enhanced targeting specificity, binding efficacy and retention stability under a physiologically-relevant flow environment.

Figure 1: Homomultivalent and Heteromultivalent binding.
Along these lines, our group has investigated the use of heteromultivalently modified nanoparticles for several disease targeted nanomedicine strategies [6–9].

In one of the simplest cases, we simultaneously decorated the surface of nanoparticles with 2 different ligands which facilitate binding to 2 different cell receptors overexpressed at the site of vascular disease. Specifically, liposomal nanoparticles were heteromultivalently modified with a GPIIb-IIIa (Receptor A)-binding peptide and a P-selectin (Receptor B)-binding peptide which allow for binding to disease site activated platelets. In an in vitro flow environment, we have shown that these heteromultivalent particles yielded a higher degree binding to activated platelets compared to particles containing only one type of targeting ligand (Figure 2) [6].

In order to better understand these experimental results, a mathematical model of nanoparticle binding to cells has been developed. Individual ligand-receptor interactions have been extensively modeled [10], [11]. Building on these models, nanoparticle
binding to target cells has been simulated, but only using homomultivalent approaches [12]. The purpose of this paper is to mathematically model the phenomenon of nanoparticle binding to a target cell via heteromultivalent interactions. Additionally, we have incorporated the effect of shear stress on nanoparticle binding to better simulate interaction conditions under physiologically-relevant flow environments such as blood flow. The results of this mathematical model should help us to better predict nanoparticle binding behavior as well as intelligently design nanoparticle systems to maximize target cell binding under flow.

Mathematical Model

**Ligand Receptor Binding Model**

The law of mass action states that if a ligand (L) binds with a receptor (R) to produce a complex (C) in a reversible manner, the rate of the forward reaction is $k_a$ and the rate of the backward reaction is $k_d$ as shown in Figure 3.

By evaluating the change in ligand, receptor and complex concentrations over time, we can get a system of first order differential equations to mathematically describe the binding kinetics of the system:

$$\frac{dC}{dt} = \frac{dL}{dt} = \frac{dR}{dt} = k_a LR - k_d C$$

Since the equations for L and R are equal, we can simplify to two differential equations that completely describe the system:

$$\frac{dC}{dt} = k_a LR - k_d C$$

(Equation 1)
\[
\frac{dR}{dt} = -k_a L R + k_d C
\]

The total receptor concentration (R_T) on the cell’s surface can be considered to be constant, therefore

\[ R_T = R + C , \quad R = R_T - C \]

Additionally, the ligand concentration (L) on the nanoparticle’s surface is constant, and the concentration of nanoparticles is in excess to the number of available receptors, therefore the ligand concentration at any given time in the system can be approximated by initial ligand concentration:

\[ L \cong L_0 \]

Applying these assumptions, we get a system where the only unknown variable is the complex concentration:

\[
\frac{dC}{dt} = k_a L_0 (R_T - C) - k_a C \tag{Equation 2}
\]

\[
\frac{dR}{dt} = -k_a L_0 (R_T - C) + k_d C
\]

to determine that stability of the system, we can compute the Jacobian:

\[
J = \begin{bmatrix}
-(R_T - C)k_a & k_a L_0 + k_d \\
(R_T - C)k_a & -(k_a L_0 + k_d)
\end{bmatrix}
\tag{Equation 3}
\]

Based on the Jacobian, the determinant of the system is

\[ Det = (R_T - C)k_a(k_a L_0 + k_d) - (R_T - C)k_a(k_a L_0 + k_d) = 0 \tag{Equation 4} \]

and the trace of the system is

\[ Tr = -R_T k_a + C k_a - k_a L_0 - k_d \tag{Equation 5} \]

At steady-state (equilibrium) conditions,

\[
\frac{dC}{dt} = 0, \quad so \quad C_{eq} = \frac{k_a L_0 R_T}{k_a L_0 + k_d}
\]
Defining the equilibrium dissociation constant to be

\[ K_D = \frac{k_d}{k_a} \]

we can further simplify the equation to become

\[ C_{eq} = \frac{L_0 R_T}{K_D + L_0} \]  

(Equation 6)

Based on this equilibrium value of \( C \), we can solve for the trace of the system

\[ Tr_{eq} = -R_T k_a + \frac{k_a L_0 R_T}{k_d + L_0} - k_a L_0 - k_d < 0 \]

Since the concentration of complexes (\( C \)) at equilibrium is constant, and the determinant = 0 and trace < 0, this system has a stable, nodal sink equilibrium. In other words, all solutions to this system will approach the steady-state complex concentration \( C_{eq} \).

\( \text{Nanoparticle Binding Model} \)

We can use the same techniques of modeling individual ligand-receptor interactions to model nanoparticle binding to target cells. The ligand (\( L \)) and receptor (\( R \)) terms remain the same, but in this case, the complex (\( C \)) term represents a nanoparticle bound to the target cell (Figure 4).

Using the same derivations as above, we get

\[ \frac{dC^*}{dt} = k_a L_0 (R_T - C^*) - k_d C^* \]  

(Equation 7)

\[ C^*_{eq} = \frac{L_0 R_T}{K_D + L_0} \]  

(Equation 8)

Figure 4: Nanoparticle-cell binding kinetics.
This model accurately predicts binding interactions between homomultivalently-modified nanoparticles and one type of target receptor. In order to model the effect of heteromultivalent interactions on nanoparticle binding kinetics, the apparent cooperative affinity needs to be considered.

*Effect of Avidity on Nanoparticle Binding*

Heteromultivalently-decorated nanoparticles exhibit enhanced binding compared to homomultivalently-decorated nanoparticles, and it is theorized that this is due to enhanced affinity. This apparent cooperative affinity is proposed to be the result of the accumulation of multiple low-affinity interactions between the binding of one ligand to its receptor and the binding of another ligand to its receptor. In other words, the apparent cooperativity seen in heteromultivalent binding arises from the increased effective local concentration of a ligand motif as a result of tethering of the other ligand motifs on the construct, increasing the chances of ligand-receptor binding. This enhanced affinity term is referred to as ‘avidity’ [13], [14]. Avidity can be mathematically modeled as a multiple of individual affinities, represented by the association rate constant, \( k_a \). The \( k_a \) of a heteromultivalent system can be derived from calculating the force of interaction as the sum in free energies of individual ligand receptor interactions as shown in Equation 9. For simplicity, we have chosen to model a heteromultivalent system consisting of two ligand-receptor pairs, but this model can be expanded for any number of pairs.

\[
\Delta G = \Delta G_1 + \Delta G_2
\]

\[
\Delta G = -RT \ln(k_{a1}) - RT \ln(k_{a2}) 
\]

(Equation 9)
Simplifying this equation, we can get the value for the avidity association rate constant, $k_{avid}$, as a function of the individual ligand-receptor $k_a$'s.

$$-RT \ln(k_{avid}) = -RT(\ln(k_{a1}) + \ln(k_{a2})) = -RT \ln(k_{a1} \cdot k_{a2})$$

$$k_{avid} = k_{a1} \cdot k_{a2} \quad \text{(Equation 10)}$$

This new avidity association constant can replace the individual $k_a$ in Equation 7.

Additionally the total ligand concentration $L_0$ of a heteromultivalent system is a sum of the concentrations of each type of ligand

$$L_0 = L_{01} + L_{02}$$

and the total receptor concentration $R_T$ is the sum of the concentrations of each type of receptor

$$R_T = R_{T1} + R_{T2}$$

Plugging these into Equation 7, we get

$$\frac{dC^*}{dt} = (k_{a1} \cdot k_{a2})(L_{01} + L_{02})(R_{T1} + R_{T2} - C^*) - k_dC^* \quad \text{(Equation 11)}$$

Equation 11 better models the kinetics of a nanoparticle heteromultivalently modified with two targeting ligands binding with two target receptors on a cell surface.

**Effect of Shear Stress on Nanoparticle Binding**

Because nanoparticle binding in the body will be influenced by shear forces of fluid convection such as the flow of blood, we wanted to incorporate the effect of shear stress on nanoparticle binding into our mathematical model. It has been shown that the bond dissociation rate ($k_d$) will increase exponentially with respect to force, as demonstrated by the Bell model (Equation 12) [15].

$$k_d(F) = k_d(0)e^{Fx/kT} \quad \text{(Equation 12)}$$
Where $F$ is the force applied to the nanoparticle-cell complex by the shear stress of fluid flow, $kT$ is the thermal energy of the system, and $x$ is the characteristic length scale, determined to be ~0.5 nm. In other words, the rate at which the nanoparticles get knocked off of the cell surface will increase with increasing shear stress. Plugging this into Equation 11, we get

$$\frac{dC^*}{dt} = (k_{a1} * k_{a2})(L_{01} + L_{02})(R_{T1} + R_{T2} - C^*) - k_d(0)e^{\frac{Fx}{kT}}C^* \quad \text{(Equation 13)}$$

The mathematical model presented in Equation 13 can be used to predict and validate heteromultivalent nanoparticle binding activity under physiologically-relevant flow conditions.

**Results and Discussion**

*Assumptions, Parameter Estimation, and MATLAB Model*

To initially evaluate the model, several assumptions were made:

- Receptor density on the cells does not change with time (constant)
- Ligands are in excess to receptors, so ligand concentration at any given time can be represented by the initial starting ligand concentration
- The nanoparticle is much smaller than the target cell
- The nanoparticle is spherical
- All of the ligands on bottom hemisphere of the nanoparticle are able to interact with the cell surface receptors
- The heteromultivalent system includes only 2 ligand-receptor pairs
- Initially, there are no nanoparticles bound to the cell (initial condition: $C(0)=0$)
The cell surface receptor density, nanoparticle ligand density, binding and
dissociation rate constants, and shear stress values of the system are known

As a proof of concept, the parameters of the system were estimated based on average
physiological values, and following representative values were used:

\[ k_{a1} = 0.1 \text{ s}^{-1} \]
\[ k_{a2} = 0.1 \text{ s}^{-1} \]
\[ L_{01} = 5 \times 10^2 \text{ligands/\mu m}^2 \]
\[ L_{02} = 5 \times 10^2 \text{ligands/\mu m}^2 \]
\[ R_{T1} = 1 \times 10^5 \text{receptors/\mu m}^2 \]
\[ R_{T2} = 1 \times 10^5 \text{receptors/\mu m}^2 \]
\[ k_{d1} = 10 \text{ s}^{-1} \]
\[ k_{d2} = 10 \text{ s}^{-1} \]
\[ F = 0.025 \text{N/\mu m}^2 \]
\[ x = 0.0005 \text{\mu m} \]
\[ k = 8.314 \times 10^{-6} \text{\mu m N} \frac{\text{K}}{\text{mol}} \]
\[ T = 298 \text{K} \]

These values were varied in order to evaluate the effects of ligand affinity (\(k_a\) and \(k_d\)),
receptor density (\(R_T\), shear stress (F), and the ratio between ligand 1 and 2 (\(L_{01}\) and \(L_{02}\))
on nanoparticle binding.
The MATLAB function ode15s was used to solve for the change in complex (nanoparticle bound to cell) and receptor concentration with respect to time. Because these interactions occur and reach steady state at such short time scales, the system was evaluated between 0-0.1 second. Figure 5 shows a representative solution of these binding kinetics. Since the values for complex concentration are the negative representation of receptor concentrations, for the rest of this report we will report complex concentrations over time only.

**Effect of Ligand Affinity on Nanoparticle Binding**

Keeping the receptor density, ligand ratio (50:50 L1:L2), and shear stress (25 dyn/cm^2) constant, the effect of varying ligand affinity (k_a and k_d) on nanoparticle binding was investigated. The affinity of ligand 1 was evaluated at 1x, 2x and 3x that of ligand 2, and the results of the simulation are plotted in Figure 6. It can be seen that increasing the affinity of one of the ligands to its respective receptor
increases the overall concentration of bound liposomes. Additionally, it decreases the rate at which the nanoparticles reach steady-state binding conditions to the platelets. The results also demonstrate that changing ligand affinity does not scale linearly with nanoparticle binding. This is because while the association rate is directly proportional to affinity, the dissociation rate is inversely proportional. Consequently, as the affinity of a ligand-receptor pair increases, the influence of $k_D$ on $C_{eq}^*$ decreases to the point where $C_{eq}^*$ is limited by the total number available receptors.

**Effect of Receptor Density on Nanoparticle Binding**

Keeping the ligand affinity ($k_a$ and $k_d$), ligand ratio (50:50 L1:L2), and shear stress (25 dyn/cm²) constant, the effect of varying receptor density ($R_{T1}$ and $R_{T2}$) on nanoparticle binding was evaluated. The density of receptor 1 was increased from 1x to 2x to 3x the density of receptor 2, and the results of the simulation are plotted in Figure 7. Overall, increasing the density of one receptor increases the degree of nanoparticle binding. Furthermore, the concentration of complexed liposomes is directly proportional to the receptor density.

**Effect of Shear Stress on Nanoparticle Binding**
To evaluate the effect of shear stress on nanoparticle binding, the force exerted on the system was varied between the physiologically-relevant shear stress values of 5-55 dynes/cm^2, while keeping ligand ratios and receptor densities constant. Figure 8a shows liposome binding to platelets under various shear stresses with ligands of equal affinities. It can be seen in the zoomed in region of the graph (Figure 8b) that increasing shear stress decreases concentration of bound nanoparticles, but the overall effect of shear stress on nanoparticle binding is small. This is because the association-related part of the model equation is greater by several orders of magnitude than the dissociation-related

**Figure 8:** Simulation output of the effect of varying shear stress on liposome binding. A) Binding of liposomes with ligands of same dissociation constants $k_d$, B) Zoomed in version of A, C) Binding of liposomes with different $k_d$ of ligands, D) Zoomed in version of C.
part. Furthermore, since force directly effects the dissociation rate constant, we also evaluated the effect of shear stress on a system in which ligand 1 has a $k_d$ that is half that of ligand 2 (Figure 8c), meaning that L1 has 2x stronger binding. The overall concentration of bound liposomes increases as a result of this increased binding strength. Additionally, there is a more narrow distribution in bound liposomes under various shear stress conditions (Figure 8d compared to 8b), suggesting that stronger binding is less influenced by the forces exerted by fluid flow.

**Effect of Heteromultivalent Ligand Decoration on Nanoparticle Binding**

Next, we used the model to compare degree of homomultivalent liposome binding to platelets compared heteromultivalent liposomes. In the case of homomultivalent nanoparticles, Equation 7 was used to model binding. Basically, the avidity association constant was replaced with individual ligand $k_a$ in the mathematical expression, and the dissociation constant ($k_d$) and concentrations of the other ligand and receptor were removed. Figure 9 demonstrates the enhanced cooperative effect of heteromultivalent interactions on nanoparticle binding. This effect is seen at various shear stress values, binding affinities, and ligand or receptor densities.

![Figure 9](image-url)
Comparing Model to Experimental Data

Lastly, we wanted to compare our mathematical model output to our experimental data (Figure 2) of liposomes modified with GPIIb-IIIa and P-selectin targeting peptides binding to activated platelets in our in vitro flow chamber setup. For this, we incorporated actual parameter values obtained from the literature for our system as follows:

\[
\begin{align*}
    k_{a1} &= 1.08 \times 10^{14} \text{ s}^{-1} \\
    k_{a2} &= 5.90 \times 10^{10} \text{ s}^{-1} \\
    L_{01} &= 2.6 \times 10^5 \text{ligands/\mu m}^2 \\
    L_{02} &= 2.6 \times 10^5 \text{ligands/\mu m}^2 \\
    R_{T1} &= 350 \text{ receptors/\mu m}^2 \\
    R_{T2} &= 2899 \text{ receptors/\mu m}^2 \\
    k_{d1} &= 0.711 \text{ s}^{-1} \\
    k_{d2} &= 4.078 \times 10^{-6} \text{ s}^{-1}
\end{align*}
\]

where ligand 1 is the P-selectin binding peptide (DAEWVDVS), and ligand 2 is the GPIIb-IIIa binding peptide (CNPRGDY(OEt)RC). The output of the mathematical model is shown in Figure 10. Although results of the simulation predict the enhanced binding of heteromultivalently

Figure 10: Simulation output of homomultivalent versus heteromultivalent liposome binding to platelets using actual parameter values.
modified liposomes compared to homomultivalent liposomes, the model does not
completely fit the data. Specifically, we did not experimentally observe such a large
difference in the binding of the two different homomultivalent liposomes formulations.
We believe that the model can be further refined to enable more accurate prediction of
experimental data by incorporating a probability term, since the probability of individual
ligand-receptor complexation is largely influenced by the formation of other ligand-
receptor pairs in the vicinity of the nanoparticle surface. Additionally, precise measures
of global $k_a$ and $k_d$ values of the nanoparticle using analytical techniques such as AFM
and SPR could lead to a better understanding of the magnitude of how the
heteromultivalent versus homomultivalent ligand modification influences the
nanoparticle’s overall binding strength.

**Conclusions and Future Work**

To conclude, we have developed a mathematical model to simulate nanoparticle binding
kinetics, incorporating the effects of heteromultivalent ligand-receptor interactions as
well as shear stress forces. The model was able to simulate reasonable trends related to
varying ligand affinities, receptor densities, and shear stress values. Furthermore, the
model simulates the cooperative effect of heteromultivalent modification on nanoparticle
binding, which can be compared to experimental data. Future modifications should be
made to the model to more accurately fit experimental data. Additionally, this model
could be expanded in the future to include more complex nanoparticle formulations with
increased numbers of heteromultivalent ligands (3, 4, etc.) as well as various ratios of
ligand concentrations.
References


Appendix II: Platelet-like Nanoparticles (PLNs): Mimicking Shape, Flexibility and Surface Biology of Platelets to Target Vascular Injuries

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Introduction

Nanoparticle-based therapies offer several advantages over free drugs including enhanced targeting and reduced off-target side effects [1–3]. However, applications of nanoparticles are limited by rapid clearance due to immune-recognition and limited targeting due to non-specific binding [4–6]. Several approaches based on synthesis of novel materials have been developed to address these challenges and some of them have yielded exciting results at pre-clinical and early-clinical stages [7], [8]. Bio-inspired design approaches have recently emerged as a novel paradigm to address the limitations of classical nanoparticles [9]. Specifically, natural circulating cells including erythrocytes, leukocytes and platelets routinely perform functions of circulation and targeting that are often desired in synthetic nanoparticles. Inspired by the superior functionality of innate circulatory cells, bio-inspired strategies aim to impart some of the essential functional biological attributes into synthetic systems. For example, RBC-
mimetic synthetic particles have been developed that mimic the shape and mechanical properties of RBCs to exhibit prolonged circulation [10]. As another example, leukocyte-mimetic particles, coated with membranes of natural leukocytes on synthetic scaffolds, have been prepared and shown to accumulate in tumors [11].

Here we report synthesis of platelet-like nanoparticles (PLNs) and their ability to target an injured vascular site to render hemostasis. Targeted delivery of nanoparticles to vascular injury sites has been actively researched owing to nanoparticle’s potential to treat various vascular disorders including cancer, inflammation, thrombosis and hemorrhage [12]. Nanoparticles comprising various materials [13], [14] and targeting moieties [3], [15] have been used to specifically target vascular inflammation [16], vascular plaque [17] and clots [18]. However, these nanoparticles possess attributes that are starkly different from circulating cells, especially platelets, which have evolved to expertly perform many of these functions.

Attempts have been made in the past to mimic certain aspects of platelet-related vascular targeting [19–27]. Earlier approaches focused on liposomes or other polymeric particles functionalized with fibrinogen or fibrinogen-derived peptide ligands [28].

![Fig 1: Platelet interactions in hemostasis and corresponding platelet-inspired design of PLN technology. Schematic showing normal hemostatic interactions that inspire PLN design.](image)
However, these approaches significantly deviate from the principles by which natural platelets function in two main ways. Firstly, they do not mimic the complex biochemical interactions that occur when natural platelets interact with and bind to the endothelium [28]. Platelets circulate in a quiescent state and bind reversibly to the injured endothelium first via interacting with endothelium-secreted von Willebrand Factor (VWF) under high shear and then undergoing stable adhesion via interacting with collagen. Platelets then become activated to trigger subsequent aggregation of other locally activated platelets via fibrinogen-mediated interactions with activated platelet integrin GPIIb-IIIa. Together, these synergistic adhesive and aggregatory interactions effectively form a hemostatic plug which halts bleeding. Secondly, liposomes and other polymeric particles do not mimic the biophysical discoid shape or the flexibility of natural platelets that are both essential in facilitating hemodynamic transport and margination of platelets towards endothelium to effectively render injury-site selective binding [29]. Our PLNs incorporate these often ignored biophysical design criteria of platelet-mimetic discoid morphology and flexibility, and integrate these design parameters with the platelet-mimetic biochemical heteromultivalent interactive functions by dendritic presentation of multiple peptides that bind simultaneously to both activated natural platelets and injured endothelial sites to promote injury-specific binding and hemostasis (Fig. 1).

Results

Synthesis and Characterization of PLNs
PLNs were synthesized using the layer-by-layer (LbL) approach [30], [31] (Fig. 2a) to yield flexible capsules [32] that are morphologically similar to natural platelets. Briefly, spherical polystyrene (PS) nanoparticles (Fig. 2bi) were coated with complementary layers of poly(allylamine hydrochloride) (PAH) and bovine serum albumin (BSA) until 4

**Fig 2: Synthesis and characterization of PLNs.** (a) Schematic showing the LbL synthesis of PLNs. Note that the schematic shows only 2 bilayers of PAH/BSA whereas 4 bilayers of PAH/BSA were used in this study. (b) Scanning electron micrographs (SEM) of (i) sacrificial 200 nm spherical polystyrene templates, (ii) (PAH/BSA)$_4$ coated polystyrene templates and (iii) final PLNs. Scale bars = 200 nm. (c) Complementary coatings of poly(allylamine hydrochloride)–AlexaFluor 594 (odd layers) and bovine serum albumin–AlexaFluor 488 (even layers) with confocal imaging of final PLNs. (d) FTIR spectra of PS templates, (PAH/BSA)$_4$ coated templates and PLNs.
bilayers, \((\text{PAH/BSA})_4\), were formed on the template PS particle (Fig. 2bii). PAH and BSA were chosen as the polycation and polyanion, respectively, due to their reliability in capsule synthesis \(\text{via} \) LbL [33] as well as use as materials for numerous biomedical applications [34–37]. PLNs (Fig. 2biii) were characterized at each step for sufficient PAH/BSA coating (Fig. 2c) \(\text{via} \) fluorimetric assays. Poly(allylamine hydrochloride)–AlexaFluor 594 (PAH-AF594) and bovine serum albumin-AlexaFluor 488 (BSA-AF488) were complementarily coated and the fluorescent intensity for each dye was determined at each coating layer. The linear relationship of independently labeled polyelectrolytes with the number of layers implies the presence, and successive uniform coating, of both PAH and BSA [38]. Coating was further confirmed qualitatively \(\text{via} \) confocal imaging of the final PLN product (Fig. 2c, inset). PS core removal was performed \(\text{via} \) incubation with tetrahydrofuran (THF) and isopropyl alcohol (IPA), at increasing THF:IPA ratios, and confirmed \(\text{via} \) FTIR (Fig. 2d, SI Fig. 1) by monitoring absorbance at wavenumbers 700 cm\(^{-1}\) and 760 cm\(^{-1}\), which represent the monosubstituted benzene rings in polystyrene [39]. PLNs comprised of \((\text{PAH/BSA})_4\) collapse into discoidal particles following core removal (Fig. 2biii). This change in shape results from the flexibility of the polymer/protein shell material collapsing onto itself since there is no longer a rigid PS core to maintain the original spherical structure (Fig. 2bi). Previous studies involving the synthesis of LbL capsules utilizing similar procedures and materials have confirmed the softness of such particles [32].

Adhesion and Aggregation of PLNs Under Flow

Role of PLN’s size and shape in determining their adhesion under flow conditions was assessed using microfluidic devices (Fig. 3a inset; see SI Fig. 2 for dimensions and
uniformity of protein coating). Devices were coated with anti-ovalbumin (anti-OVA) antibody and particles were coated with ovalbumin (OVA). OVA/anti-OVA interaction was used as a model antigen-antibody system. OVA-coated spherical particles of three sizes (200 nm, 1 μm, and 2 μm) were used to investigate the effect of particle size on shear-dependent adhesion. Larger spherical particles adhered in smaller quantities and consequently covered less surface area than their smaller counterparts (Fig. 3a), a finding consistent with literature reports [40] that is likely due to the stronger shear detachment forces experienced by larger particles under flow [41].

Shape of particles also impacted their adhesion on targeted surfaces. Since smaller particles adhered in higher quantities than their larger counterparts, 200 nm OVA-coated
spheres, OVA-coated discs stretched from 200 nm spheres and 200 nm OVA-coated PLNs were studied in the same conditions as described above. Discoidal particles (discs, PLNs) covered more surface area of coated channels (Fig. 3b) than their spherical counterpart, likely due to increased surface area contact of discoidal particles. Furthermore, PLNs covered more surface area than discs, likely due to their flexibility [32], which increases opportunities for multi-ligand interactions [12]. While spheres and discs adhere mostly as individual particles, PLNs appear to adhere as both individual and aggregates of particles adhering under shear (Fig. 3b).

In view of their superior binding properties, 200 nm PLNs were used for further studies. This specific size also helps avoid cardiopulmonary interference. Specifically, particles similarly sized or larger than lung capillaries are known to physically get trapped in lungs (the first capillary bed encountered following tail vein injection) which can impede the passage of blood, effectively impairing oxygen delivery [2], [42].

**Peptide Conjugation to PLNs**

PLNs were heteromultivalently surface-decorated with the collagen-binding peptide (CBP; \([GP\)\(O\)\(_7\)]\) which binds to exposed collagen at the wound site via helicogenic interactions [43], the von Willebrand-binding peptide (VBP; \(TRYLRIHPSQVHQI\)) which binds to VWF [44] at the injury site and the linear fibrinogen-mimetic peptide (FMP; \(GRGDS\)) which specifically binds to integrin GPIIb-IIIa on activated natural platelets [45], [46] to amplify platelet aggregation for hemostatic plug formation. These three peptide-mediated mechanisms act synergistically to promote injury-dependent site-specific stable adhesion, thereby allowing PLNs to localize at the injury site and then enhance platelet aggregation at that site via FMP. This integration of adhesion and
aggregation functions are a significant improvement over past platelet substitute designs [28]. To ensure that peptides do not detach from PLNs in vivo, the three peptide ligands were covalently coupled to PLNs. Further, to avoid non-specific interactions with the albumin-rich surface on PLNs and to increase the strength of selectivity of their respective binding, the peptides were not homogenously coupled onto the surface of PLNs, but instead were conjugated to branched dendrimers to form dendrimer-peptide structures on the surface of PLNs. Briefly, carboxyl terminated (PAMAM) dendrimers were activated via carbonyldiimidazole (CDI), washed to remove excess CDI, reacted in individual flasks with the N-terminus of each of the three peptides, and finally washed to separate unreacted peptides from dendrimer-peptide conjugates (SI Fig 3a). The outer layer of PLNs were enriched with primary amino groups by activation with CDI, followed by washing to remove excess CDI, addition of an excess diaminoethane, and finally washing to separate unreacted diaminoethane from amine-rich PLNs (SI Fig. 3b). Dendrimer-peptide conjugates were then activated via EDC chemistry, washed to remove excess EDC, and conjugated to PLNs to form stable amide bonds between the carboxyl rich dendrimer-peptide conjugates and the amine rich PLN surface (SI Fig. 3ci; see 3cii for final PLN schematic). Peptide conjugation to PLNs was quantified via fluorescent labeling of dendrimers (SI Fig. 3d) and confirmed qualitatively via confocal microscopy (SI Fig. 3e). PLN size (235 ± 4 nm) and surface charge (-16 ±1 mV), were minimally altered following conjugation with dendrimer-peptides (240 ± 8 nm and -25 ± 5 mV).

Aggregation and Adhesion of PLNs with Wound Specific Ligands and Activated Platelets Under Flow In Vitro
The platelet-mimetic adhesive and aggregatory capabilities of the PLNs were assessed in

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**Fig 4: In vitro binding of PLNs at various shear stresses.** (a) PLNs modified with CBP+VBP show specific adhesion to VWF-collagen coated surfaces (filled squares), PLNs without peptide modification show minimal adhesion to VWF-collagen coated surfaces (circles), and PLNs modified with CBP+VBP show minimal adhesion to non-targeted control BSA surfaces (filled triangles) in a parallel plate flow chamber (PPFC) at various shear values. Representative images are shown for the end of the experiment (45 minutes) for each condition. At least 10 images at for each condition at each time were used for analysis. Scale bars = 50 μm. (b) PLNs modified with FMP aggregate with natural platelets (filled squares), PLNs without peptide modification show minimal aggregation with natural platelets (circles), and PLNs modified with FMP show minimal aggregation with non-targeted control BSA surfaces (filled triangles) in a PPFC at various shear values. Representative images are shown for the end of the experiment (45 minutes) for each condition. At least 10 images at for each condition at each time were used for analysis. Scale bars = 50 μm. All images were taken at the same magnification. * Denotes statistical difference (P < 0.05) from all other groups at each time point.
in a parallel plate flow chamber (PPFC) setup (SI Fig. 4). For adhesion studies, glass slides were coated with adjacent regions of BSA (negative control surface) and 50:50 VWF/collagen (positive control surface). Green fluorescent unmodified PLNs and VBP+CBP PLNs, which should bind specifically to the VWF/collagen surface, were allowed to flow over these coated glass slides in the PPFC at both physiological and pathological wall shear stresses [18], [47] of 5-55 dynes/cm² for 45 minutes. Adhesion of targeted and non-targeted PLNs to both targeted and non-targeted surfaces was imaged and quantified (Fig. 4a). Indeed, unmodified PLNs had minimal adhesion on the VWF/collagen surface and peptide-modified PLNs had minimal adhesion on the control BSA surface. In comparison, the peptide-modified PLNs showed significantly enhanced adhesion on the VWF-collagen surface due to the specific synergistic interaction mechanisms of VBPs with VWF and CBPs with collagen. To study the aggregation capabilities of PLNs with natural platelets, platelet-rich-plasma (PRP) was incubated in presence of adenosine diphosphate (ADP), which facilitates the transition of unactivated platelets to activated platelets, with glass slides bearing BSA-coated and collagen-coated adjacent regions in order to form a layer of activated platelets on the collagen-coated region but not on the BSA-coated region. Green fluorescent unmodified PLNs and FMP-modified PLNs, which should bind specifically to the activated platelets, were allowed to flow over these slide surfaces at wall shear stresses of 5-55 dynes/cm² for 45 min. Aggregation of PLNs with natural platelets was imaged and quantified (Fig. 4b) at various wall shear stresses. FMP-PLNs showed significantly enhanced binding to the activated platelet-covered region, and minimal binding to non-targeted BSA surface, at all shear values. Furthermore, unmodified PLNs showed minimal adhesion to activated
Collectively, these in vitro flow experiments highlight the ability of PLNs to present a variety of different targeting ligands that interact specifically with target sites, while minimally interacting with physically similar, non-targeted surfaces.

**PLN Interaction and Clot Formation with Natural Platelets in an In Vitro Wound Model**

The ability of PLNs to combine both adhesion and aggregation in one particle formulation was tested in a simulated injury site environment. For these studies, human platelets (stained red) were pre-activated with ADP and subjected to flow over BSA-coated (control surface that exhibits no specific binding to VBPs and CBPs) and VWF-collagen-coated (test surface with specific binding to VBPs and CBPs) regions (Fig. 5a) at wall shear stress of 25 dynes/cm$^2$ along with the four formulations of green fluorescent PLNs, namely, (i) unmodified PLNs, (ii) ‘adhesive only’ CBP+VBP PLNs, ‘aggregatory only’ FMP PLNs or (iv) ‘functionally integrated’ biomimetic PLNs containing all 3 peptides (CBP+VBP+FMP). PLNs bearing all three peptides were most effective in adhering stably to the VWF-collagen surfaces while recruiting activated platelets, in flow, to the adhesion site (Fig. 5b). Indeed, ‘functionally integrated’ biomimetic PLNs showed significantly enhanced fluorescence co-localization with activated platelets, validating the synergistic effect of the 3 peptides in a single platelet-mimic carrier. Co-localization, which is the overlap of activated platelets (red) and PLNs (green), was quantified using multiple images for each group via the Pearson’s coefficient (Fig. 5c). This quantitative representation (Fig. 5c) of images (Fig. 5b) highlights the importance of combining both aggregation and adhesion platelet properties into a single PLN.
formulation in order to both adhere stably to wound mimicking surfaces while recruiting
activated platelets, or in other words, form a hemostatic plug.

While natural circulating platelets undergo complex shear-dependent aggregation at vascular injuries [48], [49], due in part to their discoidal shape and unique presentation of surface receptors that benefit from lateral mobility afforded by their flexibility, the majority of polymeric nanoparticles do not possess this ability. However, PLNs appear to undergo shear-dependent aggregation on targeted surfaces upon binding in all flow experiments performed (Fig. 3, Fig. 4, and Fig. 5). To investigate shear-induced aggregation, PLNs were imaged before and after flow through a capillary. Before flow, PLNs are dispersed and show limited aggregation, while after experiencing shear under flow, PLNs form larger aggregates (SI Fig. 5). This suggests that the physical properties of PLNs allow for similar aggregation abilities that their natural counterparts routinely leverage to facilitate hemostasis.

*In Vivo Hemostasis Effect and Biodistribution of PLNs*

PLNs were next investigated *in vivo* for their ability to halt bleeding in a standard tail transection model in BALB/c mice. PLNs without peptides and saline injections alone showed no decrease in tail bleeding times. PLNs functionalized with only the FMP peptide (GRGDS) lowered bleeding time by ~45% (Fig. 6a), which is comparable to results reported for past synthetic hemostat designs of particles bearing fibrinogen-based RGD or AGD peptides [23]. However, ‘functionally integrated’ biomimetic PLNs (bearing all three peptides GRGDS, TRYLRHIHPQSQVHQI, and [GPO]7) lowered bleeding time by ~65%. Further, micron sized ‘functionally integrated’ PLNs were unable to instigate hemostasis to the same extent as their 200 nm counterparts, likely due to the lower circulation time of micron sized particles and also the less efficient binding
and adhesion that larger particles exhibit under flow conditions. Non-flexible spherical 200 nm ‘functionally integrated’ PLNs containing the rigid PS core, were also unable to cause hemostasis as rapidly as the more flexible, disc-shaped, PLNs, likely due to their limited aggregation at the wound site. Organ distribution for 200 nm PLNs with and without CBP+VBP+FMP peptide functionalization showed similar organ accumulation except in the case of the tail section containing the clot (Fig. 6b). In this case, a 3-fold
increase in PLNs functionalized with CBP+VBP+FMP peptides in the tail section containing the clot was seen over unmodified PLNs. Targeting of ‘functionally integrated’ PLNs to tail clots was confirmed via fluorescent imaging (Fig. 6c). Images of the clot show that PLNs bind in aggregates (Fig. 6c) as predicted by in vitro flow experiments (Figs. 3, 4, and 5). Histology sections of liver, brain and lungs were taken at 24 hours following injections of either saline (control) or functionally integrated PLNs (SI Fig. 6). Histopathology indicated slightly increased occurrence of microthrombi in lungs in case of PLNs compared to controls, likely due to lung accumulation (Fig 6b). The liver and brain tissues showed no pathologies for either group.

Discussion

The results shown here demonstrate the ability of biomimetic PLNs to significantly reduce the bleeding time in a mouse tail transection model. PLNs were synthesized via the LbL method for precise control over their size, shape and material composition. Similar to their natural counterpart, PLNs were discoidal in shape and were functionalized with wound specific and platelet specific peptides (see SI Fig. 3 for activation and reaction schematic, peptide quantification, and PLN imaging) so as to mimic the biochemical interactions of natural platelets with both injured endothelium and each other. Both biophysical and biochemical design parameters of PLNs were investigated for their role in adhesion under physiological flow conditions. Firstly, the biophysical design parameters of PLNs, namely their discoidal shape and flexible exterior, showed individual and aggregated PLN binding to targeted surfaces under flow. PLNs were then independently assessed for their specific adhesion to both wound specific ligands and to activated platelets under physiologically relevant flow conditions.
PLNs functionalized with wound specific ligands show high specific adhesion to collagen and VWF coated slides and limited interaction with negative control BSA surfaces. Similarly, PLNs functionalized with fibrinogen interacting peptides (GRGDS) showed high specific adhesion to activated platelet-coated surfaces and minimal adhesion to negative control BSA surfaces. In both cases, PLNs which had no peptide functionalization showed minimal adhesion to both collagen and VWF coated surfaces and activated platelet-coated surfaces. Furthermore, PLNs which had targeting peptides showed minimal adhesion to negative control BSA surfaces. Following this, an in vitro wound model confirmed that PLNs functionalized with both wound adhesive and activated platelet aggregatory peptides formed clots more efficiently than PLNs functionalized with adhesive only or aggregatory only peptides. In all binding studies, PLNs adhered to targeted surfaces as both individual particles and as larger aggregates. PLN aggregation stems from a combination of their physical and chemical features. PLNs ability to aggregate under shear was investigated via capillary flow experiments. Imaging of PLNs before, during, and after capillary flow, under high shear conditions, illustrates the ability of PLNs to undergo shear dependent aggregation. We hypothesize that, much like real platelets, PLNs marginate to the wall under shear due to their physical parameters (e.g. shape and flexibility) and are likely to have higher local concentration at the wall under flow. This increase in local PLN concentration can potentially cause PLNs to interact with each and clump together if shear conditions permit. Literature in colloidal physics field has shown that colloidal particles aggregate under shear owing largely to increased local concentration of particles [50]. The in vitro
flow experiments accurately predicted the PLN clustering seen in the fluorescent histology tail sections.

In case of normal hemostatic plug formation, circulating platelets become activated and bind to the damaged endothelium due to exposure of collagen and release of VWF from the wound site. In case of hemostatic plug formation following injection of PLNs, activated circulating platelets and PLNs both bind to injured endothelium, as well as to each other, effectively forming the hemostatic plug much faster than in the absence of PLNs. Brightfield and fluorescent images show the interaction between fluorescently labeled PLNs and the clot (Fig. 6c), which was verified by via biodistribution as functionalized PLNs accumulated 3-fold higher in the clot as compared to plain PLNs. Following an injection of PLNs, the hemostatic plug formed in 35% of the time it took when no PLNs were injected. The importance of the biochemical design parameters were verified in vivo as platelet-aggregatory-only or wound-adhesive-only PLNs were unable to promote hemostasis as effectively as PLNs capable of both. Furthermore, the importance of the biophysical design parameters were verified in vivo as rigid/spherical PLNs with both wound-adhesive and platelet-aggregation abilities were unable to promote hemostasis as well as PLNs of discoidal shape. An increase in microthrombi was seen in histological lung sections in some cases following treatment with PLNs. This is likely due to increased overall activity of circulating platelets following tail transection compounded with the observed lung accumulation. To minimize these complications, a low PLNs dose of 15 mg/kg was used as compared to much higher doses reported, often exceeding 50 mg/kg, that have shown to induce cardiopulmonary issues in the form of
elevated heart rates and gasping [23]. Such complications were not observed with the lower dose of PLNs used in this study.

Various cell mimetic [10], [32] and cell-inspired [51–53] systems have been recently reported for a variety of biotechnology applications [9]. The PLNs presented here combine many unique aspects of circulatory platelets, specifically their shape and surface chemistry interactions, to perform functions that are typically exclusive to natural platelets. The LbL technique used here is widely utilized for the synthesis of a variety of LbL particles with proven biomedical applications [34], [54], [55] and is ideal for fabricating synthetic cells as it allows for unmatched control over particle design. Furthermore, the PLNs described here will benefit from the recently reported scaled up synthesis of similarly fabricated LbL particles [35]. Future studies will be focused on translating the fast acting hemostatic ability of PLNs to larger, more serious wounds, determining the dose-dependent cardiopulmonary impact following PLN injection and assessment of the extent of complement activation following PLN injection. PLNs potentially offer a new intravenous tool for the treatment of severe bleeding and hemorrhage. Also, certain peptide-decorative subsets of PLNs, for example, the FMP-decorated designs can be potentially used to target platelet-rich occlusive clot sites for site-specific delivery of fibrinolytic or anti-platelet agents which can reduce the systemic coagulopathy risks otherwise presented by such agents. Therefore, PLNs can become an effective technology not only as ‘synthetic hemostats’, but also a platform for tailoring targeted therapeutic actions in atherosclerosis, thrombosis, or restenosis in the vascular compartment.
Methods

PLN fabrication

200 nm carboxylated PS spheres (Polysciences, Warrington, PA) were suspended in 0.5 M sodium chloride. 2 mg/ml of positively-charged Poly(allylamine hydrochloride) (Sigma) was dissolved in 0.5 M sodium chloride and incubated with 12.5 mg PS particles at room temperature under constant rotation for 30 minutes. Particles were then centrifuged at 15000g for 30 minutes and resuspended in 0.5 M sodium chloride. Particles were washed 2 more times at 15000g for 30 minutes in 0.5 M sodium chloride. Following PAH coating, negatively-charged bovine serum albumin (Sigma) was coated on top of PAH layers under identical conditions. This procedure was repeated for 4 total PAH/BSA bilayers. Intermittent crosslinking with 2% glutaraldehyde (Polysciences) for 1 hour under constant rotation was performed to ensure sufficient structural integrity of the outer shells. Following glutaraldehyde exposure, the particles were incubated in 30 mM sodium borohydride to stop the crosslinking reaction. The particles were then exposed to a tetrahydrofuran-isopropyl alcohol gradient (1:3, 1:2, 1:1, 2:1, and pure THF) for 30 minutes each at room temperature under constant rotation so as to dissolve the PS core. Particles were then washed ten times with saline, so as to remove any residual solvent and stored at 4°C for no longer than 2 days.

In vitro studies microfluidic experiments

Devices (see SI text for synthesis) were washed with saline, and coated with 500 μg/ml of anti-ovalbumin monoclonal antibody at either 4°C overnight or at 37°C for 1 hour. PLNs were prepared as described above and discs were prepared using previously described film stretching methods [56]. All particles were terminally coated with 2 mg/ml PAH for
30 minutes at room temperature, crosslinked with 2% glutaraldehyde as above, and then coated with 1 mg/ml ovalbumin at 4°C overnight. Particles were washed 3x in saline and resuspended at a concentration of $5 \times 10^{11}$ particles per ml. Particles were subjected to flow, via syringe pump withdrawal, through the devices at a constant flow rate of 0.27 mL/h (shear rate of 800 s$^{-1}$ on bottom of channel) for 1 minute, and then washed with saline at 0.27 mL/h for 2 minutes to remove unbound particles. Devices were then imaged using an Andor iXON 885 fluorescent camera and at least 10 images were analyzed for binding to channel surface by calculating total surface area covered using ImageJ.

Surface area coverage was quantified as opposed to quantity of particles since sufficient hemostatic plug formation requires complete wound plugging, which is dependent not on the quantity of particles, but on the total area the particles can occupy.

*Peptide conjugation to PLNs*

Succinamic acid terminated PAMAM dendrimers, generation 5.0 (Dendritech), were activated with carbonyldiimidazole (CDI) at 1 mg/ml in acetone. Removal of CDI from activated dendrimers was performed using Amicon centrifugal filters (3 kDa) for 3 washes. Each of the three peptides were then added and coupled to CDI activated dendrimers in separate flasks via their N-terminus. Removal of unreacted peptides from dendrimer-peptide conjugates was performed using Amicon centrifugal filters (3kDa) for 3 washes. The outer layer of PLNs was activated with CDI at 1 mg/ml in acetone for 45 minutes. Free CDI was removed via centrifugation. Diaminoethane was then added to yield primary amino groups on PLN surface and purified via centrifugation. All PLNs were tested qualitatively for free amines via the Kaiser test. Finally, dendrimer-peptide conjugates were conjugated to PLNs via EDC chemistry in MES buffer at 4.5 pH for 12
hours (see SI Figure. 3a, 3b, and 3c for stepwise activation and reaction scheme). For coupling of peptides to particles still containing polystyrene cores, particles were activated with EDC at 4 mg/ml in MES buffer for 10 minutes, washed to remove excess EDC, reacted with diaminoethane to form primary amino groups on the surface, and reacted with dendrimer-peptides via EDC chemistry.

*In vitro studies of hemostatically relevant PLN interactions*

The interaction of peptide-modified PLNs with injury site-relevant proteins (VWF and collagen) and activated platelets in a flow environment was studied using a Parallel Plate Flow Chamber (PPFC) system connected with a programmable pump and recirculating loop with silicone tubing. The PPFC set-up was placed under an inverted fluorescent microscope and the experimental surfaces were imaged over time to allow quantitative fluorescence intensity analysis of particle and cell interactions. The flow rate of particle and platelet suspensions in the PPFC was controlled by a programmable pump to allow a wall shear stress range of 5-55 dynes/cm² as per the equation \( \tau_w = 6\mu Q b \frac{1}{h^2} \), where \( \tau_w \) is the wall shear stress, \( Q \) is the flow rate, \( \mu \) is the fluid viscosity (0.015 dyn/cm²), \( b \) is the PPFC chamber width (1.0 cm), and \( h \) is the distance between the PPFC plates (0.00254 cm). The resultant shear stress range is within the physiological and pathological ranges of wall shear stresses in the vasculature. For image analysis, the fluorescence intensities of 10 images per time point per shear stress were analyzed using a MATLAB script and statistical analyses were performed using ANOVA, with significance considered at \( p < 0.05 \). For the co-localization analyses between red fluorescent platelets and green fluorescent PLNs, an ImageJ (NIH) script called Just Another Co-localization Plugin
(JACoP) was used, that utilizes the Pearson’s coefficient estimation of the association strength between two color channels in the image fluorescence histogram.

**In vivo hemostasis**

All experiments were performed as per approved protocols by the IACUC of the University of California, Santa Barbara. 15 mg/kg PLNs in saline were injected *via* tail vein into healthy female BALB/c mice (18-20g; n=3-6 per group). 5 minutes after injection, 2 mm long sections of the tail, from the distal tip, were amputated. The amputated tail was immediately immersed in 14ml of sterile saline at 37˚C. The times until bleeding from the amputated tail stopped were recorded with a stopwatch.

**In vivo biodistribution**

15 mg/kg PLNs, either plain or conjugated with CBP+VBP+FMP peptides, in saline were injected *via* tail vein into healthy female BALB/c mice (18-20g; n=3-6 per group). 5 minutes following injection, 2mm long sections of the tail, from the distal tip, were amputated. 55 minutes following tail amputation, animals were sacrificed via CO₂ overdose and organs were collected. The organs were dissolved overnight in Solvable at a concentration of 100 mg organ per 1 ml of Solvable. Dissolved organ solutions were measured for their fluorescence at a concentration of 2 mg of organ per 200 μl Solvable. Background fluorescent values of each organ, from control animals receiving no injection, were subtracted from each organ value for CBP+VBP+FMP PLN and plain PLN groups.

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References


Supplemental Information

Methods

Device synthesis

Single layer, microfluidic channels were fabricated out of a glass slide and PDMS (Dow Sylgard 184). Thin PDMS films with a thickness of 50 µm were made by spinning uncured PDMS on Kapton film (DuPont Electronics) at 1500 rpm for 30 seconds. The films were baked for at least 1 hour at 80°C, where upon curing, the Kapton film and PDMS form a reversible bond, as previously reported. A laser cutter (Trotec) was used to cut channels into the PDMS film, which was then ozone bonded to the glass slide and baked for at least 4 hours at 80°C. The Kapton film was then carefully removed from the PDMS film. A thick PDMS slab, with pre-drilled inlet holes, was ozone bonded to the top of the PDMS film and baked for at least 1 hour at 80°C.

Scanning electron microscopy (SEM)

An FEI XL40 SEM at 5 kV with a 5 mm working distance was used for imaging particles. Samples were coated with palladium (at 10 kV) via a Hummer sputtering system.

Dynamic light scattering (DLS)
DLS measurements for size and zeta potential were performed in water using a Malvern Zetasizer Nano ZS.

**Capillary flow experiments**

Glass capillaries of 0.8 mm diameter and 100 mm length were connected to a syringe containing particles of identical concentrations as above. Particles were then flown through the capillary at a flow rate of 5.7 mL/h, to match the average velocity in the microfluidic device channel, and the effluent was collected and immediately imaged along with particles remaining in the syringe.

**Histology and histopathology**

100 μl saline or 15 mg/kg PLNs in 100 μl saline were injected into healthy female BALB/c mice (18-20g. n=3). Animals were sacrificed via CO₂ overdose and whole organs (liver, lung and brain) were collected and fixed in 10% formalin. Tissues were sectioned and stained with hematoxylin and eosin. The sections were imaged using an inverted light microscope and assessed by a veterinary pathologist at Mass Histology services for further analysis.

**FTIR**
All FTIR samples were suspended in identical concentrations and volumes of a water/acetone mixture. Samples were pipetted onto a zinc selenide ATR crystal and the water/acetone mixture was evaporated completely, leaving a film of the sample. Samples were then placed into an FTIR spectrometer (NICOLET 4700, Thermo Electron Corporation) and the chamber was purged with nitrogen for 30 minutes. Dry crystal backgrounds were subtracted from each sample’s spectrum.

Figures

**SI Figure 1: FTIR of PLNs and PLN composition materials.** Spectra of PAH, BSA, PS, PLNs prior to core dissolution and PLNs following core dissolution.
SI Figure 2: Device schematic. (a) Schematic of microfluidic device used for *in vitro* adhesion of spheres, discs and PLNs. (b) Brightfield and fluorescent image of microfluidic channel coated with fluorescent BSA.
SI Figure 3: Attachment of peptides to PLNs. (a) Peptide coupling to dendrimers, (b) amine modification of PLNs. (c) (i) Method of attachment of peptides to PLNs and (ii) final PLN schematic. (d) Quantification of peptides attached per PLN. (e) Qualitative confirmation of peptide attachment via confocal microscopy.
SI Figure 4: Schematic of parallel plate flow chamber. Experimental setup for PPFC in vitro adhesion and aggregation studies.
SI Figure 5: PLN Aggregation. Aggregation of PLNs before, during, and after flow through a glass capillary. Scale bars = 20 μm unless otherwise noted.
SI Figure 6: Histology. Representative histology sections of liver, brain and lung for saline injected (n=3) and PLN injected (n=3) BALB/c mice at 24 hours. Scale bars = 100 μm.
Appendix III: General Protocols
Peptide Synthesis, Cleavage, and Characterization

*Peptide synthesis using ABI synthesizer*

Start with a Knorr resin, which consists of a polystyrene bead, a linker molecule (resin), and an Fmoc N-terminal protection group.

![Knorr Resin](image)

All amino acids are conjugated in the L-conformation in our reaction, which is the naturally found conformation in the body and is represented by a capital letter.

The peptide is formed by creating amide (or peptide) bonds between the amino acids. This is achieved by creating a CONH bond from the NH$_2$ (N-terminal) of the previous amino acid and the COOH (C-terminal) of the new amino acid. Peptides are synthesized from C-N terminals.

Preparation:

1. To perform a 0.25mmol synthesis, weigh out 1mmol of each amino acid (molecular weight written on the bottle x 10^-3) and transfer to correctly labeled cartridge using funnel made from weigh paper. Be careful not to lose any AA in the transfer or contaminate any sample with prior AA powder. Attach cap using tool located in synthesizer lab. Make sure that cap is on straight!
2. Assemble reaction vessel by placing a circular filter on the end of the vessel containing tubing and tightening the cap all the way past the click. Weigh out 0.25mmol of the Knorr resin (for a 0.25mmol synthesis minding the 0.2 loading ratio use 1.25g resin) and transfer the resin to the reaction vessel. Place a second circular filter on the open end of the reaction vessel and tighten the second cap. Make sure that both ends are as tight as possible to avoid leak during synthesis.
3. Make two empty cartridges and attach cap as in number 1.
4. Check levels on the following bottles (keep in mind that all bottles must be attached since everything is pressurized with nitrogen):
   - Bottle 1: Piperidine – needs to be ¾ full
   - Bottle 5: Activator (HOBt/HBTU) – only good for 3-4 weeks, should be at least 100ml
   
   To make 0.45 M solution of HBTU and HOBt in DMF
   100mMol HBTU (37.93g)
   100mMol HOBt (13.51g)
   Add DMF to bring volume to 222ml (approx. 200ml)

   - Bottle 6: Methanol – ½ full to wash resin after synthesis
   - Bottle 7: 2M DIEA in NMP – 100ml scavenger
   
   To make 200ml 2M DIEA in NMP
   69.70 ml DIEA (d=0.724g/ml)
   130.3 ml NMP

   - Bottle 9: DCM - ½ bottle to swell bead 10x as much
   - Bottle 10: 2 bottles NMP – both full to same level. If need to fill, flip both gas switches located on the upper right portion of the side of synthesizer to “off” before opening bottles. When bottles are re-sealed, flip switches back to “on”.

5. Check the nitrogen tank – pressure on the left should read 65 psi, while the pressure on right should be >1000 psi

6. Open log book and sign in: your name, PI, date, # of cartridges, # of amino acids. Add # of AA’s to the previous entry’s in-line, bottom RV, and top RV filters. If any value is greater than the indicated values (25, 50, 75), change the filter and enter the new value for each filter (the # of AA’s).

7. Check waste container level and empty in fume hood if needed.

8. Fill out a peptide synthesis record sheet.

9. Open up the computer program and create a new sequence file, listing all of the amino acids in the correct order.

Flow tests:

1. Open chemistry file: Flow Tests 1-18.kem
2. Turn on the peptide synthesizer.
3. Make sure vents? are up for bottles 9&10 on the side of the machine.
4. Send the chemistry file to the synthesizer (communication enabled; com1; ok; send; set; close)
5. Prepare 7 empty cartridges to test mg and connect the graduated cylinder to test ml.
6. Open nitrogen tank.
7. On synthesizer, go to main menu; module test; check A on record sheet
8. Run each test and document on record sheet
a. A (bottle 10) – cylinder 3ml at start, read after test and record reading-3ml
b. B (bottle 10) – weigh empty cartridge and tare scale, weigh after test and record mass
c. a (bottle 1) – cylinder 3ml at start, read after test and record reading-3ml
d. D (bottle 5) – weigh empty cartridge and tare scale, weigh after test and record mass
e. B (bottle 10) – weigh empty cartridge and tare scale, weigh after test and record mass
f. H (bottle 8) – weigh empty cartridge and tare scale, weigh after test and record mass
g. i (bottle 9) – cylinder 3ml at start, read after test and record reading-3ml

Peptide Synthesis:

1. Load amino acid cartridges into synthesizer (N-terminus on left, C-terminus on right) and place empty start/end cartridges on each end.
2. Open chemistry file FastMoc0.25monPredPK.kem (send; send; set; close)
3. Screw reaction vial into place and connect the tubing to machine.
4. Open new run file: 1st line = chemistry file, 2nd line = sequence file. Double click on the 1st cell and change to amide resin. Substitution = 0.2 and weight = 1.25g (save; set; send; close; close; close)
5. Cycle monitor = NO; resin sampling; continue; print? NO; begin
6. Stay for 5 minutes to check for leaks and make sure everything is running properly

After Synthesis is Complete:

1. Check graphs to see if peptide synthesis looked good
2. Main menu of synthesizer, manual control; function
3. To rinse with methanol: function number 54; vortex
4. To drain: function number 42
5. Repeat 3x
6. Close computer program; turn off synthesizer
7. Remove reaction vessel from synthesizer.
8. Rinse out all cartridges with isopropanol and place on drying rack.
9. Collect resin-peptide into a 50ml falcon tube by carefully rinsing with methanol and collecting. Dispose of used filters and replace clean reaction vessel downstairs.
10. Let resin-peptide settle to bottom of falcon tube and remove methanol using a transfer pipet.
11. To ensure that most of the methanol has evaporated off, dry with air in synthesis hood.
12. Cover falcon tube with a kimwipe and place in vacuum oven overnight to remove all of the methanol
13. After removing from the vacuum oven, lyophilize the resin-peptide overnight to remove any remaining traces of moisture. Once lyophilized, the resin-peptide can be stored long-term in -20°C freezer until ready for cleaving.

**Cleavage of resin and protection groups from peptide**

Cleavage Procedure for Resin-Peptide: DATE: ______________

1. Pre-mix the appropriate cleavage cocktail in a microcentrifuge tube
2. Add this solution into the reaction vial (15 ml polypropylene falcon tube) containing a magnetic stir bar with desired amount of resin-peptide. (Empty falcon tube 1 weight: ____________)
3. Add appropriate volume of TFA (Total Volume: 1250μl) into the reaction vial (START TIME: ____________)
4. Stir constantly for 2 hours at Room Temperature. (Do not let the reaction exceed 4 hours) (END TIME: ____________)

Isolation of the final product: DATE: ______________

1. Set up cleavage apparatus. A polypropylene filter should be placed over a flask containing 5-10ml (5-10 fold excess) of ice-cold diethyl ether. (Keep the apparatus over ice for the duration of the reaction for maximum recovery of precipitate)
2. Once cleavage reaction has gone to completion (after 2 hours), transfer the contents into the cleavage apparatus and allow the solution to mix dropwise into the ice-cold diethyl ether.
3. Rinse the original reaction vial and filter support containing the resin with a small volume (usually no more than 500μl of cleavage cocktail or TFA.
4. Transfer the ether-precipitate mixture into a falcon tube, and chill in the freezer or in a dry ice/acetone bath. (Maximum recovery of the precipitate is achieved if incubated at 4°C overnight)
5. Centrifuge the ether-precipitate mixture at 750-1000 x g’s for 3-5 mins to allow the precipitate to form a pellet at the bottom of the falcon tube.
6. Carefully remove the ether layer and place into a clean 15ml falcon tube. Empty Falcon Tube 2 Weight: ____________
7. Blow Nitrogen gently for 5-10 minutes across the falcon tube to remove any excess ether.
8. Finally, add 1-2 ml of millipore water to resuspend the precipitate.
9. Freeze at -80°C for 30-mins-1 hour and lyophilize overnight. (Falcon Tube 2 Weight After: ____________)

**Peptide characterization using MALDI-TOF mass spectroscopy**

MALDI-TOF mass spec gives a spectrum of masses in the sample. The experimental value can be compared to the theoretical molecular weight of the synthesized peptide in order to determine synthesis completion. Note that the actual measured mass may be the
MW of your sample + its association with a positively charged ion such as H+ (MW = 1), Na+ (MW = 22.99), etc.

Sample prep:

Use HCCA for peptides <5 kDa and 2,5-DHB for peptides >3 kDa:

1. Dissolve sample in 100μl of MeOH.
2. Make a matrix solution by dissolving matrix powder in MeOH until it reaches saturation (until no more powder can be dissolved and you see precipitate at the bottom of the vial).
3. Add 400μl of the matrix solution to the dissolved sample and vortex.
4. End ratio of matrix:peptide should be between 10:1 and 100:1.

Running the sample on the MALDI:

1. Place sample tray in holder and pipet about 15μl of sample onto clean cell and record the position (ie. A5-A7). Let the sample air dry.
2. Log into the computer: Username: Sengupta  Password: Wickenden526 (case sensitive)
3. Open the Flex Control software.
4. Open the tray on the MALDI by pushing the green button on the machine and insert the sample tray DOUBLE EDGES IN. Close the tray by again pushing the green button.
5. Open Flex Control Method: RP_PepMix.par for peptides and lipid-peptide conjugates. Open a lift method file if you wish to fragment the sample.
6. Wait until PREPARE and DOCK in the bottom right-hand corner of the screen turn GREEN.
7. Click on the cell in the diagram that you want to measure.
8. Set the range under SPECTROMETER to Matrix Supression below 500.
9. Set Setup-offset to 85.
10. Make sure ADDED START is set at 200.
11. Set the lazer power using the scrolling scale to below 35 and adjust later to get the cleanest spectrum.
13. Save as including the name of the sample, matrix used, laser power, and date. Make sure Open in Flex Analysis box is checked.

Analysis Software on MALDI computer: Flex Analysis

1. Open Flex Analysis and open the desired mass spectrum.
2. Push the baseline subtract and smooth buttons in the top toolbar.
3. Add peak values as desired or click on the button to automatically pick peak values.
4. File; save as same name as spectrum file.
5. Export spectrum to a text file with the same name.

Analysis Software available for free download: Mmass
1. Open Mmass and open the text file that you desire to view the spectrum.
2. In this software, you can label peaks, etc.
3. Print screen the analyzed spectrum and paste into powerpoint.
4. Crop the image as desired.
5. Print screen the cropped spectrum and paste into Paint.
6. Add thicker axis lines, numbers, etc. for print quality graphs.
7. Copy and paste into new Photoshop file with Resolution=1200.
8. Save as a photoshop file as well as a JPEG.
Lipid-Peptide Conjugation and Yield Characterization

Conjugation of Lipid-PEG-peptide and Peptide Deprotection

DSPE-PEG-COOH
Chemical Formula: C_{132}H_{253}N_{2}O_{57}P
Exact mass: 2819.75 g/mol
Molecular Weight: 2821.45 g/mol

Using DSPE-PEG-COOH bought from Avanti Polar lipids, the peptide is going to be conjugated to the lipid through the formation of an amide bond. The COOH will react with the NH₂ on the N-terminal of the peptide to form CONH. Therefore, the reaction will look like the following:

\[
\text{DSPE-PEG-COOH + peptide-NH}_2 \rightarrow \text{DSPE-PEG-peptide}
\]

This conjugation will be performed by the “Single-Step EDC Coupling” method. This method was chosen because it is appropriate for small organic molecules having one or more amines without the presence of carboxylates. Since the conjugation is performed before cleaving the peptide of its protection groups on each side chain, only the N-terminal amine is reactive. Although the biologically toxic byproduct urea is produced from this reaction, complex purification steps to remove urea can be avoided by removing the urea dissolved in the aqueous buffer from the precipitated final product.

In order to calculate the amount of peptide to use in our reaction, we need to consider the overall goal of this reaction and how the peptide is conjugated. The EDC reaction recommends a 10 fold excess of peptide over carboxyl groups to ensure that the reaction goes to completion. It is important to note, however, that our peptide is attached to a knorr resin. The knorr resin we used has a loading ratio of 0.2 mmol/g, which means that for every gram of resin weighed out, we would have 0.2 mmoles of peptide.

Single-Step EDC Reaction of DSPE-PEG-COOH to protected peptide  DATE:

1. Add ~5ml of 0.05M MES Buffer; pH 5.0-5.5 into the reaction vial (15 ml polypropylene falcon tube) with a stir bar. (Empty Falcon Tube 1 Weight: _________)
2. Add appropriate volume of DSPE-PEG-COOH Stock Solution (20 mg/ml) into the reaction vial.
3. Add appropriate amount of resin-peptide into the reaction vial.
4. Add ~100 mg of EDC●HCl into the reaction vial. START TIME: ____________

5. Allow reaction to go to completion under constant, moderate stirring at room temperature. Reaction should proceed for at least 2-4 hours, preferably overnight. END TIME: ____________

Isolation of Beads: DATE: ____________

I) After reaction has gone to completion, remove the magnetic stir bar, and place reaction vial in centrifuge for 5 mins at 500-750 x g’s allowing the resin-conjugate to settle at the bottom.

II) Carefully remove the aqueous buffer and place into a separate falcon tube.

III) Reconstitute reaction vial with 5-10 ml of Milipore water and vortex to ensure complete mixture of resin-conjugate with the water.

IV) Repeat these steps for a total of three washes with Milipore. (Be sure to remove all of the frothy urea byproduct)

V) Finally, add 1 ml of Millipore water, freeze at -80 C for 30-mins-1 hour and lyophilize overnight. (Falcon Tube 1 Weight After: _________)

Cleavage Procedure of Resin-Peptide After Conjugation: DATE: ____________

5. Pre-mix appropriate cleavage cocktail in a microcentrifuge tube

6. Add this solution into the reaction vial containing a magnetic stir bar with DSPE-PEG-peptide (protected).

7. Add appropriate volume of TFA (Total Volume: 1250μl) into the reaction vial START TIME: ____________

8. Stir constantly for 2 hours at Room Temperature. (Do not let the reaction exceed 4 hours) END TIME: ____________

Isolation of the final product: DATE: ____________

10. Set up cleavage apparatus. A polypropylene filter should be placed over a flask containing 5-10ml (5-10 fold excess) of ice-cold diethyl ether. (Keep the apparatus over ice for the duration of the reaction for maximum recovery of precipitate)

11. Once cleavage reaction has gone to completion (after 2 hours), transfer the contents into the cleavage apparatus and allow the solution to mix dropwise into the ice-cold diethyl ether.

12. Rinse the original reaction vial and filter support containing the resin with a small volume (usually no more than 500μl of cleavage cocktail or TFA.

13. Transfer the ether-precipitate mixture into a falcon tube, and chill in the freezer or in a dry ice/acetone bath. (Maximum recovery of the precipitate is achieved if incubated at 4 C overnight)

14. Centrifuge the ether-precipitate mixture at 750-1000 x g’s for 3-5 mins to allow the precipitate to form a pellet at the bottom of the falcon tube.

15. Carefully remove the ether layer and place into a separate falcon tube.
16. Blow Nitrogen gently for 5-10 minutes across the falcon tube to remove any excess ether.
17. Finally, add 5 ml of millipore water to resuspend the precipitate.
18. Dialyze this solution against 1-2 L of Millipore water (1:200 to 1:400 dilution) and a 1000 MW semi-permeable membrane. For best results, replace Millipore water on the outside of the semi-permeable membrane 3 times every 3-4 hours.
19. Remove from Dialysis, and transfer contents to a 15 ml falcon tube (Empty Falcon Tube 2 Weight: _________)
20. Freeze at -80 C for 30-mins-1 hour and lyophilize overnight. (Falcon Tube 2 Weight After: _________)

Quantification of lipid-peptide conjugation yield

Lipid-peptide conjugation can be confirmed using MALDI-TOF mass spec as previously described. In addition, the conjugation yield can be determined using Ninhydrin reagent to quantify the concentration of free amines before and after conjugation. For this, a 100ul sample of lipid-peptide solution can be taken before addition of EDC and after the reaction has run to completion. The protocol for Ninhydrin calibration curve with a peptide is shown below, but the same procedure is used for a 100ul sample before and after conjugation.

Ninhydrin Calibration Curve with peptide before cleavage (start with high levels of amine 1.4-3.4 μmol)

Reagents (make fresh each day):
Solution 1: 80% Phenol Solution
1. Dissolve 40g of reagent grade phenol in 10ml of Absolute Ethanol using a 20ml glass beaker
2. Warm slightly on hot plate until solution is completely dissolved under constant stirring with a magnetic stir bar
3. Once completely dissolved, remove from heat and add 4 grams of Amberlite mixed-bed resin (MB-3)
4. Sit reaction for 45 minutes
5. Filter with Whatman Paper #1
6. Transfer filtrate into a clean 20ml glass vial

Solution 2: KCN-Pyridine Solution
1. Dissolve 65.12mg KCN in 100ml Millipore water in a 150ml glass beaker under constant stirring with a magnetic stir bar to make 0.01M KCN
2. Add 2ml of the 0.01M KCN solution into another clean 150ml glass beaker containing 100ml of pyridine and mix under constant stirring with a magnetic stir bar
3. Weigh out 4g of MB-3
4. Add to the container under constant stirring
5. Filter into a new glass vial

Ninhydrin Reagent A: Combination of Solutions 1 and 2
Ninhydrin Reagent B: 0.28M Ninhydrin Solution
1. Dissolve 500mg of ninhydrin in 10ml of Ethanol
2. Purge with Nitrogen, parafilm, and store in dark until use

Ninhydrin Reagent C: 60% Alcohol (by volume)
1. Add 24ml Ethanol into a 40ml glass vial containing 16ml Millipore water

Protocol:
1. Heat a heating block to 100°C
2. Weigh 2-5mg of peptide before cleavage into a 10 x 75 mm test tube, and from the MW calculate the moles of primary amines in the batch:
   \[
   \text{mass (g)} \div \text{MW (g/mol)} = \text{moles of NH}_2
   \]
3. Add 100µL of Reagent A and 25µL of Reagent B.
4. Add stir bar to tube
5. Equilibrate tube to 100°C
6. Stir reaction for 10min
7. Place tube in an ice bath or freezer to cool
8. Add 2ml of 60% ethanol in water and mix
9. Filter using cleavage apparatus
10. Rinse 2x with 0.5ml of 0.5M Et4NCl
11. Dilute the sample to 49.58079438µM with 60% ethanol
   \[
   \frac{49.58079438 \times 10^{-6} \text{ moles}}{L} = \frac{\text{moles NH}_2}{L}
   \]

and make serial dilutions as follows:

<table>
<thead>
<tr>
<th>Vial #</th>
<th>Stock solution (#:ml)</th>
<th>MES Buffer (ml)</th>
<th>Molarity of primary amines (µM)</th>
<th>Absorbance (AU)</th>
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<td></td>
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</tr>
<tr>
<td>15</td>
<td>11:0.125</td>
<td>1.875</td>
<td>0.0030</td>
<td></td>
</tr>
</tbody>
</table>
12. Microplate absorbance at 570nm

**Liposome Fabrication and Characterization**

1. Calculate liposome make-up using the starting materials listed in table below:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Molecular Weight (g/mol)</th>
<th>Mole Fraction</th>
<th>Moles</th>
<th>Mass (g)</th>
<th>Volume (μL) 20mg/ml stock solution</th>
<th>Volume (μL) 5mg/ml stock solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC</td>
<td>790.145</td>
<td>0.49</td>
<td>4.9×10⁻⁶</td>
<td>0.00387</td>
<td>194</td>
<td>774</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>386.65</td>
<td>0.45</td>
<td>4.5×10⁻⁶</td>
<td>0.00174</td>
<td>87</td>
<td>348</td>
</tr>
<tr>
<td>DSPE-PEG</td>
<td>2805.479</td>
<td>0.025</td>
<td>2.5×10⁻⁷</td>
<td>0.00070</td>
<td>35</td>
<td>140</td>
</tr>
<tr>
<td>DSPE-PEG-peptide</td>
<td>2805.479</td>
<td>0.025</td>
<td>2.5×10⁻⁷</td>
<td>N/A</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>DHPE-Rhodamine</td>
<td>1333.81</td>
<td>0.01</td>
<td>1×10⁻⁷</td>
<td>.000133</td>
<td>N/A</td>
<td>27</td>
</tr>
</tbody>
</table>

Keep the overall mole percent of “PEG” at 5% when factoring in a PEGalyated peptide, for example DSPE-PEG-DAEWVDVS. Also, keep the overall DSPC at 50% when factoring in fluorescence, for example DSPE-Rhodamine.

2. Multiply the volumes by the number of batches.

3. Combine all substances in a round bottom flask in the synthesis hood.

4. Blow air in the flask while rotating to form a monolayer lipid film.

5. Use the vacuum oven to remove all MeOH and Chloroform overnight.

6. Reconstitute the lipid film in 1.3ml 1x PBS per batch of liposomes. (DATE:_______________)

7. Freeze-thaw the solution by immersing the round bottom flask in liquid nitrogen until you hear a crackling noise to verify that the solution is frozen, then immerse the round bottom flask in a 60°C waterbath (using the Rotovap). Repeat at least ten times. This process causes the lipid film to form into liposomes because the
most thermodynamically favorable orientation of the lipid under these conditions is a sphere.

The freeze-thaw causes the liposomes to form; however, they formed many shells (multilaminar vesicles) instead of a single lipid bilayer (unilaminar vesicles) as we desire. So we must perform extrusion to get the desired end product of 150-200nm in diameter, spherical shaped, liposomes with a single lipid bilayer (unilaminar vesicles with an approximate diameter of 150-200nm).

8. Set up the extrusion apparatus and warm the extruder on a hot plate to 80°C. Equilibrate the liposome solution to RT, and then heat on extruder for a minute or two before performing extrusion. Extrude the liposome solution 15 times (31 total passes through the filter) and transfer to a new 15ml falcon tube. (Falcon tube weight before:_________________) Collect 10μl of the liposome solution after extrusion to ensure that you have achieved the desired size of the liposomes using the DLS (Particle Sizer).

<table>
<thead>
<tr>
<th>Trial Number</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>PDI:</td>
</tr>
</tbody>
</table>

9. For short term storage (~3 months), place liposome solution in 4°C fridge.
10. For long term storage (years), freeze the solution for 1 hr at -80°C and lyophilize. (Falcon tube weight after:_________________)
**Blood Draw and Isolation, Activation, Fixation of Platelets**

**Blood Preparation:**
1. Draw blood at a ratio of 9 parts whole blood to 1 part anticoagulant. (Draw 9 ml of blood into a syringe containing 1 ml of 0.13 M sodium citrate solution (3.8% w/v Sodium Citrate)).
2. Transfer whole blood to 15 ml falcon tubes. Do not fill falcon tube with more than 10 ml of whole blood when centrifuging. Whole blood can be used directly, or centrifuged further to obtain platelet rich plasma, platelet poor plasma, and/or a platelet suspension. A cell count can be performed using a coulter counter to determine the concentration of red blood cells and/or platelets in whole blood.
3. To obtain Platelet Rich Plasma (PRP), centrifuge whole blood at 150 x g for 15 minutes at room temperature (15°-28°C). Red blood cells will centrifuge to the bottom, platelet rich plasma will remain on top and a thin buffy coat containing white blood cells remain between the two layers.
4. Examine the plasma layer for red blood cells, and re-centrifuge at 200 x g for an additional 5 minutes if necessary.
5. Using a plastic transfer pipette, observe and carefully remove the PRP layer without disturbing the buffy coat or red blood cells, and transfer to a clean 15 ml falcon tube labeled PRP. The platelet rich plasma maybe used directly, or centrifuged further to obtain platelet poor plasma and/or a platelet suspension. A cell count can be performed using a coulter counter to determine the concentration of platelets in plasma.
6. To obtain Platelet Poor Plasma (PPP), centrifuge the PRP at 2500 x g for 25 min. Platelets will centrifuge to the bottom, forming a platelet pellet while the PPP will remain on top.
7. To obtain a platelet suspension, remove the PPP and resuspend the platelet pellet in 1-2ml of 1x PBS/1% BSA. A cell count can be performed using a coulter counter to determine the concentration of platelets in suspension.

**Activating Platelets with ADP:**
1. Bring stored reagent (lyophilized powder) to room temperature (18°-28°C) prior to use.
2. Reconstitute a vial of ADP with 0.5 ml of Millipore water to obtain a working concentration of 2×10^-4 M. (Reconstituted ADP is stable for 30 days when stored at 2°-8°C in its originally tightly sealed container).
3. Add ADP to platelet rich plasma or platelet suspension at a final concentration of 2×10^-5 M or 50μl ADP working solution to 450μl PRP/platelet suspension with a concentration of 200×10^6 platelets/ml.
4. Incubate for 5 minutes to activate platelets before use.

**Making 4% PFA and fixing platelets:**
1. Add 500mg PFA into a 50ml Erlemeyer flask containing a magnetic stir bar
2. Add 12.5ml 1x PBS pH 7.4 to flask containing PFA
3. Stir contents at 55-60°C until the PFA is completely dissolved (NOTE: temperature must be maintained inside this solution for the duration of the reaction or else PFA will crosslink)
4. Sterilize by filtering with a 0.22 micron pore-size syringe filter
5. Immediately store in a 15ml falcon tube at 4°C for up to two weeks
6. Fix platelets by incubating with 4% PFA for ~15 min at room temperature
Static Nanoparticle Binding Assay

Purpose: Determine binding of peptide-modified liposomes to activated platelets.

1. Place glass coverslips in wells of 6 or 12 well plate.
2. Gently wash all wells with 1X PBS/1% BSA
3. Add 200µl of activated platelet suspension to wells and incubate in a shaker at 80 RPM for 1 hour at room temperature.
4. Wash wells with 1X PBS/1% BSA 3 times to remove unbound platelets
5. Add 200ul of 4% PFA to wells and incubate in a shaker at 80 RPM for 1 hour at room temperature to fix adhered platelets.
6. Wash wells with 1X PBS 3 times
7. Add 200ul of fluorescently labeled liposomes in 1X PBS to wells. Fluorescent antibodies can be used as positive control. Pre-blocking with non-fluorescent antibody can be used to determine specificity in platelet-receptor binding.
8. Incubate well plate in a shaker at 80 RPM for 1 hour at room temperature.
9. Wash all wells at least 3 times with 1X PBS to remove unbound liposomes.
10. Place coverslips in a petri dish with lid partially covered and let dry in a laminar flow hood
11. Mount coverslips onto glass slides and image using fluorescence microscopy
### Flow Cytometry Nanoparticle Binding Assay

**Materials:** 5ml polystyrene tubes with cell-strainer cap: BD Falcon REF 352235

1. Draw 10mL human whole blood into a syringe containing 1mL 3.8% w/v sodium citrate
2. Aliquot 10uL whole blood into 32 microcentrifuge tubes.
3. To the “a” series of the whole blood samples, add 5ul ADP to activate the platelets. Let the platelets activate for 20 minutes.
4. Resuspend 1 batch of lyophilized liposomes in 237uL Millipore water (in order to add final lipid concentration of 500uM). Sonicate liposomes for at least 30 min and confirm size using DLS.
5. Add to all blood samples 10ul 4% PFA and allow samples to fix for about an hour.
6. Add liposomes and antibodies to samples as follows:

<table>
<thead>
<tr>
<th>Fluorescence</th>
<th>#</th>
<th>Activated sample (ADP)</th>
<th>#</th>
<th>Unactivated sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled</td>
<td>1a</td>
<td>1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AlexaFluor anti-CD62P</td>
<td>2a</td>
<td>2ul</td>
<td>2b</td>
<td>2ul</td>
</tr>
<tr>
<td>FITC anti-CD41a</td>
<td>3a</td>
<td>3ul</td>
<td>3b</td>
<td>3ul</td>
</tr>
<tr>
<td>Rhodamine-labeled Unmodified liposomes</td>
<td>4a</td>
<td>5ul</td>
<td>4b</td>
<td>5ul</td>
</tr>
<tr>
<td>Rhodamine-labeled P-selectin (D) liposomes</td>
<td>5a</td>
<td>5ul</td>
<td>5b</td>
<td>5ul</td>
</tr>
<tr>
<td>Rhodamine-labeled GPIIb-IIIa (IRGD) liposomes</td>
<td>6a</td>
<td>5ul</td>
<td>6b</td>
<td>5ul</td>
</tr>
<tr>
<td>Rhodamine-labeled GPIIb-IIIa (cRGD) liposomes</td>
<td>7a</td>
<td>5ul</td>
<td>7b</td>
<td>5ul</td>
</tr>
<tr>
<td>Rhodamine-labeled Linear dual liposomes</td>
<td>8a</td>
<td>5ul</td>
<td>8b</td>
<td>5ul</td>
</tr>
<tr>
<td>Rhodamine-labeled Cyclic dual liposomes</td>
<td>9a</td>
<td>5ul</td>
<td>9b</td>
<td>5ul</td>
</tr>
</tbody>
</table>

7. Gently tap the bottom of the tubes to mix and allow to incubate for 10 minutes.
8. Add 450uL 1x PBS pH 7.4 to each sample.
9. Transfer the samples to FACS tubes (Fischer, cat#352235)
10. Use flow cytometry analysis to characterize fluorescence values of samples bound to platelets, comparing fluorescently labeled platelets to unlabeled 20,000 events (counts) per aliquot.
11. Analyze results: expect to see more fluorescence and hence more binding of liposomes to activated platelets vs. non-activated platelets.
Parallel Plate Flow Chamber Dynamic Binding Assay

**Acid wash glass slides:**
This helps cells and polyamino acids stick to glass. It also helps your DIC pictures look prettier by getting rid of small particles stuck on the glass.

1. Heat 10-20 16 mm circular glass coverslips in a loosely covered 50 ml glass beaker containing 30 ml of 1M HCl at 50-60°C for 4-16 hours. It is important that the coverslips are not sticking to each other in order to ensure proper cleaning.
2. Cool to room temperature.
3. Wash the glass coverslips 3 times with Millipore water, removing any residual HCl.
4. Fill beaker with 30 ml of Millipore water and sonicate in a water bath for 30 mins.
6. Discard Millipore water, and repeat step 4 with 50% Millipore:50% EtOH Solution.
7. Discard 50% Millipore: 50% EtOH solution, and repeat step 4 with 100% EtOH.
8. Discard 100% EtOH solution and replace with fresh 100% EtOH for storage. (Coverslips may be stored in 100% EtOH until use)
9. Prior to use, discard 100% EtOH, and transfer coverslips to a petri dish with lid partially covered and let dry in a laminar flow hood.

**Slide Preparation:**

**Collagen coat glass slides**
This should be done during the centrifugation and activation of the PRP.

1. Using the largest circular gasket, draw a circle on one end of the glass slide with a marker.
2. Distribute collagen solution evenly inside of circle and subsequently remove with a pipet.
3. Repeat coating for a total of 3 times.
4. Remove the gasket and allow the collagen coated slide to dry in the hood for approx. 10 mins.
5. Place the slide in a petri dish and cover with 1x PBS/1% BSA and allow to fix for at least 30 minutes.

**Activated platelet coated glass slides**

1. Incubate approx. 200ul of ADP activated PRP on the collagen coated surface for 45-60 min.
2. Gently wash with 1x PBS and fix by incubating in 4% PFA for an hour.
3. Gently wash the slide again with 1x PBS and store in 1x PBS/1% BSA at 4°C for no longer a week until use.
Avidin coated glass slides
1. Using the largest circular gasket, draw a circle on one end of the glass slide with a marker.
2. Distribute 5mg/ml avidin solution (Thermo Scientific cat# 21121) evenly inside of circle and subsequently remove with a pipet.
3. Repeat coating for a total of 3 times.
4. Remove the gasket and allow the avidin coated slide to dry in the hood for approx. 10 mins.
5. Place the slide in a petri dish and cover with 1x PBS/1% BSA and allow to fix for at least 30 minutes (store in 1x PBS/1% BSA at 4° for no longer than 24 hours until use).
6. Before use, rinse 1x with PBS.

Parallel Plate Flow Chamber Experiments:

0:00  Reconstitute 1 batch of fluorescent particles with 10mL PBS and sonicate for 10mins
       Set up Parallel Plate Flow Chamber with appropriate flow rate

0:15  Start Parallel Plate Flow Chamber (Run 5 mins) with fluorescent particles

0:20  Stop time and remove slide
       Gently wash slide with Milipore Image (10 images per substrate)
       Place slide back on Parallel Plate Flow Chamber

0:45  Start Parallel Plate Flow Chamber (Run 10 mins) with fluorescent particles

0:55  Stop time and remove slide
       Gently wash slide with Milipore Image (10 images per substrate)
       Place slide back on Parallel Plate Flow Chamber

1:20  Start Parallel Plate Flow Chamber (Run 15 mins) with fluorescent particles

1:35  Stop time and remove slide
       Gently wash slide with Milipore Image (10 images per substrate)
       Place slide back on Parallel Plate Flow Chamber

2:00  Open loop, Start Parallel Plate Flow Chamber (Run 5 mins) with PBS
<table>
<thead>
<tr>
<th>Time</th>
<th>Action</th>
</tr>
</thead>
</table>
| 2:05 | Stop time and remove slide  
Gently wash slide with Milipore  
Image (10 images per substrate)  
Place slide back on Parallel Plate Flow Chamber |
| 2:30 | Start Parallel Plate Flow Chamber (Run 10 mins) with PBS |
| 2:40 | Stop time and remove slide  
Gently wash slide with Milipore  
Image (10 images per substrate)  
Place slide back on Parallel Plate Flow Chamber |
| 3:05 | Clean up Parallel Plate Flow Chamber |
| 3:30 | END |
Mice Tail Transection Hemostatic Assay

1. Extrude liposomes before experiments, resulting in a mean diameter between 150-200nm, verified by DLS.
2. Inject mice via the tail vein with 10mL/kg (mouse weight) of saline only or saline containing 30mg/kg (mouse weight) of nanoparticles.
3. Thirty minutes after injection, completely transect 0.5cm from the end of the tail and submerse in 37 degrees C saline.
4. Measure time until cessation of bleeding.

For biodistribution studies

1. Harvest the lungs, spleen, and liver 3, 24, and 72 hours post-injection.
2. Homogenize the tissue in saline.
3. Spin down at 10,000rpm for 10 minutes.
4. Collect the supernatant and analyze for nanoparticle fluorescence using a fluorescent plate reader. The fluorescence intensity can be used to determine fluorescent particle accumulation in the tissue.
Drug Encapsulation and Release

*PLA₂-triggered CF Release from Liposomes:*

**Materials:**

5mM Tris-HCl Buffer
1. Measure out 500ml Millipore Water
2. Weigh out 393.9mg Tris-HCl (MW: 157.56 g/mol) and add to water
3. Mix until dissolved
4. Adjust pH to 7.4 with 1M NaOH

5mM Tris-HCl + 1mM CaCl₂ Buffer
1. Measure out 50ml of 5mM Tris-HCl Buffer
2. Weigh out 5.55mg CaCl₂ (MW: 110.98 g/mol) and add to Tris-HCl Buffer
3. Mix until dissolved

10% TritonX-100
1. Measure out 45ml of Millipore water
2. Add 5ml of TritonX-100
3. Mix (fast) until dissolved

100mM Carboxyfluorescein (CF) in 5mM Tris-HCl Buffer
9. Measure out 50ml of 5mM Tris-HCl Buffer
10. Weigh out 1.882g of 5,6-Carboxyfluorescein (MW: 376.33 g/mol) and add to Tris-HCl Buffer (will not dissolve completely)
11. Freeze and lyophilize solution
12. Add 1M NaOH to the lyophilized powder until all CF is dissolved (should be red translucent solution)
13. Adjust the pH to 7.4 with 0.1M HCl
14. Add additional Millipore water if necessary until original volume (50ml)

125ug/ml PLA₂
1. Prepare a stock of 1mg/ml PLA₂ in Millipore water
2. Measure out 140ul of Millipore water into a microfuge tube
3. Add 20ul of 1mg/ml PLA₂ stock to the vial and vortex to mix

**Procedure:**

CF encapsulation in Liposomes:
1. Make 6 batches of lipid films using unmodified liposome protocol. **Date:**
2. Rehydrate lipid film with 100mM CF solution (1ml per batch) and perform 10 cycles of freeze thaw. **Date:**
3. Sonicate liposomes for 15-20 minutes and confirm size distribution using DLS.
4. Ultracentrifuge the liposome solution at 100,000rpm for 20 minutes to separate unencapsulated CF from the liposomes.
5. Remove supernatant and measure absorbance (492nm) (______________) and fluorescence (Ex: 492nm, Em: 517nm, Gain: 25) (______________) using plate reader to determine encapsulation efficiency:

\[
EE (%) = \left[1 - \frac{\text{amount unencapsulated}}{\text{starting amount}}\right] \times 100
\]

6. Resuspend liposome pellet in 5mM Tris-HCl + 1mM CaCl\(_2\) at a concentration of 2ml per batch (0.1 umol lipid/ml).

PLA\(_2\)-triggered CF Release from Liposomes

Date: ___________

1. Add 490ul of liposome solution to tubes labeled as shown in the table below.
2. To half of the tubes, add 10ul of 125ug/ml PLA\(_2\).
3. To the other half of tubes, add 10ul of Millipore water.
4. Place the tubes on a shaker in an incubator set at 37°C (Marchant Lab).
5. At each time point remove a tube from the incubator and take 2-100ul samples and add to black-walled 96 well plate (to measure released CF).
6. Add 20ul of 10% TritonX-100 solution to the tubes and vortex to disrupt liposome membrane and release all remaining CF into the solution.
7. Take 2-100ul samples and add to black-walled 96 well plate (to measure total CF).
8. Read the absorbance (492nm) and fluorescence (Ex: 492nm, Em: 517nm, Gain: 25) at each time point immediately after isolating:

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Absorbance</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
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<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. Calculate percent release at each time point using the following equation:

\[
\text{Release} (%) = \left[\frac{F_t - F_0}{F_{\text{triton}} - F_0}\right] \times 100
\]
Clot Lysis Assays

In Vitro Clot Lysis Assay – Static Conditions

Materials:
1x PBS pH 7.4
1.25 KU/ml streptokinase in PBS (50ul of 25 KU/ml streptokinase + 950ul PBS)
0.5M CaCl in Millipore water
0.5nM thrombin in Millipore water
3mM S-2251 substrate in Millipore water
400ul of 1 batch/ml unmodified liposomes + 25 KU/ml streptokinase
12.5 ug/ml PLA₂ in Millipore water

Clot Lysis Assay Procedure:

1. Centrifuge whole blood at 250 x g for 15 minutes, remove PRP supernatant, and transfer into clean 15ml falcon tube
2. Label and record the weight of 10 - 1.5ml microfuge tubes as follows:

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Sample Name</th>
<th>Condition</th>
<th>Tube Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative lysis control</td>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Positive lysis control</td>
<td>1.25 KU/ml streptokinase</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Unmodified liposomes w/o PLA₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Unmodified liposomes w/ PLA₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Starting clot mass</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Add 50 ul 0.5M CaCl (final concentration in plasma=0.05M), 50ul 0.5nM thrombin (final concentration in plasma=5nM), and 121ul 3mM S-2251 substrate (final concentration in plasma=400uM) to each tube
4. Transfer 500ul PRP into each of the 1.5ml microfuge tubes and allow the tubes to incubate at 37°C for 45 minutes until clot formation
5. Completely remove the serum from the clot at the bottom and freeze and lyophilize tube #5.
6. To tube 1, add 200 ul PBS
7. To tube 2, add 200 ul of 1.25KU/ml streptokinase
8. To tubes 3&4, add 200 ul of unmodified liposomes loaded with SK 1 batch/ml
9. To tubes 4 add 40 ul of 12.5 ug/ml PLA₂ (final plasma concentration=2.5ug/ml)
10. To tubes 1,2,3 add 40 ul of Millipore
11. Incubate the tubes at 37°C for 60 minutes to allow the clot lysis to occur
12. Remove the supernatant from the tubes and transfer to new 1.5ml microfuge tubes labeled the same way
13. Freeze and lyophilize tubes.
14. Record the weight of the remainder of the clots

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>Remaining Clot Weight (mg)</th>
</tr>
</thead>
</table>
Percentage of clot lysis can be calculated as follows:

\[
\% \text{ Clot lysis} = \frac{\text{Original clot weight} - \text{remaining clot weight}}{\text{Original clot weight}} \times 100
\]

15. Take 100ul samples from the supernatant and transfer to 96-well plate
16. Read SK-induced S-2251 cleavage absorbance at 405nm

**In Vitro Clot Lysis Assay – Flow Conditions**

Materials:
3.8% sodium citrate
5mM Tris-HCl + 1mM CaCl\(_2\) Buffer
0.857 mg/ml (3000 units/ml) streptokinase in PBS
0.5M CaCl in Millipore water
0.5nM thrombin in Millipore water
1000ul of 1 batch/ml unmodified liposomes
1000ul of 1 batch/ml unmodified liposomes + 3000 units/ml streptokinase
1000ul of 1 batch/ml platelet-targeted (60G:40D) liposomes
1000ul of 1 batch/ml platelet-targeted (60G:40D) liposomes + 3000 units/ml streptokinase
12.5 ug/ml PLA\(_2\) in Millipore water
1mg/ml Calcein AM cytoplasm stain – Invitrogen cat # C3099

Acid wash glass slides:
This helps cells and polyamino acids stick to glass. It also helps your DIC pictures look prettier by getting rid of small particles stuck on the glass.

10. Heat 10-20 glass microscope slides with concave well in a loosely covered 50 ml glass beaker containing 30 ml of 1M HCl at 50-60\(^\circ\)C for 4-16 hours. It is important that the slides are not sticking to each other in order to ensure proper cleaning
11. Cool to room temperature
12. Wash the glass slides 3 times with Millipore water, removing any residual HCl
13. Fill beaker with 30 ml of Millipore water and soincate in a water bath for 30 mins.
14. Discard Millipore water, and repeat step 4
15. Discard Millipore water, and repeat step 4 with 50% Millipore:50% EtOH Solution
16. Discard 50% Millipore: 50% EtOH solution, and repeat step 4 with 100% EtOH
17. Discard 100% EtOH solution and replace with fresh 100% EtOH for storage. (Coverslips may be stored in 100% EtOH until use)
18. Prior to use, discard 100% EtOH, and transfer slides to a petri dish with lid partially covered and let dry in a laminar flow hood

Collagen coat glass slides:
6. Distribute collagen solution evenly inside of concave well in slide and subsequently remove with a pipet.
7. Repeat coating for a total of 3 times.
8. Allow the collagen coated slide to dry in the hood for approx. 10 mins.
9. Place the slide in a petri dish and cover with 1x PBS/1% BSA and allow to block for at least 30 minutes and room temperature, and store at 4°C until use (up to ~1 week).

Clot Lysis Assay Procedure:
17. Draw 30ml of blood from aspirin-refraining donor into syringe with 3.8% sodium citrate at a ratio of 1:9 anticoagulant:blood (1ml sodium citrate per 10ml syringe)
18. Transfer blood to 15ml falcon tube
19. Centrifuge the whole blood at 150 x g for 15 minutes, remove PRP supernatant, and transfer into clean 15ml falcon tube
20. Incubate half of the PRP with calcein (10ul calcein per 5ml PRP) for 20 min at 37°C to stain cytoplasm green
21. Centrifuge all of the PRP at 2500 x g for 25 minutes, remove calcein stained PPP, and resuspend calcein platelet pellet with clean PPP
22. Add 50 ul 0.5M CaCl (final concentration in plasma=0.05M) and 50ul 0.5nM thrombin (final concentration in plasma=5nM) per 500ul PRP

<table>
<thead>
<tr>
<th>Trial Number</th>
<th>Sample Name</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative lysis control</td>
<td>PBS</td>
</tr>
<tr>
<td>2</td>
<td>Positive lysis control</td>
<td>0.857 mg/ml streptokinase</td>
</tr>
<tr>
<td>3</td>
<td>Unmodified liposomes w/o PLA_2</td>
<td>Equivalent weight SK in liposomes</td>
</tr>
<tr>
<td>4</td>
<td>Unmodified liposomes w/ PLA_2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Unmodified-SK liposomes w/o PLA_2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Unmodified-SK liposomes w/ PLA_2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Targeted liposomes w/o PLA_2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Targeted liposomes w/ PLA_2</td>
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<tr>
<td>9</td>
<td>Targeted-SK liposomes w/o PLA_2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Targeted-SK liposomes w/ PLA_2</td>
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</tr>
</tbody>
</table>

23. Transfer 200ul PRP to each concave well acid washed, collagen coated microscope slides and allow the slides to incubate at room temp for 45 minutes to 1 hour until clot formation
24. Based on trials above, combine in a 15 ml falcon tube 500μl of liposome formulation, 100μl of 12.5 μg/ml PLA₂ (final plasma concentration=2.5μg/ml), and bring volume up to 12 ml with 5mM Tris-HCl + 1mM CaCl₂ buffer
25. Assemble the slide on PPFC, and transfer liposome solution to PPFC reservoir
26. Run PPFC at 5 dynes/cm² for 30 minutes, take images of calcein (green) platelet clot dissolution and liposome (red) binding to the clot every 5 minutes while under flow
27. Take video (~5 minutes total) for each condition
Bibliography


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