THE EFFECT OF PARTICLE SIZE AND SHAPE ON THE \textit{IN VIVO} JOURNEY OF NANOPARTICLES

By

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Submitted in partial fulfillment of the requirements

For the degree of Doctor of Philosophy

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Acknowledgements

I would first like to acknowledge the mentorship of my research advisor, Dr. Efstathios Karathanasis. Stathis has been a fabulous mentor and teacher. I greatly appreciate his constructive criticism and advice and would not be the engineer I am today without his mentorship. I could not imagine doing my Ph.D. in another lab.

I also would like to thank all of the members of my Ph.D. committee for their support and advice – Dr. James Basilion, the chair of my committee, Dr. Mark Griswold, Dr. Harihara Baskaran, and Dr. Stanton Gerson. I am also thankful for Dr. Jeffrey Duerk’s service in my committee during my first few years of graduate school.

I am extremely thankful to have worked with Dr. Pubudu Peiris in the lab. He has been a second mentor to me in the lab and has helped me navigate many difficult portions in my Ph.D. career.

Also, I would like to thank everyone who I have worked with while at Case. First, I would like to thank members of the lab for their help, past and present: Aaron Abramowski, Tarik Akyuz, Avik Banerjee, Zachary Berman, Andrew Camann, Partha Deb, Gilad Doron, Elizabeth Doolittle, Justin Einstein, Amy Goldberg, Priya Govender, Elliott Hayden, Arman Khayyat, Aaron Lin, Aaron Mayer, Jesse Martin, James McGinnity, Kaitlyn Murray, Jenna Pansky, Swetha Rao, Alex Roman, Erik Schmidt, Shruti Shah, Isha Sharma, Christopher Shoup, Sohaj Singh, Jacob Sullivan, Meilyn Sylvestre, Morgan Tam, Emily Tran, Samantha Tucci, and Peter Vicente. I am grateful for the support of the Case Center for Imaging Research, including Dr. Chris Flask, Joseph Molter, and Bernadette Erokwu. In addition, I am grateful for the help of our
collaborators Dr. Agata Exner, Dr. Ketan Ghaghada, Dr. Ruth Keri, Dr. William Schiemann, and Dr. David Wilson. I would like to give specific recognition to Lisa Bauer for her work on the RF-triggered drug release. Also, I am extremely thankful for the help of Hanping Wu, David Prabhu, Patiwet Wuttisarnwattana, Mohammed Qutaish, Joseph Meyers, and Joseph Young in the multimodal imaging studies.

Last but not least, I would like to acknowledge my funding support through the NIH Interdisciplinary Biomedical Imaging Training Grant (5T32EB007509).
List of Abbreviations

VEGF: vascular endothelial growth factor
EPR: Enhanced Permeation and Retention effect
ABC: ATP-binding cassette
ROS: reactive oxygen species
MMP: matrix metalloproteinases
EPC: endothelial progenitor cell
RES: reticuloendothelial system
IFP: interstitial flow pressure
GMP: Good Manufacturing Practice
GLP: Good Laboratory Practice
IND: Investigation of New Drug
DES: deep eutectic solvents
PRINT: Particle Replication in Non-wetting Templates
PLA: Poly-lactic acid
PEG: Poly (ethylene glycol)
AR: aspect ratio
RBC: red blood cells
AFAC: active fractional area of a nanocarrier
iCAM: intracellular adhesion molecule
TNF-α: tumor necrosis factor α
BAEC: bovine aortic endothelial cells
EGFR: epidermal growth factor receptor
CCP: clathrin-coated pits
LDL: low density lipoprotein
DPPC: 1,2-dipalmitoyl-sn glycerol-3-phosphocholine
TEM: transmission electron microscopy
DLS: dynamic light scattering
PDMS: Polydimethylsiloxane
nCE-μCT: nano-Contrast-Enhanced micro-computed tomography
FMT: fluorescence molecular tomography
NIR: near-infrared
DSPE-mPEG: 1,2 – Distearoyl-phosphatidyl ethanolamine-methyl-polyethylene glycol
HU: Hounsfield units
DCE-CT: dynamic contrast-enhanced computed tomography
fBV: fractional blood volume
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
PlGF: placental growth factor
TGF-β: transforming growth factor-β
HPC: haematopoietic progenitor cells
DTSSP: 3,3'-Dithiobis(sulfosuccinimidyl propionate)
TCEP: tris(2-carboxyethyl)phosphine
RGD-NC: cyclo (Arg-Gly-Asp-Phe-Cys) targeted nanochain
GFP: Green fluorescent protein
BLI: Bioluminescence imaging
DOX: doxorubicin
nChain: multicomponent nanochain (iron oxide spheres + doxorubicin liposome)
RF: radiofrequency
Lip: α,β3 integrin-targeting 30 nm liposomes
NT-Lip: non-targeting liposome
TNBC: triple-negative breast cancer
ICP-OES: inductively coupled plasma optical emission spectroscopy
PET: positron emission tomography
SPECT: single photon emission computer tomography
The Effect of Particle Size and Shape on the In Vivo Journey of Nanoparticles

Abstract

by

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Although several formulations of nanomedicines are approved to treat cancer, their therapeutic efficacy has been limited in the clinic. The delivery of nanoparticles, which is driven by blood flow, is hindered by high interstitial pressures in primary tumors. Moreover, clinically approved nanoparticles are not well designed to target metastasis, which is the leading cause of death from cancer. To effectively treat tumors, it is essential to improve a nanoparticle’s ability to marginate (drift) to the blood vessel wall, overcome interstitial pressures, and bind to overexpressed receptors at a tumor. We assert that nanoparticle size and shape are both design parameters which must be optimized to target and treat tumors effectively. Shape, in particular, heavily influences a nanoparticle’s pharmacokinetics, margination, and binding avidity to receptors. To evaluate the effect of size and shape on nanoparticle margination, the wall deposition of different classes of nanoparticles was compared under flow in a microfluidic chamber. With the knowledge that flow influences nanoparticle intravascular transport, we then employed an in vivo multimodal imaging protocol to evaluate the effect of blood flow on the intratumoral deposition of untargeted and targeted nanoparticles of unique sizes. These studies established that convection heavily influences the deposition of large nanoparticles, while active targeting to cell receptors improves the retention of smaller
nanoparticles. Furthermore, these studies allowed us to derive design rules to improve the site-specific performance of nanoparticles for hard-to-treat cancers. For example, in contrast to primary tumors, micrometastatic lesions lack the hyperpermeable vasculature that allows nanoparticles to passively accumulate in the tumor interstitium. Thus, we developed a chain of iron oxide nanoparticles targeted to the αvβ3 integrin, which is overexpressed on the vascular wall in metastatic lesions. The chain-shaped nanoparticle was identified to have high margination behavior and binding avidity, which enabled it to detect liver and lung micrometastases in a metastatic breast tumor model. Attachment of a doxorubicin liposome to the nanochain and use of a radiofrequency triggered drug release mechanism created an approach to treat metastatic breast cancer. This work demonstrates that rational selection of a nanoparticle’s size and shape can positively impact the efficacy of nanoparticle chemotherapies against the aggressive forms of cancer.
CHAPTER 1

Introduction
1.1 Historical perspective of cancer nanomedicine

Numerous nanoparticle formulations have been developed to deliver anticancer agents to tumors. The first nanotherapeutics to be approved were Doxil and Myocet, which are PEGylated and unPEGylated formulations of doxorubicin liposomes, respectively, approved by the FDA for the treatment of metastatic breast cancer. Today, liposomal daunorubicin (DaunoXome) and liposomal vincristine (Marqibo) have been also approved for the treatment of Kaposi’s sarcoma and leukemia, respectively. These nanoparticle drug carriers provide several benefits for patients. First, a nanoparticle extends the circulation half-life of the drug, which provides it with more opportunities to deposit and accumulate at the site of a tumor. Encapsulation of a potent chemotherapeutic into a liposome extends the circulation half-life of the active drug up to 2-3 days. A second benefit is that nanoparticles can carry very high doses of chemotherapeutic to a tumor. Through employment of a remote loading mechanism, specific chemotherapeutics (e.g. doxorubicin, irinotecan) can be loaded stably into nano-carriers with very high encapsulation efficiency [1, 2]. The third and probably the most important benefit of nanoparticles is a significantly improved safety profile. Nanoparticles prevent healthy tissue from exposure to highly cytotoxic chemotherapeutics. In particular, cardiotoxicity is decreased, which increases the permissible dose to the patient.

Although many nanoparticle formulations are clinically approved or undergoing clinical testing, their efficacy has been very limited. A review of clinical studies revealed that the median progression free survival of first line Myocet treatment to be 18 months. Meanwhile, when Myocet is administered as a treatment for tumor unresponsive to first line therapy, the median progression free survival is only 6 months [3]. Even in
conjunction with cyclophosphamide and trastuzumab, the use of Doxil to treat metastatic breast cancer resulted in an average overall survival rate of 34 months [4]. While patients treated with liposomal daunorubicin suffer from less alopecia and neuropathy than patients treated with a combination of free doxorubicin, bleomycin, and vincristine, overall survival remains at approximately 1 year [5]. Recent studies have also tested the clinical efficacy of liposomal paclitaxel and cisplatin [6]. Prior to the liposomal formulation, paclitaxel was administered in patients in conjunction with castor oil, which caused adverse side effects. The encapsulation of paclitaxel into a liposome increased the maximum tolerable dose for patients from 200 mg/m² to 325 mg/m². Increasing the dose of paclitaxel provided a modest improvement in outcomes for patients with pancreatic adenocarcinoma. When these patients were treated with cationic paclitaxel liposomes in conjunction with gemcitabine, the 12-month survival rate doubled from when they were treated with gemcitabine alone. In contrast, replacement of free cisplatin with liposomal cisplatin only improved the survival rate of patients with non-squamous cell non-small-cell lung cancer by 2 months [7]. Researchers have also attempted to increase the therapeutic efficacy of nanoparticle formulations by using active targeting to improve nanoparticle specificity to tumors. For example, a liposomal formulation, which encapsulated a transgene that could restore the function of tumor suppressor gene p53, was targeted to the transferrin receptor [8]. The methodology successfully delivered a significant amount of exogenous p53 to metastatic lesions in patients, but median survival still remained under one year.
1.2 The inconvenient truths about nanotechnology

Despite all of the benefits that nanoparticles bring to chemotherapeutics, they are still not very successful in treating patients in the clinic. Why are they not effective? To answer this question, it is necessary to understand the mechanisms in which nanoparticles deliver chemotherapeutics to a tumor. For a liposome to successfully deliver chemotherapy to a tumor, there must be a high amount of irregular vascular morphology at the tumor site. To obtain a larger blood supply to feed itself, tumors create new blood vessels which are characterized by small diameters, high branching, and increased tortuosity [9]. Another hallmark of tumor blood vessels is elevated vascular permeability. Accompanying the process of tumor angiogenesis is the increased production of vascular endothelial growth factor (VEGF). This growth factor increases vascular permeability to construct a scaffold for endothelial cells and facilitate the extravasation of plasma proteins [10]. Endothelial cells in tumor blood vessels, however, are characterized by irregular morphology and a lack of organization. Pericytes, which form another layer of the blood vessel wall, also do not associate well with the endothelial cells. The end result is a network of leaky blood vessels with pore diameters that are commonly less than 2 µm [11]. These pore sizes easily permit the passage of nanoparticles into the tumor interstitial space, which is a phenomenon commonly referred to as the Enhanced Permeation and Retention (EPR) effect (Fig 1.1). Once a nanoparticle deposits inside a tumor, it will slowly deliver drug either passively or through an environmentally triggered mechanism (e.g. pH).
Unfortunately, tumor physiology limits the transport of nanoparticles through the EPR mechanism. While the blood circulation is well developed in a tumor, the lymphatic system within a tumor is poorly developed. As a result, the tumor microenvironment is characterized by extremely high interstitial pressures, which prevent fluid flow out of the blood vessel. For small molecule therapeutics (<5 nm) that primarily depend on diffusive transport, this is not very problematic. For nanoparticles, which are 2-3 orders of magnitude larger than molecular therapeutics, the interstitial pressure gradient establishes a significant barrier to transport [10, 11]. The large size of nanoparticles enables their transport to be dictated heavily by convection. Blood flow is a major determinant of nanoparticle delivery into a tumor. If the blood flow can overcome the interstitial pressure gradient, then nanoparticles can move into the tumor interstitial space. Moreover, the blood flow will prevent the nanoparticles from exiting the tumor back into the blood circulation. If the blood flow is insufficient to overcome the interstitial pressure gradient inside the tumor, nanoparticle delivery will be very limited. Adding to the
complexity of nanoparticle delivery is the variability in tumor angiogenesis throughout a
tumor. As tumors grow larger, their centers tend to become necrotic and avascular. In
contrast, the tumor periphery remains highly angiogenic and hypervascular. As a result,
blood flows are highly variable throughout the tumor. This has implications for the
delivery of nanoparticles, as variable flow will result in an uneven distribution of
nanoparticles throughout a tumor. In addition, the drug must be then able to escape the
nanoparticle, diffuse to the tumor cells, and become internalized. The rate of drug leakage
from a nanoparticle is slow, which limits the dose received by the cancer cells.
Nanoparticles also fail to travel far away from the blood vessel they extravasate from,
which limits the treatment of tumor cells that reside far from the vasculature. If a
chemotherapeutic is able to reach a tumor cell, it must be able to overcome drug
resistance mechanisms. ATP-binding cassette (ABC) transporters facilitate drug efflux,
which lowers intracellular drug concentrations and lowers therapeutic efficacy [12]. It is
necessary to either inhibit the transporters or deliver a sufficient concentration of drug to
overwhelm the drug transporters to achieve a therapeutic effect.

1.3 Nanotechnology and critical unmet clinical needs

So far, the successes and failures of delivering nanoparticles to preclinical primary
tumors have been discussed in great length. This focus of preclinical development on
primary tumors, however, is misguided because primary tumors are not even the primary
cause of mortality in clinical practice. This stems from the fact that traditional
chemotherapy is not designed to consider the microenvironment of micrometastatic
disease. Thus, the results obtained from the mouse studies are disconnected from clinical
practice. Typically, when a patient is diagnosed with cancer, the first-line treatment
includes surgery to remove the primary tumor, followed by chemotherapy to eradicate any residual disease, including micrometastases at distant organs. The nanoparticle-based drug delivery may be useful in well-vascularized tumors with several millimeters in diameter, it is ineffective against micrometastases, which presents small clusters of malignant cells dispersed within variable tissue types. In reality, the vast majority of cancer-related mortality is due to metastatic disease, in which cancer cells metastasize to sites, which include distant lymph nodes, the brain, and the liver. A dismal statistic is the 5-year survival rate for distant-stage breast cancer, which is 23%. If the breast cancer is diagnosed when it is confined to the primary site, however, the 5-year survival rate improves to 84% [13].

Nanoparticle treatment of metastasis is even less effective than the nanoparticle treatment of primary tumors. This is because the tumor microenvironment at a metastatic lesion is even less hospitable to EPR-driven nanoparticles than the primary tumor microenvironment. In the case of breast cancer, the birth of a metastasis occurs when tumor cells from the primary site go through the epithelial-mesenchymal transition (EMT). This allows the tumor cells to traverse the tumor microenvironment towards the blood vessels which supply nutrients to the tumor. To enter the bloodstream, the cancer cells disrupt the vascular endothelial barrier by either releasing VEGF, reactive oxygen species (ROS), or matrix metalloproteinases (MMP). Once they enter the bloodstream, they must circulate until they are able to find a suitable metastatic niche for colonization [14]. Overexpressed integrins on the surface of the circulating tumor cells enable binding to extracellular matrix proteins in the metastatic niche. CD44 ligation between metastatic tumor cells mediates the initial growth of what is termed a “micrometastasis”.
The growth of the tumor remains suppressed until endothelial progenitor cells (EPCs) are recruited to the metastatic site. At this point, an angiogenic switch is triggered, which facilitates vascular development and growth of the lesion from a micrometastasis to a macrometastasis.

Ideally, a therapeutic regimen would attack and destroy micrometastatic lesions with minimal side effects to the rest of the body. First off, detection of micrometastatic lesions (<100 mm³) is extremely difficult with current technologies. In addition, there is little angiogenesis when a metastatic lesion is at this early, treatable stage. Without angiogenesis, the vasculature that feeds the tumor remains intact and impermeable to nanoparticles. By the time vasculature at a metastasis become angiogenic and leaky, cancer will also be highly invasive, wide-spread and difficult to treat. If nanoparticles are to be used to treat metastasis in the clinic, a new design and delivery strategy is critical to increase therapeutic efficacy.

1.4 Our concept to design site-specific nanomedicines

Tumor microenvironments are unique and present different opportunities and challenges for targeting. Blood flow and interstitial pressures play a critical role in the transport and deposition of nanoparticles, which is essential for them to deliver their payload to malignant cells. In addition, overexpression of receptors necessary for the proliferation and growth of tumors act as biomarkers which may be targeted. Thus, a single design of a nanoparticle may not be best to treat all types of tumors. In fact, recent advances in engineering have enabled many degrees of freedom for the design of nanoparticles. To enhance deposition in tumors, engineers have extensively studied the
effect of size and surface chemistry on tumor biodistribution. Nanoparticle shape, however, has recently emerged as a design parameter which may be critical for the improvement of nanoparticle targeting. Shape plays a major role in not only nanoparticle pharmacokinetics, but also intravascular transport, binding, and accumulation at the site of a tumor.

The approach in this dissertation is to optimize nanoparticle targeting to metastasis through rational selection of both the particle size and shape. To arrive at a tumor-specific nanoparticle design, it is first essential to understand the relationships between nanoparticle size and shape with its transport. In particular, a transport phenomenon of interest is margination, which is the escape of a nanoparticle from the bloodstream and its lateral movement towards the vessel wall. This is an essential process which must occur for nanoparticles to interact with the vascular endothelium or accumulate in the tumor interstitium. It is also important to study the relationships between nanoparticle design and blood flow, which is coupled to factors in the tumor microenvironment that affect nanoparticle transport. Determination of the relationships between nanoparticle design and transport can guide the design of nanoparticles with an enhanced ability to locate and bind to metastatic lesions. Because slow drug release also hinders the efficacy of nanoparticle chemotherapies, a controlled method to enhance drug release and the drug’s distribution at a tumor site is also essential.

1.5 Objectives

To test our central concept, our research approach consists of the following four objectives:
Objective 1: Evaluation of the effect of nanoparticle size, shape, and flow rate on nanoparticle margination.

Margination (lateral movement towards the blood vessel wall) is a transport event that is critical for nanoparticle delivery. Using an *in vitro* microfluidic flow setup, the effect of nanoparticle design on its ability to escape blood flow and marginate will be investigated.

Objective 2: Evaluation of the effect of blood flow on the deposition of different classes of nanoparticles.

Blood flow is an important transport parameter which influences the transport of nanoparticles. A multi-modal imaging approach was developed to measure and relate regional blood flow to nanoparticle deposition. Analysis was then conducted to determine the effect of blood flow on the deposition of untargeted and targeted nanoparticles of different sizes.

Objective 3: Design of a nanoparticle to target metastasis.

A linear assembly of iron oxide nanoparticles conjugated to the RGD peptide (nanochain) was formulated. The nanochains were tested for their ability to marginate under flow and deposit in lesions of the 4T1 metastatic breast tumor model.

Objective 4: Assessment of the therapeutic efficacy of a nanoparticle targeted to metastasis.

The nanochains were modified with a doxorubicin liposome and used to treat the 4T1 mouse model of metastatic breast cancer. A mechanism to release drug from the nanochain using a focused radiofrequency field was developed and tested. A treatment
strategy incorporating the triggered release of drug form the targeted nanochains was then evaluated for its ability to augment drug delivery to the tumors, disperse wide-spread cytotoxic agents to the entire metastatic site and improve the prognosis for the tumor bearing mice.
CHAPTER 2

The Effect of Particle Size and Shape on Nanoparticle Transport

Adapted from Nanomedicine January 2014, Vol. 9, No. 1, Pages 121-134 with permission of Future Medicine Ltd

Randall Toy, Pubudu M. Peiris, Ketan B. Ghaghada, Efstathios Karathanasis
2.1 Why is the size of a nanoparticle important?

The effect of particle size on nanoparticle pharmacokinetics, margination, extravasation, and binding has been comprehensively studied. Early studies evaluating the effect of liposome size on pharmacokinetics have identified the size range of 60-100 nm to be ideal for maximizing blood circulation half-life [15-17]. Very small nanoparticles have a tendency to be removed by renal clearance mechanisms, while liposomes greater than 100 nm in size have a high tendency to be recognized and phagocytosed by macrophages in the reticuloendothelial system (RES). In a library of gold nanoparticles with optimized PEG surface functionalization of sizes between 20-90 nm, the maximum half-life was observed for a particle with a diameter of ~60 nm [18]. Nanoparticles which are ~60 nm in diameter also have favorable margination when compared to nanoparticles that are 100 - 130 nm in diameter [19]. This can be attributed to the higher effect of convection on the transport of larger nanoparticles, which have difficulty escaping the blood flow. In contrast, smaller nanoparticles that rely more on diffusive transport are able to leave the blood flow more easily. Caution must be taken with size selection, however, as pharmacokinetic and margination performance does not necessarily correlate to faster extravasation kinetics. For instance, the apparent permeability of a nanoparticle to the vascular endothelial wall decreases as the radius of the particle increases [20]. On the other hand, larger nanoparticles have higher long term tumor accumulation. Due to the high diffusive component of transport for smaller nanoparticles, there may be significant particle washout from the tumor over time [21]. Cellular uptake is also favored for larger nanoparticles. Studies have shown that the uptake of 50 nm nanoparticles is 2-3 fold higher than the uptake of 10 nm
nanoparticles[22, 23]. Large ligand-functionalized nanoparticles also have higher binding strength when compared to small ligand-functionalized nanoparticles.

With consideration of both pharmacokinetics and tumor deposition, a novel strategy would be to fabricate a nanoparticle which changes it size once it reaches the tumor microenvironment. Zwitterionic 16 nm gold nanoparticles, which aggregate to sizes between 50-300 nm in the mildly acidic environment of a tumor, have been developed with this strategy in mind. The nanoparticles had both the benefits of long blood circulation ($t_{1/2}$=8 h) and significant tumor uptake after 24 h [24].

2.2 Why is the shape of a nanoparticle important?

The overall transport of a circulating nanoparticle is due to movement from applied convective forces and, to a much lesser degree, Brownian motion [25]. First, the transvascular transport (e.g. extravasation) of nanoparticles is partially governed by the rate of fluid flow and filtration along a capillary, which depends upon the hydrostatic pressure gradient (i.e. the difference between the vascular pressure and interstitial flow pressure (IFP)) [26]. The typical IFP of solid tumors is typically much higher than that of normal tissues due to the higher vessel leakiness and reduced lymphatic drainage. Thus, the typical decreased blood flows and increased IFP in tumors dictate the degree of resistance to extravasation of nanoparticles. In this context, as the particle size increases, faster blood flow patterns are required to overcome high IFP in tumors [21]. In fact, a recent clinical study demonstrated that transient increase of blood pressure (and subsequently tumor blood flow) resulted in enhanced delivery of nanoscale drugs to tumors that otherwise have modest EPR [27]. A preclinical study [28] showed that hyperthermia-induced increase of tumor blood flow enabled the extravasation 400-nm
liposomes into human ovarian carcinoma, which otherwise is impermeable to even 100-nm liposomes.

However, an even more important process than transvascular transport is the one that has to occur before. One of the pivotal steps dictating the transport of flowing nanoparticles is their margination (i.e. radial drift) towards the blood vessel walls. Near-the-wall margination is not just desirable, it is required for a nanoparticle to interact with the tumor vascular bed and have subsequent meaningful interactions. Obviously, this is critical not only when the site of interest is the tumor interstitium (e.g. cancer cell targeting), but also the tumor vasculature (e.g. vascular targeting). Since nanoparticles are primarily transported in the tumor microcirculation via convective means, margination is not favored. Contrary to spherical nanoparticles, oblate-shaped nanoparticles are subjected to torques resulting in tumbling and rotation, which increase the lateral drift of nanoparticles towards the blood vessel walls in microcirculation [29-31].

In addition to transport, the shape of nanoparticles has been shown to dictate the interaction of nanoparticles with cell membranes. First, the shape of nanoparticles has been shown to dictate their clearance by macrophages of the reticuloendothelial organs. Recent studies have indicated that the oblate shape of particles favors their circulation in the blood due to lower uptake by macrophages [23, 32-34]. Subsequently, this prolongs the blood residence of nanoparticles and increases their chances of reaching their target site. Besides macrophages, the nanoparticle shape seems to also dictate endocytosis by normal and cancer cells. Furthermore, targeting nanoparticles using receptor-ligand systems is also governed by shape. To maximize the specificity towards sites of interest, cancer cell targeting or vascular targeting strongly depends on the targeting avidity of
nanoparticles. Compared to nanospheres, oblong-shaped nanoparticles are able to form a greater number of multivalent occurrences [35]. This is essential for targeting especially in the case of vascular targets, since geometrically enhanced targeting can effectively offset hemodynamic forces that tend to detach the nanoparticle from the endothelium [36].

Importantly, understanding the design rules for improved margination and vascular targeting of nanoparticles can facilitate diagnosis and treatment of the most aggressive forms of cancer. While potent cytotoxic agents are available to oncologists, the clinical utility of these agents is limited by their toxicity to normal tissues, which leads to use of suboptimal doses for eradication of metastatic disease [37]. Additionally, the small size, high dispersion to organs, and low vascularization of micrometastases makes them nearly inaccessible to drugs. To date, applications of nanomedicine have mainly focused on primary tumors. While the EPR strategy may be effective in well-vascularized primary tumors larger than 100 mm³, it is ineffective with micrometastatic disease, which presents small clusters of malignant cells within variable tissue types [38, 39]. Considering that the vast majority of cancer deaths are due to metastatic disease [40], the most effective strategy to reach micrometastases may be to design nanomedicines capable of highly selective delivery to micrometastases via vascular targeting of biomarkers on the endothelium associated with metastatic disease. Thus, “shaping” nanomedicine can provide new opportunities to address important and unmet clinical needs.

2.3 How much control can we have over the shape of nanoparticles?

Numerous fabrication methods have enabled the creation of non-spherical nanoparticles with a wide range of different sizes and shapes with high precision (Table
These nanoparticles are manufactured from a wide range of materials and also vary in flexibility. Using different methods, particles are generated with two-dimensional polygonal shapes [41-48], three-dimensional polyhedral shapes [49-53], rod shapes [54-60], branched structures [61-64], and other complex shapes such as snowflakes [62], flowers [62], thorns [62], hemispheres [65, 66], cones [66, 67], urchins [68, 69], filamentous particles [33], biconcave discoids [70], worms [71], trees [72], dendrites [73], necklaces [74], and chains [36, 75-77]. Importantly, the ability to produce drug product with a high level of consistency and reproducibility is critical in the pharmaceutical industry. Needless to say, the field of nanotechnology presents its own challenge, since it is a highly diverse new area. However, various nanomanufacturing methods (e.g. liposomal drugs, iron oxide nanoparticles) have already demonstrated reproducibility and scalability, which enabled the manufacturing process to be transferred to a Good Manufacturing Practice (GMP) facility for production of Good Laboratory Practice (GLP)- and GMP-grade material. In this context of translation of nanomedicine from bench to bedside, besides the obvious importance in clinical trials, scalable and consistent nanomanufacturing accelerates preclinical tests including Investigation of New Drug (IND)-enabling safety and toxicity studies as well as efficacy in large animal models. Along those lines, adjusting existing methods can fabricate both spherical and non-spherical nanoparticles with similar ease. For example, the gold nanoparticle synthesis method employing deep eutectic solvents (DES) produces a wide variety of different particle shapes through small adjustments in stoichiometry [62]. Another top-down fabrication method, Particle Replication in Non-wetting Templates (known as PRINT), uses lithography to produce nanoparticles of all shapes with the same ease [78].
Furthermore, a nanofabrication technology based on Dip-Pen nanolithography has enabled commercial manufacturing of nanoparticles of various shapes and structures from a variety of different materials [79]. In addition, by adapting a solid-phase chemistry strategy, nanospheres can be assembled into oblong chain-like nanoparticles of various aspect ratios, which can be scaled up relatively easily [75].

![Diagram of biological processes influencing nanoparticle delivery.](image)

**Figure 2.1 Biological processes which influence nanoparticle delivery.** A nanoparticle must be able to circulate, marginate, and bind to a vascular target or extravasate into the tumor interstitium before it can be internalized by a cancer cell.

While nanoparticle size and surface characteristics have been reviewed elsewhere [80], this chapter focuses exclusively on the importance of shape as an essential property of nanoparticles that plays an ultimate role in various biological processes [78, 81, 82]. In the proceeding sections, we will illustrate how a nanoparticle’s shape influences its biodistribution, ability to marginate and escape the blood flow, and binding affinity to the receptors it targets (Figure 2.1).
### Table 2.1: Non spherical nanoparticles of various shapes fabricated in recent years.

<table>
<thead>
<tr>
<th>Shape</th>
<th>Types</th>
<th>Materials</th>
<th>Methods</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-D</td>
<td>Polygonal</td>
<td>Au, Ag, Pt, Pd, Triacrylate resin, PLA(^6), PEG(^#)-diacrylate, poly(pyrrole)</td>
<td>PRINT(^\text{®}) lithography, microfluidics, photopolymerization</td>
<td>[41-48]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-D</td>
<td>Polyhedral</td>
<td>Au, Ag, Pt, Pd, PLA, poly(pyrrole), PEG-(diacrylate),</td>
<td>PRINT(^\text{®}), Step flash imprint lithography, Microfluidics</td>
<td>[49-83]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rod-like</td>
<td></td>
<td>Au, Ag, Pt, Fe, Cu, Cr, Co metals, alloys, and oxides</td>
<td>Stretching spherical particle, Electrochemical deposition, self assembly, Solution-phase chemical reduction</td>
<td>[54-60]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branched</td>
<td>Monopod, Bipod, Tripod, Tetrapod, Star-shaped, Octapod</td>
<td>Au, CdS, CdSe, CdTe, MnS, and ZnO</td>
<td>Thermal and chemical vapor deposition, Solution-phase chemical reduction</td>
<td>[61-63]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>Snowflakes, Flowers, Thorns, hemispheres, Cones, Urchins, Filamentous particles, Biconcave discoids, Worms, Trees, Dendrites, Necklaces, Chains</td>
<td>Au, Ag, Pt, Fe, Cu, Ru, Co, metals, alloys, and oxides, Si and Si oxides</td>
<td>Hydrothermal, PRINT(^\text{®}), Solid phase synthesis, self assembly, wet chemical synthesis, Solution-phase chemical reduction,</td>
<td>[33, 36, 62, 65-76, 84]</td>
</tr>
</tbody>
</table>
For a nanoparticle to successfully travel to and bind to its biological target, it must first be able to travel in the bloodstream while evading uptake by macrophages, particularly in the reticuloendothelial system. Nanoparticle size was first identified as a critical parameter which affects the rate of macrophage uptake [85, 86]. Particles less than 5 nm are rapidly cleared from the circulation through extravasation or renal clearance [87, 88], and as particle size increases from the nanometer range to ~15 μm, accumulation occurs primarily in the liver and the spleen [89, 90]. In addition, studies have shown that PEG-coated spherical nanoparticles with neutral charge exhibit increased blood residence times [91]. While these studies with spherical particles have resulted in valuable insights, nanoparticle shape has also been established as an important parameter that dictates the rate of macrophage uptake [34]. In a study comparing the uptake of six distinct classes of nanoparticles with different shapes, a contact angle parameter was formulated, which was quantitatively related to particle internalization velocity. If a particle with a very high aspect ratio (~20) aligns with its long axis parallel to the cell membrane, it will internalize more slowly than a particle which aligns with its short axis parallel to the cell membrane (Figure 2.2). In support of these findings, computational simulations evaluating particle engulfment demonstrated that spheroidal particles internalized 60% more quickly when they were engulfed with their tips first [92].
Interestingly, nanoparticles that have a high rate of attachment may sometimes have a slow internalization rate [32]. An experiment which decoupled the attachment and internalization rates of nanoparticles found that prolate ellipsoids (major axis 0.35 - 2 μm, minor axis 0.2 – 2 μm) had both the highest attachment rate and slowest internalization rate in comparison to spheroidal (radius 0.26 – 1.8 μm) and oblate ellipsoidal nanoparticles (major axis 0.35-2.5 μm, minor axis 0.2- 2 μm).

Figure 2.2 Effect of θ (contact angle) on the rate of nanoparticle internalization. Rod-shaped nanoparticles internalize most quickly when their major axis is perpendicular to the cell membrane. As the rod is oriented more tangentially to the cell membrane, the rate of internalization decreases. This is owing to the increased difficulty to ‘wrap’ the nanoparticle. Since spherical nanoparticles are symmetric, they internalize at a rate independent of θ.

Biodistribution studies have also demonstrated that the uptake of spherical particles is favored over the uptake of particles with high aspect ratios in macrophages. For example, the liver uptake of 100-nm-long nanochains with an aspect ratio of about 4 was significantly lower than that of nanospheres with a 100-nm diameter [76]. Taking under consideration that nanoparticles are primarily cleared by the reticuloendothelial system, the low uptake of nanochains by the liver correlated to prolonged blood residence. Subsequently, the nanochains outperformed the 100-nm nanospheres in terms of extravasation into tumors, since nanoparticle extravasation into tumors is directly
proportional to their blood residence time [93]. Another study evaluated the blood circulation of filomicelles, which are elongated assemblies of polymer micelles that have the ability to change in size in vivo over time [33]. Shear forces from blood flow act on portions of the filomicelle not in contact with the cell; these forces pull the nano-carrier away from the cell before it can be internalized. In the liver and spleen, the uptake of gold nanorods (size = 10 x 45 nm, AR = 4.5) was shown to be less than the uptake of gold nanospheres [94]. A biodistribution study with mesoporous silicon nanoparticles with an aspect ratio of 5 (length of 720 nm) had 50% of the liver uptake rate of particles with an aspect ratio of 1.5 (length of 185 nm) [95]. In the spleen, however, the uptake after 2 hours was nearly 3 times higher for nanorods with the aspect ratio of 1.5. Thus, it can be concluded that both nanoparticle size and aspect ratio play a role in organ-specific uptake. In addition, the rate of phagocytosis for a nanoparticle of a particular geometry may be organ-dependent.

2.5 Modifying nanoparticle shape to enhance margination

After a nanoparticle successfully evades phagocytosis via macrophages and reaches its target site, it must be able to escape the blood flow and marginate towards the wall of the blood vessel. Nanoparticle margination is dictated by forces which influence particle translational and rotational motion, which include buoyancy, gravity, drag, van der Waals interactions, electrostatic double layer interactions, and steric repulsive interactions. Under a balance of these forces, spherical nanoparticles tend to follow the streamlines of the flow they are traveling in [96, 97]. The tendency for a spherical nanoparticle to marginate is dependent on its size [98, 99]. The transport of large spherical nanoparticles is heavily driven by convection, which results in a higher difficulty to escape the flow
and move towards the vessel wall. On the contrary, the transport of smaller nanoparticles exhibits a relatively higher diffusion component, which allows them to move laterally in the blood vessel with greater ease. This was demonstrated in a study which found that a 65 nm liposome had a 3.4 times higher margination rate than a 130 nm liposome [99].

Unlike spherical nanoparticles, rod-shaped nanoparticles experience lateral drift that varies depending on the angle of their orientation (Figure 2.3) [96, 100]. The tendency for rods to drift may be explained by the variable drag forces and torques that are exerted on rods under flow, which influence their ability to marginate. In fact, discoidal (aspect ratio (AR) = 0.5), hemisphere, and ellipsoidal particles (AR = 0.5) also have higher drift velocities than spheres [30]. For different classes of discoidal particles, the drift velocity increases as the particle aspect ratio deviates further away from one. Of the ellipsoids, hemispheres, and discs, it was found that discoidal particles most prominently follow highly oscillatory trajectories that lead to increased interactions with the vessel wall.

Figure 2.3. Effect of shape on nanoparticle margination. Spherical nanoparticles tend to remain in the center of the flow. Variable forces and torques exerted on rods under flow allow
them to marginate and drift towards the vessel wall, where they are able to bind to wall receptors or extravasate through gaps between endothelial cells.

In light of these findings, researchers have developed *in vitro* methods to study the effect of nanoparticle shape on margination [30, 31, 99, 101-103]. Primarily, studies have compared the margination of four classes of particles: spheres, rods, ellipsoidal particles, and discoidal particles. These studies are typically conducted in parallel plate flow chambers and microfluidic flow chambers, which allow experimentation to be conducted at controlled flow rates [98, 99]. One recent study compared the margination of nanoparticles below 200 nm in diameter in rectangular microfluidic flow networks [99]. It was observed that when shear rate was decreased, the margination of nano-spheres increased almost two-fold. Shape also played a major role in enhancing nanoparticle margination; nano-rods (56 nm x 25 nm; AR ~ 2) had 7-fold higher accumulation than nano-spheres under the same shear rate. Similar results were observed when the margination rate of an oblong iron oxide nanochain (100 x 20 nm; AR=5) was compared to a 20-nm iron oxide nanosphere in a microfluidic flow network setup [36]. At a flow rate of 50 µL/min, which is in the range of expected blood flow in tumor microcirculation, the nanochain exhibited 2.3-fold higher margination than the IO sphere. Differences in blood hematocrit also have an effect on nanoparticle margination [102, 104]. Another study evaluated the effect of shape and flow rate simultaneously using mesoporous spherical, rod-like, and disc-like particles [101]. When particle width was held constant, discs marginated two times more than rods. Differences in adhesion are likely to be the dominant cause for lower particle adhesion, as high shear rates easily dislodge adhered particles. Yet, we also postulate that high shear rates hinder particle
margination because it is more difficult for nanoparticles to escape fast flows. Vessel geometry is also a critical consideration for the evaluation of nanoparticle margination; in general, particles deposit at higher levels at a vessel bifurcation than along a straight vessel [103]. Shape, however, can further enhance the increase in deposition at the vessel junction – flowing ellipsoidal discs displayed a 2-2.5 fold higher ratio of bifurcation to straight vessel wall attachment than spheres. Thus, the advantages of oblate shapes may be even greater in the complex vessel geometries commonly found in vivo.

Recent studies have also focused on improving the in vitro experimental design to more accurately represent physiological conditions. As laminar and shear flows are highly idealized, nanoparticle margination has also been recently evaluated under pulsatile flows and recirculating flows [102]. Both particle size and aspect ratio were again identified as key determinants that affect the level of margination. For 1 and 2 μm particles in pulsatile flow, high aspect ratio rod-shaped particles (9 < AR <11) had 2 times higher binding than spherical particles. A similar enhancement in binding was observed when the same classes of particles were subjected to recirculating flow. The trajectory of nanoparticles in vivo is also impacted by particle interactions with circulating red blood cells (RBC) and leukocytes. Red blood cells, which are shaped like symmetric circular discs, have a tendency to remain in the middle of the blood flow, which creates a “cell free” layer that depends on the blood flow rate. In contrary, the oblong shape of leukocytes allows them to escape the blood flow, which allows them to move within the RBC-free layer [105]. Nanoparticles in circulation collide with both red blood cells and leukocytes, which influence their margination behavior. Experimentally, it has been found that increasing hematocrit from 30% to 45% enhances the margination
and binding of 2 μm particles 2-fold [104]. At higher hematocrits, the ratio of rod to sphere margination and binding is also increased [102].

2.6 Tailoring nanoparticle shape to maximize binding avidity

After marginating to the wall of a blood vessel, a nanoparticle must be able to bind to its target of interest, which is usually a cluster of receptors that are over-expressed on the cell surface. Unlike small molecule targeting agents that display a single ligand, nanoparticles are able to exhibit multiple ligands on their surface, which results in an avidity-based targeting [106]. As a result, several low binding ligands can work in tandem to create a high-affinity nanoparticulate construct for targeting multiple receptors over-expressed on target cells [107]. Thus, while the ligand-bearing nanoparticles have high affinity, several other factors are known to influence their binding to cell-surface receptors. For example, increasing the nanoparticle’s size (i.e. decreasing its curvature), while keeping the surface ligand density constant, will increase the number of bonds between the nanoparticle and the cell. Another effective way to enhance binding avidity is to alter the nanoparticle shape, which changes the way in which ligands are presented on the particle surface (Figure 2.4). Nanoparticles of different shapes have a different active fractional area (AFAC), which results in variability in binding avidity. It is important to note that the flexibility and density of the ligands on the particle surface also affects binding avidity [108-111]. The use of flexible polymeric chains, such as polyethylene glycol (PEG), for presenting ligands result in a larger volume for interaction with cell surface receptors and therefore a high probability for the formation of receptor-ligand bonds [112]. The length of the polymer has also been shown to influence binding of nanoparticles [109, 113, 114]. For a 100 nm particle, tripling the molecular weight of
the PEG used will also triple the AFAC. In certain cases, where the presence of ligands is known to reduce the blood half-life of nanoparticles, the nanoparticles are decorated with an additional layer of ‘stealth’ polymer. In such cases, an understanding of the interplay between the two different types of polymer chain length and their surface density becomes important to ensure sufficient binding efficiency to target receptors while maintaining the desired blood half-life of the targeted nanoparticles [115].

Figure 2.4 Effect of shape on nanoparticle binding avidity. Shape, ligand length, and polymer flexibility all play a role in the active fractional area of a nanocarrier (AFAC). For a sphere, the AFAC is defined as \((L - d_b)/D_c\). For particles with equal surface area, \(L \neq d_b\), and shape affects AFAC. \(d_b\): Binding distance between the nanoparticle and the receptor; \(D_c\): Diameter of the nanocarrier; \(L\): Length of the ligand

Both in vitro and in vivo studies have demonstrated the influence of particle shape and size on the binding efficiency of nanoparticles [69, 116]. A recent study has shown that anti-intracellular adhesion molecule (iCAM) spheres are internalized more quickly by endothelial cells than anti-iCAM discs of comparable size [69]. These spheres also
were processed into lysosomal compartments and degraded more quickly than their discoidal counterparts. Rods targeted to ovalbumin, on the other hand, have twice as many specific binding interactions as ovalbumin-targeted spheres [116]. Binding avidity has also proven to be critical for vascular targeting. Successful vascular targeting requires that a nanoparticle can escape the blood flow and drift towards the blood vessel walls, followed by strong attachment to the targeting site offsetting the blood flow forces that tend to detach the particle. The targeting avidity of an $\alpha_v\beta_3$ integrin-targeting oblate shaped nanochain was assessed under flow in a microfluidic device coated with tumor necrosis factor $\alpha$ (TNF-$\alpha$)-treated bovine aortic endothelial cells (BAEC) [36]. At only 5 min, the nanochain achieved 9.5-fold higher attachment compared to their $\alpha_v\beta_3$ integrin-targeting spherical counterparts (20 nm in diameter).

For the successful maximization of binding affinity through the adjustment of particle shape, ligand density, and ligand flexibility, the dynamics of the targeted receptor must also be considered. Receptors are often distributed and organized in a clustered, heterogeneous pattern or a semi-homogenous pattern involving a combination of individually and clustered distribution. The folate receptor, a GPI-anchored receptor over-expressed in a variety of solid tumors, is distributed within small nano-sized lipid raft clusters [117, 118]. Transferrin receptor, a trans-membrane receptor over-expressed in brain tumors, is known to be clustered in coated pits as well as diffusively distributed on the cell surface [119]. Furthermore, some of the receptors, such as epidermal growth factor receptor (EGFR), are known to alter their surface distribution and enter into a clustered distribution upon ligand binding [120]. Since the binding event is often followed by receptor-mediated endocytosis, identifying the receptor internalization
pathway is also important for understanding the route for intracellular entry of targeted nanoparticles [121-123]. The receptor-mediated internalization pathways can broadly be divided into: (1) clathrin-coated pits (CCP) pathway (2) caveolae-mediated pathway, and (3) lipid rafts pathway. A number of transmembrane proteins such as transferrin receptor, EGFR and low density lipo-protein (LDL) receptor are endocytosed via the CCP pathway. The folate receptor is internalized via the lipid rafts (also known as microdomains) pathway. In certain cases, the degree of internalization also depends on the cellular content of the exogenous ligand. For instance, the internalization dynamics of the folate receptor are governed by the cellular folate content [124]. An increase of intracellular folate content from 0.5 pmol to 1 pmol per million cells will result in a 75% decrease in folate binding affinity. Finally, the steric hindrance effects and access of receptor sites that arise due to the size and shape of nanoparticles should also be taken into consideration when evaluating binding and internalization [125].

Overall, we should carefully consider that binding of targeted nanoparticles to surface receptors is maximized at an optimal number of ligands per nanoparticle [126]. Surface presentation of very few ligands cannot achieve sufficient binding avidity, whereas too many ligands create steric hindrance and consume more receptors than necessary. Excess receptor consumption downregulates the expression of receptors on the cell surface, which decreases the number of binding sites for additional nanoparticles. At the same time, it is also necessary to consider the force necessary to translocate targeted nanoparticles of different shapes from the cell surface [127]. Increasing the maximum contact area of the nanoparticle by doubling its radius will lead to a 3-fold increase in the necessary force for particle translocation. Thus, an optimal combination of nanoparticle
shape and ligand density has to be considered to facilitate targeting based on geometrically-enhanced multivalent docking and subsequent nanoparticle endocytosis.

2.7 Effect of nanoparticle shape on tumor deposition and therapeutic efficacy

Given that the shape of a nanoparticle affects its blood circulation, ability to marginate, and binding affinity, it is expected that a nanoparticle’s shape will also affect its rate of tumor deposition and therapeutic efficacy. Nanoparticle size has been extensively shown to affect the rate of nanoparticle intratumoral deposition through the EPR effect and subsequent therapeutic efficacy [16, 128]. These studies identified 100 nm as the optimal diameter for the deposition of spherical nanoparticles into tumors, which is dependent on vascular pore size, pharmacokinetics, and the ability to overcome high interstitial pressures through flow driven convection. In addition to different tumor microvascular systems (e.g. size of gaps on the endothelium, interstitial pressures, microvessel density, vasculature geometry), different tumor types have distinct vascular wall pore shapes. Therefore, the nanoparticle’s aspect ratio may also determine unique extravasation rates and patterns into different tumors [129]. In an LS174T tumor, quantum dots extravasated four times more than carbon nanotubes with equivalent surface area; in an U87MG tumor, however, only the carbon nanotubes could extravasate. Higher aspect ratio also increased the delivery of nanoparticles to overexpressed vascular targets in a tumor. The ability of nanochains to perform vascular targeting was evaluated in vivo in the orthotopic 4T1 mammary adenocarcinoma model in mice [36]. At t=45 min after injection, vascular targeting of the nanochains resulted in a 2-fold higher tumor targeting than their spherical variant. At that time point, vascular targeting resulted in more than 40% of the administered nanochains being localized in the primary tumor.
The tailoring of nanoparticle shape also has improved the efficacy of tumor therapy. In particular, the use of an oblate shape increases the targeted delivery of antibody-displaying nanoparticles [130]. For example, trastuzumab coated nanorods exhibited a 5-fold greater cellular growth inhibition of when BT-474 breast cancer cells compared to equivalent nanospheres at the same nanoparticle dose [130]. This can be likely attributed to the 66% increase in binding and uptake by the cells of the nanorods compared to the nanospheres.

Besides cell culture studies, similar findings have been seen in tumors in vivo. Recently, it was shown that an 100-nm-long oblong chain-like nanoparticle (termed nanochain) composed of three iron oxide nanospheres and one doxorubicin-loaded liposome exhibited a 2-fold higher extravasation into tumors in a mammary adenocarcinoma model compared to spherical liposomal doxorubicin [76]. In addition, as a result of the multicomponent design of the nanochain, radiofrequency-triggered drug release from the particles resulted in the therapeutic efficacy being three-fold higher than treatments using spherical nanoparticles [76, 131]. Interestingly, the chain-shaped nanoparticle responded in a unique manner in the presence of a magnetic field. When magnetic nanoparticles are subjected to an external, oscillating magnetic field, there are two relaxation mechanisms (Brownian and Néel relaxation) that govern their magnetization response in an effort to align with the applied field. Brownian relaxation, the physical rotation of the entire nanoparticle, is typically the dominant relaxation mechanism for nanoparticles larger than about 25 nm. In the case of nanochains, Brownian relaxation is restricted by the bonds between the constituent nanospheres, such that Brownian motion may be observed as a mechanical “vibration” of the chain, rather
than true rotational motion. Néel relaxation (reorientation of the particle’s magnetic moment with the applied field) is dominant for nanoparticles smaller than 15 nm. The size of the constituent iron oxide nanospheres in the nanochains places them in between the Brownian and Néel regimes, resulting in mechanical vibration that causes rapid and significant drug release. Therefore, beyond the shape and the size, composition, surface charge and polymer coating of nanoparticles, non-spherical nanoparticles result in new unique properties. The use of non-spherical gold nanoparticles over spherical gold nanoparticles is also advantageous in the application of photothermal triggered therapy. When nanorods were used in place of nanospheres, the photothermal absorption efficiency of the particle increased by 10-fold, which resulted in improved therapeutic efficacy [132].

2.8 Conclusions

Shape plays an instrumental role in determining the \textit{in vivo} fate of a nanoparticle. This chapter highlighted studies assessing how shape affected a nanoparticle’s ability to evade immune uptake, escape the blood flow to travel to its target, and bind to its target with high affinity. Although spherical nanoparticles have been traditionally employed for tumor targeting due to their relative ease of fabrication, many recent findings suggest that non-spherical nanoparticles (e.g. rods, discs, hemispheres, ellipsoids) may target tumors more effectively. To make definitive conclusions on the best choice for nanoparticle shape, more comprehensive studies encompassing reticuloendothelial system clearance, margination, and cell attachment must be conducted. The next chapter explores the effect of nanoparticle shape on \textit{in vitro} margination in microchannels of comparable
geometry to capillaries \textit{in vivo}. In addition, the effect of nanoparticle size and flow rate on margination is evaluated.
CHAPTER 3

The Effects of Particle Size and Shape on Margination of Nanoparticles in Microcirculation

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Randall Toy, Elliott Hayden, Christopher Shoup, Harihara Baskaran, Efstathios Karathanasis
3.1 Introduction

Being at the crossroads of chemistry, material science, engineering, and medicine, nanomedicine exploits the unique features of nanoparticles to design improved anticancer interventions [133, 134]. An advantage of being at the nanoscale is the ability to combine more than one function by enabling the design of multifunctional nanoparticles that seek (i.e. passive and active targeting), image (i.e. loaded with contrast agents), and damage (i.e. loaded with anticancer agents) [135]. Not surprisingly, this has led to the development of numerous types of nanoparticles for imaging and therapy such as liposomes, dendrimers, other lipid-based and polymeric nanoparticles, and metal nanoparticles (e.g. iron oxide and gold) [136]. While most of the early generation particles were spherical, recent advances have exploited the engineerability of nanoparticles to shape them with defined geometrical, physical and chemical properties. For example, oblate- and rod-shaped nanostructures have been fabricated suitable for biomedical applications such as nanorods [137], nanochains [84], nanoworms [71, 138], and nanonecklaces [74]. Evidently, nanotechnology has the ability to shape matter into various classes of particles with very different characteristics including size, shape, density, and surface chemistry.

Notably, those characteristics of nanoparticles have been shown to play a central role in their transport in the abnormal tumor microcirculation [30]. Successful delivery of nanoscale agents requires that the particle enters the tumor microcirculation, navigates through the tumor leaky vasculature into the tumor interstitium and is delivered to cytoplasmic targets in cancer cells. However, nanoparticles en route to their target face numerous biobarriers created by the tumor abnormal physiology. Abnormal tumor
features, including physically compromised vasculature, erratic blood flow, abnormal extracellular matrix, and high interstitial fluid pressure, can limit the effective delivery of nanoparticles.

One of the pivotal steps dictating the intratumoral fate of nanoparticles is their margination (i.e. lateral drift) towards the blood vessel walls. Near-the-wall margination is not just desirable; it is required for the particle to be able to interact with the tumor vascular bed. Subsequently, the particle will have the chance to either target tumor-specific vascular biomarkers or extravasate through the tumor leaky endothelium into the tumor interstitium. Even though tumors display blood flows significantly slower than that of normal circulation, nanoparticles, due to their size, are primarily transported in the tumor microcirculation via convective means and therefore margination is not favored, which is not the case for small molecules. In order for a particle to escape the blood flow streamlines resulting in margination, forces that depend on the particle characteristics such as gravity, buoyancy, diffusion or torque are required [29, 139]. Apparently, only the latter two factors are important for nanoparticle margination [31, 98]. Therefore, key characteristics of nanoparticles, such as density, size and shape, have important consequences on the delivery of nanoparticles to tumors.

In a recent experimental study [98], 50 nm polystyrene nanospheres exhibited significantly higher margination on a parallel plate flow chamber compared to 100 or 200 nm spheres. Other computational and experimental studies have shown that oblate-shaped particles encourage margination due to torque forces [31, 140]. In addition, the tumor hemodynamics and vascular permeability has also been shown to significantly influence the transport of nanoparticles [25, 141, 142]. However, it is well-known that
tumors represent a very heterogeneous population with several parameters varying not only among same type of tumors but even spatially within the same tumor [143-147]. Taking under consideration this variability, one intriguing argument is to consider that each tumor displays unique biobarsriers to nanoparticles.

Table 3.1 Particle characteristics.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle density (g/mL)</th>
<th>Diameter or length (nm)</th>
<th>Aspect ratio (width/length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130 nm PBS-loaded liposome</td>
<td>1</td>
<td>130</td>
<td>1 (sphere)</td>
</tr>
<tr>
<td>100 nm PBS-loaded liposome</td>
<td>1</td>
<td>100</td>
<td>1 (sphere)</td>
</tr>
<tr>
<td>65 nm PBS-loaded liposome</td>
<td>1</td>
<td>65</td>
<td>1 (sphere)</td>
</tr>
<tr>
<td>65 nm iodine-loaded liposome</td>
<td>2.4</td>
<td>65</td>
<td>1 (sphere)</td>
</tr>
<tr>
<td>Iron oxide nanosphere</td>
<td>5.1</td>
<td>60</td>
<td>1 (sphere)</td>
</tr>
<tr>
<td>Gold nanosphere</td>
<td>19.3</td>
<td>60</td>
<td>1 (sphere)</td>
</tr>
<tr>
<td>Gold nanorod</td>
<td>19.3</td>
<td>56</td>
<td>0.45 (rod)</td>
</tr>
</tbody>
</table>

To understand the fundamental relationship of flow with particle characteristics, in this work we studied the margination of nanoparticles in microchannels (175 µm wide) under physiologic flow rates expected in tumor microcirculation. The effect of particle density ($\rho_p$), size and shape were evaluated using different classes of nanoparticles (shown in Table 1): 1) spherical liposomes ($\rho_p$~1) with diameters of 65, 100, and 130 nm, 2) a spherical iodine-loaded liposome ($\rho_p$~2.4) with a diameter of 65 nm, 3) an iron oxide nanoparticle ($\rho_p$~5) with a diameter of 60 nm, 4) a gold nanoparticle ($\rho_p$~19) with a
diameter of 60 nm, and (5) a gold nanorod ($\rho_p \sim 19$) with a length of 56 nm and an aspect ratio of $\sim 0.45$. Another important aspect of this study was that each nanoparticle population exhibited a very narrow size distribution, the same surface coating (i.e. polyethylene glycol), and nearly zero surface charge.

3.2. Materials and Methods

3.2.1. Materials

The phospholipid 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Genzyme Pharmaceuticals (Cambridge, MA). DSPE-PEG$_{2000}$-NH$_2$ was obtained from Laysan Bio (Arab, AL). Cholesterol was purchased from Sigma (St. Louis, MO). Gold nanospheres and gold nanorods were purchased from Nanopartz Inc. (Loveland, CO). Iron oxide nanoparticles were obtained from Ocean Nanotech (Springdale, AR). Alexa Fluor 488 N-succinimidyl ester was obtained from Invitrogen (Carlsbad, CA). The rest of the reagents were of analytical grade (Fisher Scientific, Cleveland, OH).

3.2.2. Fabrication of liposomes

A lipid composition of DPPC, cholesterol and DSPE-PEG$_{2000}$-NH$_2$ in the molar ratio of 55: 40: 5 respectively was used. The lipids were dissolved in ethanol and hydrated with phosphate buffered saline (PBS) at 60°C followed by sequential extrusion in a Lipex Biomembranes Extruder (Northern Lipids, Vancouver, Canada), to size the liposomes to 130 or 100 or 65 nm. Samples were then dialyzed for two days against PBS using a 100 kDa molecular weight cutoff dialysis membrane.
A particle density of 2.4 g/mL was achieved by encapsulating a concentrated iodinated solution into the liposome using established methods [146, 147]. Briefly, a highly concentrated iodine solution (650 mg I/mL) was prepared by dissolving ioxapaxol powder (lyophilized from Visipaque 320; GE Healthcare, Milwaukee, WI) in ultrapure water under stirring and heating at 70°C. The lipid composition was similar to that described previously. The liposomes were extruded to size them to 65 nm. Free, un-encapsulated iodine was replaced by PBS using a 2-day dialysis. The final iodine levels were quantified through spectrophotometry at 245 nm.

Finally, the liposome formulations were fluorescently tagged by conjugating Alexa 488 NHS on the amines at the distal end of the surface PEG polymer. An excess (2x) of the fluorophore was used to assure coverage of all the amines on the surface of the particle (~900, 2000, 3500 amines per 65, 100, 130 nm liposome respectively). Free fluorophore was removed by dialysis against PBS for two days. We employed a typical 5 mol% PEG coverage which has been shown to provide in vitro and in vivo stability [148-151]. The sizes of the liposome formulations were confirmed by dynamic light scattering (DLS) with a Brookhaven Particle Size and Zeta Potential Analyzer (Brookhaven Instruments, Holtsville, NY). Their zeta potentials were measured using the Brookhaven Particle Size and Zeta Potential Analyzer.

3.2.3. Preparation of metal nanoparticles

The metal particles were obtained from commercial vendors as mentioned in the materials section. Gold nanospheres displayed a diameter of 60 nm with very low size dispersity which was confirmed by transmission electron microscopy (TEM) and DLS
measurement. The gold nanorod’s length and transverse diameter were 56 and 25 nm, respectively. The surface of the gold sphere and rod were decorated with about 70 and 200 amines per particle. The iron oxide nanospheres exhibited a core diameter of 50 nm (based on TEM) and a hydrodynamic diameter of 60 nm (based on DLS measurements). The modification of the surface with a PEG coating has been well-documented for the gold sphere and rod [152, 153] and the metal nanoparticles [154, 155]. Conjugation of Alexa 488 onto the surface of the particles was achieved using a method similar to that of liposomes followed by measurement of the zeta potential.

### 3.2.4. Fabrication of Polydimethylsiloxane (PDMS) Microchannel Devices

Standard photolithography techniques were used to transfer the design of the flow microchannel to negative templates of photoresist (SU-8 2075) on a 4” silicon wafer [156]. These templates were then used to embed the designs in PDMS [157]. PDMS has advantages such as ease of availability, fabrication and manipulation. The channel had a length of 5 cm and a cross section with dimensions of 175x100 µm (WxH). Fluid access to the networks was provided via 1 mm OD Silastic tubing connected to the PDMS membrane. Phase contrast and relief contrast techniques were used to measure and verify channel dimensions of the PDMS networks.

### 3.2.5 Experimental setup

Image acquisition was performed using an Olympus IX-71 inverted fluorescent microscope (Olympus Corporation, USA). All acquired images were 12-bit grayscale and taken at 20X magnification. At every imaging time point, three regions of interest
were imaged at different portions of the microchannel (entrance, center, and exit). PDMS microchannel devices were incubated with fibronectin at 100 µg/mL overnight. Channels with fibronectin were flushed thoroughly with PBS before the start of the experiment. Fluorescent images of the channel were acquired before the introduction of nanoparticles. Then, nanoparticles at a concentration of $3 \times 10^{11}$ particles/mL were infused into the microchannels with a Harvard syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 50 µL/min for 8 minutes. Following nanoparticle flow, channels were washed with PBS at a flow rate of 50 µL/min for 30 minutes. Images were acquired in each phase to qualitatively assess particle deposition during the experiment. The non-adherent nanoparticles were collected from the outlet of the microchannel during both the nanoparticle flow and PBS wash phase of the experiment. Adherent nanoparticles were collected separately by flushing the system with ethanol. Particle concentration was measured using a fluorescent plate reader (BIOTEK, Winooski, VT: excitation wavelength = 485 nm, emission wavelength = 528 nm). The volumes of the collected nanoparticles were measured so that the number of adherent and non-adherent nanoparticles could be calculated from mass balance. Percent deposition was then calculated by dividing the total number of adherent nanoparticles by the sum of the number of adherent and non-adherent nanoparticles, multiplied by 100. It should be noted that we performed pilot studies to evaluate potential photobleaching of the fluorophore and whether this should be taken into account. Alexa-488 photobleaching was assessed by analyzing images of a channel filled with fluorescently-tagged liposomes under static conditions (no flow), exposed continuously to light from the microscope, acquired over a 20 minute time period. It was found that the fluorescence decreased approximately 1%
per minute of direct light exposure. In each microchannel experiment, overall image acquisition time was less than 2 minutes. Since quantification of the nanoparticle deposition rates were based on the collection of a large number of particles from the entire channel, the effect of photobleaching on the fluorescence measurements was considered negligible.

3.2.6. Statistical Analysis

To determine the significance in the differences between groups, an unpaired one-tailed Student $t$ test analysis was performed (SPSS 15, Chicago, IL). A p-value less than 0.05 was used to confirm significant differences.

3.3. Results and Discussion

3.3.1. Preliminary evaluation of the microchannel setup

In order to study the tendency of nanoparticle margination in microcirculation, we fabricated a microfluidic device consisting of a straight 5 cm-long microchannel with dimensions of 100 x 175 µm (height x width), which replicates the dimensions of an arteriole or venule [158, 159]. To truly evaluate the relation of the physical characteristics of a nanoparticle to its margination under flow, we need a surface which captures marginating particles in a broad non-specific manner. Therefore, active targeting of a ligand-presenting particle to a “receptor-coated” microchannel would be undesirable as the shape and size of particles results in a variable degree of binding strength for each particle [35], and therefore delineation of the chemical effects from the physical effects will be difficult. To avoid undesirable specific binding events, the channel was coated
with fibronectin, an extracellular matrix protein that has been shown to significantly increase cell attachment on PDMS [160-162]. It has been shown that cells can adhere to a fibronectin treated PDMS surface when the surface is incubated with a solution of 10 µg/mL fibronectin [160]. To further ensure adhesion of the nanoparticles to the wall upon contact, we incubated the microchannel with an excess of fibronectin (i.e. 100 µg/mL). Previous studies have shown that fibronectin at these concentrations forms a layer of 4-5 nm thick, which is insufficient to influence the flow pattern within the device [163].

Figure 3.1. Schematic of the experimental setup to measure nanoparticle margination.
Figure 3.2. A typical saturation experiment is shown for the 65 nm liposomes. At t<0, the microchannel was filled with PBS. At t=0, $3 \times 10^{11}$ nanoparticles/mL were flowed through the microchannel at 50 µL/min. At t=30 min, the lumen was continuously flushed with PBS for 30 minutes. At t=60 min, the microchannel was flushed with ethanol. A series of 12-bit images were captured using a fluorescence microscope which enabled the dynamic semi-quantitative analysis showed in the plot. Representative images of each phase are shown in the bottom.

Figure 3.1 shows a schematic of the experimental setup. To study the adhesive strength of the fibronectin-coated microchannel, the nanoparticle suspension was infused via a syringe pump into a PBS-filled microchannel at a flow rate of 50 µL/min, which is the expected blood flow in a vessel with the microchannel’s dimensions [158]. The nanoparticle suspension was switched to PBS to flush out the nanoparticles that were deposited on the walls. In order to optimize the experimental conditions, we initially
asked three important questions: (1) How long did it take for the nanoparticles to reach a constant concentration in the lumen during the initial transition time? (2) What was the required time for deposition of nanoparticles to completely cover the channel’s surface (i.e. saturation of the channel’s surface)? (3) Once the nanoparticles deposited on the walls, did they detach and return back to the flow? Fluorescence microscopy was used to semi-quantitatively interrogate the fate of the fluorescently tagged nanoparticles in the channel in a real-time fashion. **Figure 3.2** shows results from a ‘saturation experiment’ performed with the 65 nm liposomes. Images were acquired from the center of the microchannel. Fluorescence signal was found by determining the average pixel value of an ROI drawn around one segment of a microchannel (175 x 400 µm). In the beginning of the experiment, the nanoparticles required about 5 minutes to reach a constant concentration in the lumen of the channel. The initial sharp signal increase due to the transition from PBS to nanoparticles in the lumen was followed by a substantially slower increase in the fluorescence signal. The latter slower increase suggests continuous deposition of nanoparticles on the channel walls. In the third phase, the removal of the nanoparticles from the lumen of the channel with PBS caused a rapid decrease in the signal. Once the channel was completely filled with PBS, it was observed that the signal from the channel remained constant (t=40-60 min). Importantly, the levels of the signal were significantly higher compared to the background (i.e. t<0) indicating adhesion of particles to the lumen wall. Not surprisingly, ethanol, which solubilizes liposomes, was able to completely clean the channel as indicated by the return of the signal to the background levels. Therefore, we concluded the nanoparticles adhered on the fibronectin coating without detaching and returning back to the flow. In the bottom of **Figure 3.2**,
representative microscopy images are shown. It should be noted that the saturation experiments for the other nanoparticles yielded similar trends.

It should also be noted that we employed microchannels with a rectangular cross-section in contrast to the circular one of the microvasculature. The flow profile in channels of rectangular or circular cross-section is expected to exhibit dissimilar patterns. The primary difference is anticipated to be in the ‘dead space’ of the corners of the rectangular channel. Using fluorescence microscopy, we interrogated the topology of the nanoparticle deposition in the microchannels with rectangular cross-section and we detected negligible signal from the corners indicating that few particles deposited in these regions. Therefore, we can conclude that our findings can be fairly extrapolated to cylindrical microchannels as well. Notably, in an ongoing project we are working on the fabrication of cylindrical microchannels with more complex structure (i.e. more bifurcations).

Furthermore, it should be noted that all the nanoparticles used in this study displayed a very mild charge. The fluorescently tagged liposomes and gold particles displayed a slightly positive zeta potential (about 5 mV), whereas the iron particles exhibited a slightly negative zeta potential (about -10 mV). Therefore, the nanoparticle margination and deposition on the fibronectin-coated channel could not be attributed to ionic interactions.

3.3.2. Effect of nanoparticle size

The concentration of nanoparticles used in this study was chosen based on a representative dose of a clinically used nanoparticle chemotherapeutic (i.e. liposomal
An intravenous dose of 10 mg doxorubicin per kilogram of body weight results in an initial concentration of about $5 \times 10^{12}$ liposomes per mL of blood. In our experiments, the number of particles per mL for all the nanoparticles was fixed at about 10% of the initial “clinical” concentration ($i.e.$ $3 \times 10^{11}$ nanoparticles/mL).

Based on the results from saturation experiments, we chose 8 minutes as the adhesion time for subsequent margination and adhesion experiments. The nanoparticles were allowed to run through the system for 8 min followed by PBS flushing and finally ethanol wash. The channel was semi-quantitatively monitored in real-time using fluorescence microscopy as before. The timeline of a typical margination and adhesion experiment with the 65 nm liposomes is shown in Figure 3 based on the semi-quantitative microscopy. Upon collection of only the wall-deposited nanoparticles using a final ethanol wash, the deposition of the nanoparticles was quantified using a fluorescence reader.

**Figure 3.3.** A typical margination experiment is shown for the 65 nm liposomes. At $t<0$, the microchannel was filled with PBS. At $t=0$, $3\times10^{11}$ nanoparticles/mL were flowed through the
microchannel at 50 µL/min. At t=8 min, the lumen was continually flushed with PBS for 30 minutes. A series of 12-bit images were captured using a fluorescence microscope which enabled the dynamic semi-quantitative analysis showed in the plot. At t=39 min, the nanoparticles deposited in the microchannel were collected by an ethanol flush. Those collections were quantified with a fluorescence reader which was the basis of the quantitative analysis presented in the following figures.

Figure 3.4. Dependence of the nanoparticle margination on their size. (a) The size distribution of each formulation as measured by dynamic light scattering. (b) Comparison of the deposition of 65, 100 and 130 nm in diameter liposomes encapsulating PBS at a flow rate of 50 µL/min (n=3-6; * indicates p<0.05; t-test, two-tailed).
In order to evaluate the dependence of margination on the size of the particles at the nanoscale, three different liposome formulation encapsulating PBS were tested. The size distributions shown in Figure 3.4a indicate that each liposome formulation represented a distinct population with a narrow size distribution. Not surprisingly, the smaller liposomes exhibited the highest deposition (Figure 3.4b). For margination to occur, the particle has to escape the flow streamline. Since the contribution of buoyancy or gravity can be assumed negligible for these nanoparticles [30], diffusion of the particle should outweigh its momentum for margination and deposition to occur. Since the diffusion coefficient is inversely proportional to the size of the particle (Table 3.2), the higher diffusion component of the smaller spheres is implied to enhance their margination.

3.3.3. Effect of nanoparticle density and shape

The effect of particle momentum was further examined by comparing same-sized particles with different masses. Based on the Stokes-Einstein equation, particles with the same diameter should have the same diffusion coefficient (as shown in Table 3.2). Therefore, nanospheres with a diameter of 65 nm of different constituting materials will diffuse equally. However, the momentum (and inertia) of particles of the same size strongly depends on the particle’s density. While the degree of diffusion of every 65 nm sphere was the same, at similar flow rates, the gold sphere is expected to “carry” more momentum due to its large density. Therefore, a gold sphere should face more difficulty to marginalize compared to a particle of the same size but lower density (e.g. liposome). The relative contribution of diffusion to momentum can be expressed by the inverse of a
modified Péclet number, as shown in the right column of Table 3.2 where $\rho_p$ is the particle density, $\rho$ is the density of water, $L$ is the length of the channel, $u$ is the maximum mean fluid velocity, and $D$ is the diffusion coefficient of the particle. As expected, increases in this dimensionless number as a result of density changes led to an increase in deposition. Figure 3.5a shows a clear dependence of the deposition on the particle density. Notably, a very “light” particle (e.g. liposome with $\rho_p \sim 1 \text{ g/mL}$) can result in 57 times greater deposition than a very “heavy” one (e.g. gold particle with $\rho_p \sim 19 \text{ g/mL}$). In addition, particles that consist of the same shell (i.e. liposome) but encapsulate material of different density (e.g. water versus concentrated iodine) resulted in significant differences in deposition.

We should mention that we compared soft and hard nanoparticles. The axial migration of liposomes (soft particles) depends on their elasticity, since deformability of the liposome membrane can lead to a lift force under flow. However, the liposomes used in our studies, DPPC-membrane liposomes are known to be very rigid particles (and thus have long circulation times). The Young's modulus of DPPC liposomes is about 110 MPa and the bending rigidity is about 330 $k_B T$. We, therefore, did not consider this phenomenon to contribute to our results.

To further investigate the effect of momentum on margination, we hypothesized that a faster flow rate should result in a decrease of nanoparticle margination. Theoretically, a quadrupling in the flow rate (i.e. 200 µL/min) should decrease the contribution of diffusion and subsequently margination by a factor of 4 as indicated by the dimensionless number (i.e. diffusion/momentum) in Table 3.2. Not surprisingly, Figure 5b shows that the deposition of a 65 nm liposome was about 3 times lower at the
faster flow rate. One could hypothesize that the deposition under the slower and the faster flow rates are similar. The lower deposition at the faster flow rate could actually be attributed to the fact that the adhesive strength of fibronectin is not sufficient to retain deposited nanoparticles onto the channel’s walls under the faster flow. It should be noted that we performed a saturation experiment under the faster flow rate, which showed no detachment, and therefore we can conclude that there is lower margination at 200 µL/min.

Table 3.2. Theoretical estimation of the the diffusion and convection components for each particle. Based on the Stokes-Einstein equation, the diffusion coefficient and the inverse of modified Peclet number was calculated

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Diffusion coefficient (×10^{-9} m^2/s)</th>
<th>diffusion/momentum = ( \frac{D}{\rho_p/\rho_{water}} \times 10^{-4} ) uL</th>
</tr>
</thead>
<tbody>
<tr>
<td>65 nm PBS-loaded liposome</td>
<td>6.76</td>
<td>5.68</td>
</tr>
<tr>
<td>100 nm PBS-loaded liposome</td>
<td>4.39</td>
<td>3.69</td>
</tr>
<tr>
<td>130 nm PBS-loaded liposome</td>
<td>3.38</td>
<td>3.52</td>
</tr>
<tr>
<td>65 nm iodine-loaded liposome</td>
<td>6.76</td>
<td>2.11</td>
</tr>
<tr>
<td>65 nm PBS-loaded liposome*</td>
<td>6.76</td>
<td>1.42</td>
</tr>
<tr>
<td>60 nm Fe sphere</td>
<td>7.32</td>
<td>1.20</td>
</tr>
<tr>
<td>60 nm Au sphere</td>
<td>7.32</td>
<td>0.31</td>
</tr>
</tbody>
</table>

* The flow rate in this experiment was 4 times faster (200 µL/min) than the rest of the studies (50 µL/min)
Figure 3.5. Dependence of the nanoparticle margination on their density and shape. (a) Comparison of the deposition of spheres with a diameter of about 65 nm and different particle
density at a flow rate of 50 µL/min. **(b)** Comparison of the deposition of 65 nm liposomes at different flow rates. **(c)** Comparison of the deposition of a 60 nm gold sphere and a 56 nm-long gold rod at a flow rate of 50 µL/min (n=3-6; * indicates p<0.05; t-test, two-tailed).

The effect of shape has also been shown to play a critical role in the margination of nanoparticles [30, 31]. In contrast to spherical particles, oblate-shaped particles are subjected to torques resulting in tumbling and rotation [29, 30]. These complex dynamics of non-spherical particles cause translational as well as rotational motions. In order to obtain a comparative indication of the effect of shape, the deposition of a gold nanorod was tested and compared against a gold sphere with comparable diameter (**Figure 3.5c**). Similarly to previous theoretical and experimental studies [30, 31], the rod displayed about 8 times higher deposition than the sphere.

In **Figure 3.6**, the overall performance of all the nanoparticles is shown. Taking under consideration that the experimental conditions were identical for all the particles, the influence of the physical characteristics of the particles can be appreciated. While the size and shape has been shown to strongly affect margination, the particle density seems to play an even more essential role. Within the short timeframe of the experiment (*i.e.* 8 min), the “lighter” sphere (liposome encapsulating PBS) displayed about 57 and 7 times higher deposition than the “heavier” gold sphere and rod of similar dimensions, respectively. It should be noted that the *in vivo* performance of a nanoscale agent depends on many complex factors, such as its blood residence time, and the loading of active compound (*e.g.* therapeutic or imaging agent). However, the relation of margination with the particle physical characteristics should not be neglected in the design of nanoparticles as they might be key contributors to the *in vivo* outcome.
Figure 3.6. Overall dependence of the nanoparticle margination on their size, density and shape. The deposition of each nanoparticle was normalized with respect to the particle exhibiting the lowest deposition (i.e. 60 nm gold sphere).

3.4. Conclusions

In conclusion, a systematic in vitro analysis provided valuable findings regarding the margination tendency of flowing nanoparticles. In good agreement with previous studies [30, 31], the smaller particles and rods favored margination. Notably, the experimental protocol allowed us to resolve differences in margination tendency of particles at the nanoscale (i.e. about 30 nm difference in the diameter of nanoparticles). More importantly, the particle density had an even stronger influence, as the lighter particles marginated further than the denser particles. Finally, the effect of particle size and density on margination and deposition can be described by a single dimensionless number (inverse of modified Peclet number). While the current experimental setup generated valuable conclusions, the transport of nanoparticle in a medium resembling the
complexity of blood using a microchannel network of more than one channel is a subject of ongoing studies.

The studies in this chapter provided valuable insight, but they neglect many factors of tumor complexity that include vessel tortuosity, vessel bifurcations, and interstitial pressures. To fully understand how flow affects nanoparticle transport, it is necessary to relate flow to nanoparticle deposition \textit{in vivo}. In the next chapter, multimodal imaging will be used to determine the relationship between blood flow and the intratumoral deposition of liposomes of different sizes.
CHAPTER 4

Multimodal In Vivo Imaging Exposes the Voyage of Nanoparticles in Tumor Microcirculation

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Randall Toy, Elliott Hayden, Andrew Camann, Zachary Berman, Peter Vicente, Emily Tran, Joseph Meyers, Jenna Pansky, Pubudu M. Peiris, Hanping Wu, Agata Exner, David Wilson, Ketan B. Ghaghada, Efstathios Karathanasis.
4.1 Introduction

Although nanoparticles should localize themselves with high specificity in solid tumors while reducing off-target delivery due to the so-called EPR effect,[76, 165] clinical data indicate that nanoparticle-based chemotherapy is not consistently effective.[135, 136, 166] This has been attributed to the fact that nanoparticles have to overcome various tumor-related biobarriers (e.g. premature and tortuous blood vessels, high interstitial pressure, erratic blood flow), which highly vary between tumors of even the same stage.[143-145, 167] For example, the studies that led to the clinically adapted liposomal doxorubicin identified the 100-nm size as an optimal ‘compromise’. [17, 134, 168-170] However, the uniqueness of each tumor was not taken under consideration. We have previously shown that a principal reason for differential tumor responsiveness to 100-nm liposomal doxorubicin is the differential microvascular permeability. [147, 171, 172] These findings suggest that a single design of nanoparticles may not achieve equally high extravasation in all the regions of a tumor.

The deposition of nanoparticles into tumors is a complex process, which is governed by characteristics of the tumor microenvironment such as blood flow, physical gaps of the endothelial fenestrations, interstitial fluid pressure (IFP), and microvessel density. The overall transport of a nanoparticle is due to movement from applied convective forces and to a lesser degree Brownian motion.[25] Thus, the extravasation of nanoparticles is partially governed by the rate of fluid flow and filtration along a capillary, which depends upon the hydrostatic pressure gradient (i.e. the difference between the vascular pressure and IFP).[26] Decreased blood flows and high IFP are indicative of tumor’s degree of resistance to extravasation of nanoparticles.[11] Thus, it is
essential to understand the forces, which drive a nanoparticle to overcome the numerous biobstacles within a tumor. Our previous *in vitro* studies in microfluidic channels demonstrated that the margination of flowing nanoparticles (i.e. lateral drift towards the vessel walls) depends on their size and the flow rate.[19] This indicates that the intravascular and transvascular transport of nanoparticles in a tumor’s region is governed by the relationship of particle size to the hemodynamics of that tumor’s region. Due to the convective transport of nanoparticles, as the particle size increases, faster blood flow patterns are required to overcome high IFP in tumors. Thus, we suggest that one nanoparticle formulation does not fit all the regions of a tumor, which motivated us to study tumor development and nanoparticle transport in real-time on a microscopic basis.

Here, we developed *in vivo* imaging tools to non-invasively evaluate the regional expression of functional, molecular and morphological biomarkers that affect the deposition of a nanoparticle inside a tumor. To simultaneously monitor the spatiotemporal progression of tumor vasculature and its vascular permeability, we developed a quantitative *in vivo* imaging method using a 100-nm iodinated liposomal contrast agent and high-resolution micro-CT. We term this method nano-Contrast-Enhanced micro-CT (abbreviated as nCE-μCT). The nCE-μCT method provides a unique opportunity to non-invasively monitor the tumor vasculature development and accurately measure vascular permeability to the 100-nm liposomal agent at very high resolution (28 μm).[147, 173, 174] Our initial step was to explore how vascular permeability is related to regional tumor blood flow and the expression of an angiogenic marker (i.e. αvβ3 integrin) using multimodal imaging. In addition to nCE-μCT, tumor blood flow and molecular imaging was performed using standard contrast-enhanced computed
tomography (i.e. perfusion CT) and small animal fluorescence molecular imaging (i.e. FMT), respectively. While nCE-µCT can monitor the intratumoral deposition of a liposome at microscopic resolutions, one practical limitation stems from the fact that only a single liposome formulation can be tracked in the same tumor. To be able to simultaneously image the intratumoral deposition of different formulations, we then used FMT imaging. Following perfusion CT for quantitative assessment of tumor’s regional blood flow, FMT was used to image the in vivo fate of cocktails containing liposomes of different sizes (30, 65, and 100 nm in diameter) labeled with different NIR fluorophores. Co-registration of the imaging sets from the different modalities enabled the extraction of correlations between the regional deposition of differently sized liposomes and the blood flow in different regions of the same tumor. We also evaluated whether the tumor retention of liposomes is dictated by targeting them to a receptor overexpressed by the cancer cells. Thus, besides the EPR effect, we evaluated the relationship of tumor blood flow to active targeting and liposome size. Since tumor microenvironment is variable not only among different cancer types but also among tumors of the same cancer type and stage, we used two highly aggressive mammary adenocarcinoma animal models: the rat 13762 MAT B III model and the mouse 4T1 model.

The multimodal, noninvasive and longitudinal nature of this imaging approach enabled us to quantitatively assess the extravasation of nanoparticles in tumors in terms of their relation to regional blood flow, nanoparticle size and active targeting to overexpressed receptors. While the convective transport of nanoparticles is well acknowledged,[25, 142] we found that the nanoparticle deposition in different tumor
regions varied dramatically (anywhere from one to three orders of magnitude) depending on the particle size, receptor-targeting and blood flow.

4.2. Methods

4.2.1 Animal model and care protocols

All procedures were conducted in accordance with institutional, US, and international regulations and standards on animal welfare and approved by the institutional animal care and use committee at Case Western Reserve University, Cleveland, OH. Female Fischer F344 rats (Harlan, Indianapolis, IN) and BALBc/4j mice (Jackson Laboratories, ME) were used. The 13762 MAT B III (American Type Culture Collection, Manassas, VA) rat tumor model was used for the longitudinal CT imaging study. For multi-modal imaging and the assessment of liposome deposition of different sizes, the 4T1 mammary adenocarcinoma mouse tumor model was used. The tumors were orthotopically implanted by injecting $5 \times 10^5$ 13762 MAT B III cells or 4T1 cells into the #9 mammary fat pad of the rat or mouse, respectively. After imaging, animals were anesthesized and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde in PBS. The tumors were soaked in 30% sucrose (w/v) in PBS at 4°C and then cryosectioned. Slides were stained with both DAPI and a TUNEL stain (Promega) to qualitatively assess cell apoptosis.

4.2.2 Fabrication of iodinated liposomal contrast agent

The long circulating liposomal-iodinated contrast agent was fabricated using established methods [174]. A highly concentrated iodine solution (525 mg I/mL) was
prepared by dissolving iodixanol powder in deionized water at 60 C and mixed with lipids (56:4:40 Dipalmitoylphosphatidylcholine(DPPC): 1,2-Distearoyl-phosphatidyldiethanolamine-methyl-polyethyleneglycol (DSPE-mPEG):cholesterol) dissolved in ethanol. Liposomes were sequentially sized using an extruder (Lipex). After extrusion, the resulting solution was diafiltered using a MicroKros module (Spectrum Laboratories, CA) of 500 kDa molecular weight cutoff to remove unencapsulated iodixanol. Liposome size was characterized through dynamic light scattering (mean size ~100 nm) and iodine concentration (110 mg I/mL) was verified through UV spectrophotometry (\(\lambda=245\) nm).

4.2.3 X-ray computed tomography scanning parameters and protocols

Computed tomography imaging was performed using the Inveon micro-CT system (Siemens Healthcare, AG). All scans were Hounsfield calibrated and had a tube voltage of 80 kVp, tube current of 500 μA, 180 projections, and a 512 x 512 x 512 reconstruction matrix. Two CT scanning protocols were developed: a 99 μm pixel resolution protocol with a 120 ms exposure time and an 85 x 85 x 98 mm field of view and a 28 μm pixel resolution protocol with a 400 ms exposure time and a 43 x 43 x 30 mm field of view. The rat was imaged on days 0, 2, 4, 6, and 8 after tumor inoculation with both scanning protocols. In an independent subset of rats, the daily clearance of the contrast agent was monitored through CT measurement of blood pool contrast, in Hounsfield Units or HU (Figure 4.1), which exhibited a linear relationship. This was used to calculate the necessary follow-up doses of contrast agent to maintain a blood pool concentration of 35 mg I/mL (2.24 g/kg), which corresponds to about 1,500 HU. Thus, a
small injection volume of the liposomal contrast agent (200-350 μL) was typically administered every two days to compensate for a signal drop of 270 HU.

Figure 4.1. Blood clearance of the 100-nm liposomal contrast agent in rats with orthotopic 13762 MAT B III tumors. The time–attenuation curve in the aorta was measured at multiple time points using a micro-CT system after systemic administration of the liposomal contrast agent encapsulating 110 mg/mL iodine (dose: 2.25 g/kg iodine; n=3 animals). Signal is shown in Hounsfield Units (HU). The pre-injection attenuation in the aorta (t<0) was ~150 HU. Imaging was performed immediately after post-injection (t=0) and 1, 4 and 7 days post-injection.

4.2.4 Multimodal imaging of tumor blood flow, vascular permeability, integrin expression and microvessel density

A BALB/cJ mouse was maintained on a low fluorescence diet two weeks prior to imaging. Blood flow measurements were obtained through dynamic contrast enhanced computed tomography (DCE-CT). A dynamic perfusion scan (80 scans, 0.5 s rotation time, 12 consecutive slices) was taken with a 24 slice CT scanner (Sensation Open, Siemens Medical Systems, 80 kV, 150 mAs, detector width = 1.2 mm, slice thickness = 2.4 mm). Five seconds after scan initiation, a 100 μL bolus of Visipaque contrast agent
(GE Healthcare, 320 mg I/mL) was administered through tail vein injection. Siemens Body Perfusion CT syngo software, employing the Patlak perfusion model and a user defined arterial input function, was used to calculate regional blood flow at an in-plane resolution of 152 x 152 μm². The mouse was then imaged in the FMT before tail vein administration of the 100-nm iodinated liposomal contrast agent (250 mg/kg iodine) and an integrin-targeting fluorescent agent (Perkin Elmer, MA). Imaging was conducted with FMT and μCT 24 hours after cocktail administration. Following these scans, the mouse was injected with a high dose of iodinated liposome contrast agent (3 g/kg) and euthanized. The mouse was re-imaged in the μCT to obtain a high resolution (28 μm), high contrast angiogram.

4.2.5 Correlation of tumor blood flow to intratumoral deposition of different liposomes

In an independent subset of mice with 4T1 or rats with MAT B III tumors, tumors were allowed to grow for 2 weeks. Blood flow maps of the tumors were acquired with perfusion CT using methods previously described. Following blood flow map acquisition, a cocktail containing liposomes of different and distinct sizes was injected intravenously once at t=0. Each class of liposomes was labeled with a different near-infrared (NIR) fluorophore (Vivotag 750, 680, 635, respectively; Perkin Elmer, MA) for quantitative imaging using FMT. The administered cocktail contained equal number of each liposome class. Animals were imaged using FMT at 14 or 24 hours after injection of the cocktail containing the different liposomes.
4.2.6 Image Analysis

All images were analyzed using the AMIRA software (Visage Imaging, CA). Manual segmentation and thresholding was used to isolate individual blood vessels. The multi-modal images were manually registered to the best of our ability using regional landmarks applied to the skin of the animal. Regional liposome extravasation, integrin expression, and blood flow were quantified on the microCT, FMT, and DCE-CT images, respectively, using the AMIRA MaterialStatistics function. To assess liposome deposition at different blood flows, FMT images at 4 channels were registered to a three-dimensional volume of blood flow. Zones of blood flow were chosen \textit{a priori} using a histogram of regional blood flows from a full tumor. 3D regions of interest encapsulating these zones were selected by masking of blood flow images in AMIRA. The volume of these regions of interest ranged from 10-60 mm$^3$ in size. Deposition of each class of liposome was measured at different zones of blood flow. In addition, we also measured the fractional Blood Volume (fBV) in each ROI, which indicates the source of liposomes in the blood available for extravasation.

4.2.7 Histological evaluation

After the last imaging acquisition, tumors were collected for histological studies. The animals were anesthetized with an IP injection of ketamine/xylazine and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde in PBS. Tumors were explanted and post-fixed overnight in 4% paraformaldehyde in PBS. The tissues were soaked in 30% sucrose (w/v) in PBS at 4 °C for cryosectioning. Serial sections of 12 μm thickness were collected using a cryostat (Leica CM 300). To visualize
the tumor microvasculature, the tissue slices were immunohistochemically stained for the endothelial antigen CD31 (BD Biosciences, Pharmingen). The tissues were also stained with the nuclear stain DAPI. The tissue sections were imaged at 5 and 10 on the Zeiss Axio Observer Z1 motorized FL inverted microscope. To obtain an image of the entire tissue section, a montage of each section was made using the automated tiling function of the microscope.

4.2.8 Statistical Analysis

Correlations between regional tumor blood flow, regional vascular permeability, and regional nanoparticle extravasation in the same animal were assessed independently using the Pearson correlation coefficient. Means were determined for each variable in this study and the resulting values from each experiment were subjected to one-way analysis of variance with post hoc Bonferroni test. A $P$ value of less than 0.01 was used to confirm significant differences. Normality of each data set was confirmed using the Anderson-Darling test.

4.3 Results

4.3.1 Longitudinal imaging of early tumor angiogenesis and vascular permeability

Following an injection of a 100-nm liposome encapsulating a high cargo of an iodinated contrast agent, we performed high resolution imaging of a rat using a micro-CT system. Imaging immediately after injection of the agent (early-phase post-injection) provided an accurate angiogram, since the majority of the detected signal should only be attributed to intravascular contrast agent. Due to the high intravascular signal (~1500 HU in the aorta) and spatial resolution (28 μm), Figure 4.2 provided a micromorphological
angiogram, which gave detailed information of the microvasculature. We have performed early-phase post-injection nCE-μCT imaging to a large group of animals (n>15) resulting in consistently high-resolution images with high signal-to-noise ratio. Based on a study by Jain et al., mammary adenocarcinomas in rodents present a capillary network composed of vessels with a mean length of 67 μm, a mean diameter of 10 μm, and a mean intercapillary distance of 49 μm. Thus, nCE-μCT imaging provided comparable resolution to the features of tumor microvasculature allowing us to non-invasively obtain tumor parameters that were relevant to our study.

To observe angiogenesis at its earliest stages of development, a rat was injected with the 100-nm iodinated liposomal contrast agent and longitudinally imaged by micro-CT for 8 days after tumor inoculation. A set of scans with a large field of view (85 x 85 x 98 mm) and resolution of 99 μm allowed us to compare blood vessel formation and structure in a mammary fat pad inoculated with a tumor and a healthy mammary fat pad (Figure 4.3a). Following systemic administration of the iodinated liposomal contrast agent, late-phase post-injection imaging enabled us to quantitatively track the development of tumor vasculature in a time-dependent manner. From the second day after inoculation, changes in feeding vessel size and vascular permeability, as evidenced by higher signal from increased nanoparticle extravasation, could be observed. In order to observe the developmental changes of the tumor vasculature in higher detail, we performed 28 μm-resolution nCE-μCT scans. Figure 4.3b shows that tumor vasculature began to rapidly grow 2 days after tumor inoculation. Newly formed vessels in the tumor were observed to be highly irregular and tortuous in comparison to vessels in the healthy mammary fat pad. In addition, we have established a method for quantification of the
extravasation of the iodinated liposomes without the interference of signal from the circulating liposomes in the blood.[146, 147, 171] By applying thresholded colormaps to volume rendered images, manual segmentation was used to isolate regions with low extravasation (purple) from high extravasation (green). Importantly, the intratumoral deposition of the 100-nm liposome displayed significant differences from one region to the next. While Figure 4.3 shows an example from a single animal, this spatiotemporal variability in the development of tumor vasculature and its vascular permeability has been observed in the entire group of animals (n=4).

Figure 4.2. An example of a high-resolution angiogram using nCE-μCT. A rat bearing a 13762 MAT B III breast tumor inoculated into the mammary fat pad was injected with a high dose of iodinated nanoparticle contrast agent resulting in a blood pool concentration of 35 mg/mL iodine. Imaging was done 4 days after tumor inoculation using with nCE-μCT (high-resolution scan, 28 μm isotropic voxel). Major vascular structures were identified, including the: (A) Uterine vein and artery, (B) iliolumbar vein and artery, (C) lumbar branches of iliolumbar vein and artery, (D) iliac branch of iliolumbar vein, (E) left colic vein, (F) inferior mesenteric vein, (G) superior
hemorrhoidal vein, (H) hypogastric vein and artery, (I) inferior epigastric vein and artery, (J) common iliac vein, (K) external iliac vein and artery, (L) internal iliac vein and artery, (M) l. internal iliac vein, (N) iliolumbar vein and artery.[175] In all cases, the vein is the larger of the two labeled vessels.

Figure 4.3. An example of longitudinal imaging of the progression of tumor microvasculature and its permeability to a 100-nm liposome using nCE-μCT. (a) Large field of view and low resolution (99 μm) images of the initial steps of tumor development using longitudinal nCE-μCT imaging. A rat with a 13762 MAT B III tumor inoculated orthotopically in the mammary fat pad was imaged at 99 μm resolution before and on days 2, 4 and 8 after tumor inoculation. Maximum intensity projections (MIPs) show the lower abdominal region of the rat at
the site of tumor inoculation. The white arrows label the area with tumor vasculature development and subsequent nanoparticle contrast agent extravasation. (b) Small field of view and high resolution (28 μm) images of the initial steps of tumor development using longitudinal nCE-μCT imaging. A rat with a 13762 MAT B III tumor inoculated orthotopically in the mammary fat pad was imaged before and on days 2, 6, and 8 after tumor inoculation. Column 1: A thresholded colormap was applied to volume rendered images in AMIRA, where manual segmentation was used to isolate regions with low extravasation (purple) from high extravasation (green). Column 2: Volume rendered images without manual segmentation. Column 3: Manually segmented extravasation in the absence of blood vessels.

Due to the longitudinal nature of the study and exposure to x-ray radiation for multiple days, we evaluated whether imaging with the micro-CT system significantly affected tumor viability in terms of apoptosis of cancer cells. The high-resolution micro-CT scan exposed the rats to ~4 Gy x-ray radiation. Post-mortem TUNEL staining was performed on multiple histological sections per animal to compare the level of apoptosis in irradiated and non-irradiated tumors (n=3 animals per condition). There was no significant difference in the apoptotic index (ratio of apoptotic to non-apoptotic cancer cells) between the two conditions, with the value being less than 1%. Using detailed histological analysis, we previously showed that the apoptotic index of mammary tumors in rodents was about 1% in the same timeframe as the one used here, which is consistent to our findings in this study.[76]
4.3.2 The effect of blood flow on the intratumoral deposition of liposomes in the rat MAT B III model

While nCE-μCT allowed us to quantitatively monitor the intratumoral deposition of the 100-nm liposome at microscopic resolutions, only a single liposome formulation was tracked. To simultaneously measure the intratumoral deposition of different formulations in the same tumor, we employed FMT imaging in a group of rats bearing MAT B III tumors (n=5). Initially, a tumor blood flow map was generated through quantitative assessment of tumor’s regional blood flow using standard clinical perfusion CT at an in-plane resolution of 152 x 152 μm². Following perfusion CT, FMT was used to image the in vivo fate of a cocktail containing two liposomes with different sizes (65 and 100 nm in diameter; Figure 4.4a) labeled with different NIR fluorophores. Figures 4.4b and c show representative images of the blood flow map and the FMT-based liposome deposition map in the same tumor indicating the wide regional variability of blood flow and liposome deposition within the same tumor. Co-registration of the three-dimensional maps from the two different modalities allowed us to quantify the blood flow and deposition of the two liposome classes on a regional basis within the same tumor. While blood flow exhibited high variability in this tumor model, the blood flow range was not very wide compared to other types of tumors. Even with this relatively narrow blood flow range, Figure 4.4d illustrates that higher deposition for both liposome classes was favored in the regions with fast blood flows (e.g. 175 mL/min/100mL). Furthermore, the 100-nm liposome outperformed its 65-nm counterpart in the regions with fast flow. The opposite occurred in the regions with slow flow, which favored the 65-nm liposome more than the 100-nm one. To confirm the in vivo findings, we
performed post-mortem histological evaluation. Care was taken to obtain tissue sections from the same location of the tumor with the same orientation as that of the blood flow map obtained using perfusion CT. A representative histological image is shown in Figure 4.4e, which indicates that greater number of 100-nm liposomes was found in tumor locations that exhibited high blood flow than regions with slow flow. These patterns were observed in multiple histological sections. As with most nanoparticles with sizes in the 100 nm range, the liposomes exhibited near-perivascular accumulation in the tumor interstitium. Complex cellular arrangements and components of the extracellular matrix in tumors create additional geometric restrictions that contribute to the diffusion limitations of nanoparticles.[176] Even after successful extravasation and deposition in the near-perivascular region, nanoparticles remain proximal to the vessel wall. Dreher et al. showed that dextran with molecular weight of 2 MDa could only penetrate 5 μm from the vessel wall in 30 min.[177] Liposomes with sizes ranging from 50-150 nm were shown to accumulate predominantly within 40-50 μm from the rim of avascular multicellular spheroids of about 400 μm in diameter consisting of prostate cancer cells.[178]
Figure 4.4 Dependence of liposome deposition into tumors on the liposome size and blood flow. (a) The size distribution of two different liposome classes (65 and 100 nm) as measured by dynamic light scattering. (b) Perfusion CT was performed on rats with 13672 MAT B III tumors to generate 3D maps of blood flow in tumors. (c) Following tumor blood flow mapping, the tumor deposition of two different liposome classes (65 and 100 nm) was non-invasively measured using FMT imaging at 24 h after injection. To image the two liposome classes in the same tumors, distinct NIR fluorophores were used to distinguish each class of liposome inside the tumor. A representative 3D FMT image is shown as an example. (d) The intratumoral deposition of liposomes with two different sizes is shown as a function of regional blood flow in tumors. The injection dose of each liposome class contained equal number of particles. The deposition was normalized to the fractional blood volume (fBV) of each region. The data of blood flow and liposome deposition is presented as mean ± SD in a given 3D ROI (n=4 animals). (e) Fluorescence image of a histological section of a 13762 MAT B III tumor shows the microdistribution of 100-nm liposomes (5x magnification; red: 100-nm liposome; blue: nuclear
stain (DAPI); green: endothelium (CD31)). Images of entire histological sections of the organs were obtained using the automated tiling function of the microscope. Care was taken to obtain tissue sections from the same location of the tumor with the same orientation as that of the blood flow map obtained using perfusion CT. Insets: The location of liposomes is shown with respect to blood vessels (10x magnification).

4.3.3 Multimodal imaging of functional tumor biomarkers in the mouse 4T1 model

Since tumor microvascular network varies widely among different types of tumors, we also tested the deposition of liposomes in an additional aggressive mammary adenocarcinoma model: the mouse 4T1 model. Initially, we employed the high-resolution nCE-μCT method to image the tumor microvasculature and the intratumoral deposition of the 100-nm iodinated liposomal contrast agent. In addition to performing nCE-μCT imaging, we also measured regional blood flow and integrin expression in the same tumor (Figure 4.5a). FMT imaging enabled the quantification of the fluorescence signal from an integrin-targeting agent in 3D volumes. Using the three imaging modalities (micro-CT, perfusion CT and FMT), imaging was performed 24 hours after administration of an $\alpha_v\beta_3$ integrin-targeting NIR fluorescent agent and the 100-nm iodinated liposomal contrast agent in order to allow time for the two agents to interact with the tumor endothelium. Previous work has fully characterized the $\alpha_v\beta_3$ integrin-targeting NIR fluorescent agent showing its high specificity for targeting $\alpha_v\beta_3$ integrin receptors.[179] In addition, to illustrate the variable pattern of vascular permeability, we generated a map with bins of low, medium, and high extravasation of the liposomal contrast agent. Finally, a tumor blood flow map was generated through the standard
clinical perfusion CT at an in-plane resolution of 152 x 152 μm². Co-registration of the three-dimensional maps from the three different modalities and quantification of the integrin expression, vascular permeability and blood flow allowed us to explore potential correlations among morphological, functional and molecular biomarkers. For example, these measurements revealed that increased extravasation of the liposomal contrast agent correlated to increasing levels of integrin expression (Figure 4.5b) and high blood flows (Figure 4.5c). On the other hand, a nonlinear correlation was found between tumor blood flow and integrin expression (Figure 4.5d). Thus, high-resolution images of blood flow and liposome distribution in tumors can be analyzed to extract correlative extravasation patterns of differently sized liposomes in different regions of the same tumor.
Figure 4.5. Multimodal *in vivo* imaging of vasculature, vascular permeability, integrin expression and blood flow in an orthotopic 4T1 mammary tumor in mouse. (a) Following nCE-μCT, FMT (after injection of integrin-targeting probe), and standard perfusion CT of the same animal with a mammary tumor, the angiogram (28μm resolution) was overlaid with the map of vascular permeability, a map of integrin expression (FMT imaging), and a map of tumor blood flow (perfusion CT). Correlations were assessed between (b) vascular permeability and integrin expression, (c) vascular permeability and blood flow, and (d) blood flow and integrin expression. Error bars indicate standard error of the mean value of the measurement within each ROI (n=1 animal).
4.3.4 Effect of blood flow on the intratumoral deposition of different classes of liposomes in the mouse 4T1 model

Equipped with a set of imaging tools capable of simultaneously assessing nanoparticle deposition and blood flow in unique regions of a tumor, we then explored how nanoparticles of different sizes behave in 4T1 tumors as a function of blood flow. In this study, we further explored this relation by testing three sizes of liposomes. Similarly to the rat tumor model, we used FMT imaging to non-invasively track the intratumoral deposition of a cocktail of three liposome classes with different sizes (labeled with different NIR fluorophores) into the same tumor (orthotopic mouse mammary 4T1 tumor). The distinct NIR fluorophores were used to distinguish each class of liposome inside the tumor (an example is shown in Figure 4.6a). As shown in Figure 4.6b, the three liposome classes exhibited distinct, narrow size distributions (termed as 30-nm, 65-nm and 100-nm liposome). Following perfusion CT imaging of the same animals (n=5 animals), the blood flow rates were analyzed and 3D regions of interest (ROIs) were drawn with their volume ranging from 10-60 mm³. We should note that each tumor presented 5-6 different blood flow zones. The fractional blood volume (fBV) varied from 1-25% of the ROI’s volume. Since fBV dictates the availability pool of liposomes for extravasation in any given ROI, the nanoparticle deposition was normalized to the fractional blood volume (fBV) of each region. As shown in Figure 4.6c, all the classes of liposomes displayed higher extravasation in the regions of faster flow than slow flow. Most importantly, considering tumor regions in terms of blood flow rate, the deposition of liposomes exhibited variable patterns in different tumor regions. The patterns were observed to be different for liposomes of different sizes. In general, the larger liposomes
effectively extravasated in fast flow regions, while the opposite occurred in the slow flow regions (with the exception of the 30-nm liposome). The faster flow significantly benefited the extravasation of the 100 nm liposome. In fact, their deposition was ~2 orders of magnitude higher in fast flow regions compared to slow flow regions. The blood flow had a weaker effect on the extravasation of the 65 nm liposomes. However, in the slow flow regions, the 65-nm liposome significantly outperformed the larger liposomes. The smallest liposomes (*i.e.* 30 nm) did not follow the size-dependent pattern, which suggests that the retention of smaller liposomes into the slow flow regions was much lower than that of larger liposomes.

**Figure 4.6.** Dependence of liposome extravasation into tumors on the liposome size and blood flow on a region-by-region basis. (a) The intratumoral deposition of the different
liposome classes (i.e. different sizes, targeted or non-targeted) was measured in an orthotopic mouse mammary tumor (4T1) using fluorescence molecular tomography (FMT). To image all four liposome classes in the same tumors, distinct NIR fluorophores were used to distinguish each class of liposome inside the tumor. A representative FMT image is shown as an example. (b) The size distribution of three different liposome classes (30, 65 and 100 nm) as measured by dynamic light scattering. (c) The intratumoral deposition of liposomes with the 3 different sizes is shown as a function of regional blood flow in tumors. Following tumor blood flow mapping using perfusion CT, the tumor deposition of the three different liposome classes (30, 65 and 100 nm) was non-invasively measured using FMT imaging at 24 h after injection. The injection dose of each liposome class contained an equal number of particles. The deposition was normalized to the fractional blood volume (fBV) of each region. The data of blood flow and liposome deposition is presented as mean ± SD in a given 3D ROI (n=5 animals; 5-6 tumor regions (flow zones) per animal per liposome class). The scale of the y-axis is logarithmic.

Furthermore, the liposome size is a critical factor that determines blood circulation, which in turn relates to tumor deposition.[17] Since the administered dose containing the cocktail of different liposomes was 150 mg of lipids per kg of body weight, the reticuloendothelial system was not saturated and therefore the clearance rate of each liposome class may have varied.[180, 181] However, there is no significant difference in the blood residence time of liposomes in the size range and timeframe used in our study.[15]

Since we expected the 30-nm liposomes to follow the same trend and achieve the highest deposition in the slow flow regions, we further investigated whether the issue with the 30-nm liposomes is related to their retention in the tumor. A targeted variant of each liposome class was synthesized by incorporating an EGFR-targeting peptide onto
the distal end of the PEGs on the liposomal surface. We compared the 30-nm and 100-nm EGFR-targeting liposomes to their non-targeted variants (Figure 4.7a). While targeting of the 100-nm liposomes did not improve their deposition in slow or high flow regions, active targeting substantially increased the deposition of the 30-nm liposome in slow flow tumor regions (~12-fold increase). Imaging at a later time point (t=48 hrs) showed that EGFR-targeting further enhanced the retention of the 30-nm liposomes (~40-fold increase) in slow flow regions compared to their non-targeted variants (data not shown).

Figure 4.7. Dependence of liposome extravasation into tumors on the liposome size, active targeting towards the EGF receptor, and blood flow on a region-by-region basis. (a) Following tumor blood flow mapping using perfusion CT, the intratumoral deposition of four different liposome classes (30 and 100 nm with or without EGFR-targeting ligands) was quantitatively measured in the orthotopic mouse (4T1) mammary tumor using FMT imaging at 24 h after injection. To image all four liposome classes in the same tumors, distinct NIR fluorophores were used to distinguish each class of liposome inside the tumor. The intratumoral deposition of liposomes is shown as a function of regional blood flow in tumors. (b) Comparison of the four liposome classes is shown at two different time points (i.e. 14 and 24 h after injection).
The deposition was normalized to the fractional blood volume (fBV) of each region. The data of blood flow and liposome deposition is presented as mean ± SD in a given 3D ROI (n=5 animals; 5-6 tumor regions (flow zones) per animal per liposome class). The scale of the y-axis is logarithmic.

**4.4 Discussion**

The 100-nm iodinated liposomal contrast agent was used to assess spatiotemporal changes in tumor microvascular development and vascular permeability to nanoparticles at a resolution comparable to the microvascular features of tumors.[11, 182] Using nCE-μCT imaging, high intravascular signal from the contrast agent enabled the observation of tumor vessel development at the capillary level. Angiography immediately after injection of the 100-nm iodinated liposomal contrast agent (early-phase post-injection) enabled accurate observation of microvascular characteristics, since the 100-nm liposome behaves explicitly as an intravascular contrast agent. Late-phase micro-CT imaging at multiple time points allowed us to monitor the time-dependent regional deposition of the 100-nm liposomal contrast agent into the tumor. In conjunction to the high iodine cargo of the liposomes, the linear relationship between CT signal enhancement and iodine concentration facilitated time-dependent quantification of liposome deposition into tumors.[146, 147, 171] It was found that tumor regions exhibited variable patterns of nanoparticle extravasation over time (Figure 4.2b). These temporal differences in vascular permeability imply that the efficacy of a single nanoparticle formulation may differ with time.
Previous clinical[27] and preclinical[28] studies demonstrated that transient increase of blood pressure (and subsequently tumor blood flow) resulted in enhanced delivery of nanoscale drugs to tumors that otherwise have modest EPR. Thus, we then explored if blood flow could gauge the likelihood that a nanoparticle would accumulate in a particular type of tumor. While perfusion CT cannot resolve the dynamics of an individual microvessel, it has sufficiently high resolution (in-plane: 152 x 152 μm²) and penetration depth to conduct regional flow analysis.[183, 184] We performed these studies in two mammary adenocarcinoma models: the rat 13763 MAT B III and the mouse 4T1. Even in these ‘controlled’ tumor models of the same cell type, stage, and tumor size, regions with very different blood flows were identified which varied widely in topology from one tumor to the next, which is consistent with previous studies.[185, 186] While nCE-μCT provided high resolutions, it only allowed to study the intratumoral deposition of a single liposome formulation. Therefore, we utilized small animal fluorescence imaging (FMT) to quantitatively and simultaneously image four different liposome formulations (labeled with a different NIR fluorophore) in the same tumor. Finally, we employed image processing to co-register the volume-rendered blood flow and liposome deposition maps. This enabled simultaneous volumetric measurement of regional blood flow, microvascular characteristics and liposome deposition, which allowed us to extract correlative patterns of the extravasation rates for each liposome class at different flow zones.

Our central hypothesis was that fast blood flow can help liposomes to overcome high IFP in tumors. We also anticipated that especially the larger liposome classes would not be able to overcome the high tumor interstitial fluid pressures under very slow blood
flow resulting in minor extravasation (even when endothelium may be very leaky).[25, 141, 142] In both animal models, blood flow in a tumor region affected the degree at which liposomes deposited in that specific region. These differences were more profound in the 4T1 model, because these tumors exhibited a wider range of blood flows (20-400 mL/min/100 mL). In the case of the largest liposome class (100 nm), Figure 5c shows that a significantly higher deposition (i.e. 340-fold) was observed in tumor regions with high flow than slow flow. While liposomes of any size extravasated in regions of both fast and slow flow, faster flow had a more dominant effect on aiding larger liposomes to overcome high interstitial pressures inside a tumor. We should emphasize that convective forces predominantly govern the transport of nanoparticles. While the contribution of diffusive transport is small, its relative contribution increases as the liposome size decreases. Therefore, the deposition of smaller liposomes depends less on blood flow than larger liposomes. Indeed, the 65 nm liposome class exhibited a similar trend to the 100 nm liposome with their deposition in high flow regions being 180-fold higher than slow flow regions. However, due to the relatively higher diffusion of the 65-nm liposome compared to the 100-nm variant, the 65-nm liposome required less convective forces than the 100-nm liposome to achieve similar deposition. These findings are in agreement with the mathematical analysis by Decuzzi et al., which showed that for a blood vessel of a fixed radius, nanoparticle deposition increases at higher flows.[25]

Interestingly, our in vivo studies showed that the smallest liposomes (i.e. 30 nm) did not follow the size-dependent pattern. Out of the three liposome classes, we were expecting the 30-nm liposome to exhibit the highest deposition in the slow flow regions. Due to the relatively high diffusivity of the smallest liposomes, we hypothesized that
extravasated 30-nm liposomes re-enter the tumor microcirculation at much higher rates than larger liposomes do. Indeed, active targeting of the 30 nm liposome substantially increased its deposition in slow flow tumor regions (~12-fold increase), which may be attributed to increased retention due to active targeting that prevented the washout of the smaller nanoparticles from the tumor interstitium back to blood circulation. On the other hand, the deposition of 30-nm liposomes with or without targeting ligands in tumor regions of fast blood flow was statistically insignificant. This can be attributed to the fact that the higher intravascular pressure in regions of fast blood flow prohibits washout of 30-nm (targeted or non-targeted) liposomes back to bloodstream at much higher degree than the regions of slow blood flow. Targeting the 100 nm liposome showed no benefits at any flow rate. Because the relative contribution of diffusion is even smaller for the 100 nm liposome than the 30-nm variant, we believe that the deposited 100-nm liposomes remained in the tumor interstitium with or without a targeting moiety. Several prior studies have shown that while active targeting typically enhances the intracellular transport of the nanoparticles, there is no gain in the retention of nanoparticles with sizes of about 100 nm.[187-193]

Given that a tumor is heterogeneous in both its hemodynamics and its pathology, our study indicates that a single “one-size-fits-all” treatment might not be the most effective approach. We chose as a case study, the liposome, due to its clinical adaptation as a chemotherapeutic agent. In the ‘80s and ‘90s, an enormous number of studies concluded that a PEGylated unilamellar liposome composed of rigid phosphatidylcholine and cholesterol with a diameter between 50-150 nm displayed increased accumulation in tumors and antitumor activity.[17, 194] The 100-nm liposome was chosen as the optimal
compromise between loading efficiency of liposomes (increases with increasing size) and ability to extravasate (decreases with increasing size).[151, 195] However, a close examination of the published literature indicates a variable in vivo performance among liposomes of different sizes, exhibiting significant overlap between the in vivo performance of liposomes of different sizes.[17, 128, 196, 197] The results of our study suggest that there is a liposome size that maximizes deposition into a specific tumor region. One could envision that an a priori evaluation of regional blood flow in tumors can facilitate an ‘exclusive’ design of a cocktail of differently sized liposomes tailored to the hemodynamics of the different regions of a tumor. While liposomes of any size extravasate in all the regions, larger liposomes are a better ‘match’ for fast flow regions (and vice versa in the case of smaller liposomes). Most importantly, the outcomes from such tumor region-specific therapy could be generalizable to other types of nanoparticles, since other types of nanoparticles (e.g. polymeric, iron oxide, gold) can be fabricated in different sizes.[198, 199]

4.5 Conclusion

Considering the complexity of the microenvironment of tumors, multimodal in vivo imaging shed some light on the deposition of circulating nanoparticles in tumors. In conclusion, we show that an optimal nanoparticle size can be predicted in terms of maximum deposition in a specific tumor region, if the blood flow rate of that region is known. Furthermore, a critical liposome size exists below which active targeting substantially improves the intratumoral retention of nanoparticles, especially at slow blood flow regions. Thus, the use of an easily measured phenotypic biomarker (tumor
blood flow) [186, 200, 201] could facilitate the selection of a specific set of different nanoparticles to maximize the overall deposition into a specific tumor. This implies that therapeutic regimens could be individualized to the regional hemodynamic profile of a patient’s tumor to maximize the drug deposition in a tumor not just on a patient-by-patient but on a region-by-region basis.

This chapter provided exciting insights on how to tailor nanoparticle design to target primary tumors. Yet, the majority of deaths from cancer are due to metastatic disease that lacks the hyperpermeable, leaky vasculature which facilitates the passive targeting of nanoparticles. Thus, a new approach is needed to target nanoparticles effectively to metastatic lesions. Using the lessons learned from the nanoparticle margination and in vivo multimodal imaging studies, a nanoparticle’s geometry will be selected for a vascular targeting strategy that enables nanoparticle deposition at the site of a metastasis.
CHAPTER 5

Design of a Chain-like Nanoparticle to Target Metastasis

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Pubudu Peiris, Randall Toy, Elizabeth Doolittle, Jenna Pansky, Aaron Abramowski, Morgan Tam, Peter Vicente, Emily Tran, Elliott Hayden, Andrew Camann, Andrew Mayer, Bernadette Erokwu, Zachary Berman, David Wilson, Harihara Baskaran, Chris Flask, Ruth Keri, Efstathios Karathanasis.
5.1 Rational design of a nanoparticle to target metastasis

Exploitation of the mechanisms used by metastatic cancer cells to develop a suitable microenvironment can provide the foundation for a new targeting strategy for nanoparticles. Inflammatory factors secreted by the primary tumor, including vascular endothelial growth factor A (VEGFA), placental growth factor (PIGF), and transforming growth factor-β (TGF-β) cause the upregulation of inflammatory markers at pre-metastatic sites [14]. These inflammatory markers lead to the clustering of bone marrow-derived haematopoietic progenitor cells (HPCs), which secrete premetastatic factors that promote inflammation and degradation of the local extracellular matrix. Metastatic tumor cells can then localize in these regions by targeting activated integrins, which include P-selectin, E-selectin, and the αvβ3 integrin. This abundance of biomarkers enables the use of a vascular targeting scheme for nanoparticles. If nanoparticles can target and bind to these metastatic niches with high avidity, they will be well-positioned to deliver therapeutics to the metastasis.

The design of vascular targeted nanoparticles requires special considerations. Like with passively targeted nanoparticles, it is imperative to use a nanoparticle design that maximizes margination so the nanoparticle has an opportunity to scavenge the blood vessel wall for receptors. It is also critical that a nanoparticle be designed so it has very high binding avidity to its target. One design parameter that plays a vital role in determining nanoparticle binding avidity is surface ligand density. This was demonstrated by a recent study with folate receptor targeted liposomes, where it was shown that both an excess and deficit of ligands reduced nanoparticle binding and subsequent uptake [17]. Another design parameter that plays a major role in determining
both a nanoparticle’s margination ability and binding avidity is its shape. As discussed in previous chapters, variable drag forces and torques cause asymmetric nanoparticles to tumble in the blood flow, which leads to their movement to the blood vessel wall. Modification of the shape of a nanoparticle also influences the number of binding interactions it can have with the vascular endothelium. In this chapter, a chain-shaped nanoparticle will be evaluated for its ability to accumulate in a metastatic lesion. The chain shape was chosen on the premise that its oblate shape and high flexibility would increase margination and binding avidity to the target site. The $\alpha_v\beta_3$ integrin was chosen because it is highly overexpressed by metastatic tumor cells for the purpose of attachment and subsequent extravasation in the metastatic niche. In particular, the cyclic RGD (cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC)) will be used to target the $\alpha_v\beta_3$ integrin. To evaluate the particle’s ability to locate metastatic lesions in vivo, RGD-functionalized nanochains were injected into mice inoculated with 4T1 mammary orthotopic tumors. The 4T1 model was chosen because its metastatic progression mimics the progression of human triple-negative metastatic breast cancer, which tends to spread to sites such as the lung and liver. It is important to note that the $\alpha_v\beta_3$ integrins are minimally expressed on normal blood vessels [202].

5.2 Methods

5.2.1 Synthesis and characterization of the nanochain particles

The nanochains were synthesized following our previously published method.[75] Briefly, solid-phase chemistry was used to partially modify the surface functionality of
IO nanospheres. CLEAR resin (Peptides International Inc, Louisville, KY) functionalized with amines was modified with a homobifunctional cleavable cross-linker reactive towards amines (DTSSP). Amine-functionalized IO nanospheres were introduced, allowed to bind to the solid support and then cleaved off using a reducing agent (TCEP). The same type of resin was used and the modified spheres with surface asymmetry were introduced in a step-by-step manner. After recovering the chain via a reducing agent, the suspension was further cleaned using dialysis. The nanoparticles were characterized in terms of their size (DLS), structure (TEM), and magnetic relaxivity (Bruker minispec relaxometer). The cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC) was conjugated onto PEG(3400) via maleimide chemistry. In addition to conjugation of the cRGDe peptide, the nanochain particles were tagged with an NIR fluorophore (Vivotag 680) to be detectable by FMT imaging or fluorescence spectroscopy or microscopy.

5.2.2 Cellular Uptake and Margination Studies

Cellular uptake studies were performed by seeding the BAEC cells at a density of 2 × 10^5 cells/well in 6-well plate 24 h before incubation with the nanoparticles. Prior to incubation, cells were washed three times with fresh medium and then incubated with the RGD-NC nanoparticles for 15, 30, 45, 60 and 180 minutes at a concentration of 1.3 × 10^11 particles/mL. At the end of each incubation time, the cells were washed three times with fresh medium, fixed with paraformaldehyde, stained with Prussian blue, and imaged using fluorescence and bright field microscopy. Quantification of the signals were used as a measure of the intracellular content of nanoparticles. Under identical conditions, cellular uptake experiments were also performed with the 4T1 cells.
For the margination experiments under flow conditions, PDMS microchannel devices were incubated with fibronectin at 100 µg/mL overnight. Channels with fibronectin were flushed thoroughly with PBS before the start of the experiment. Fluorescent images of the channel were acquired before the introduction of nanoparticles. Then, nanoparticles at a concentration of $2.15 \times 10^{12}$ particles/mL were infused into the microchannels with a Harvard syringe pump (Harvard Apparatus, Holliston, MA) at a flow rate of 50 µL/min. Following nanoparticle flow, channels were washed with PBS at a flow rate of 50 µL/min for 30 minutes. Images were acquired in each phase to qualitatively assess particle deposition during the experiment. The non-adherent nanoparticles were collected from the outlet of the microchannel during both the nanoparticle flow and PBS wash phase of the experiment. Adherent nanoparticles were collected separately by flushing the system with ethanol. Particle concentration was measured using a fluorescent plate reader. The volumes of the collected nanoparticles were measured so that the number of adherent and non-adherent nanoparticles could be calculated from mass balance. Percent deposition was then calculated by dividing the total number of adherent nanoparticles by the sum of the number of adherent and non-adherent nanoparticles, multiplied by 100. It should be noted that we performed pilot studies to evaluate potential photobleaching of the fluorophore and whether this should be taken into account. Photobleaching of the Alexa fluorophore was assessed by analyzing images of a channel filled with fluorescently-tagged nanoparticles under static conditions (no flow), exposed continuously to light from the microscope, acquired over a 20 minute time period. It was found that the fluorescence decreased approximately 1% per minute of direct light exposure. In each microchannel experiment, overall image
acquisition time was less than 2 minutes. Since quantification of the nanoparticle deposition rates were based on the collection of a large number of particles from the entire channel, the effect of photobleaching on the fluorescence measurements was considered negligible.

For the targeting avidity experiments under flow, PDMS microchannel devices were incubated for 2 h with fibronectin overnight before cell seeding. A BAEC cell suspension was prepared by harvesting cells from a culture flask with trypsin solution, pelleting the cells by centrifugation (300 g, 5 min), removing the supernatant, and resuspending in culture media. This cell suspension was pipetted into the microchannel, and incubated at 37°C and 5% CO2. Once the microchannel’s surface was covered by endothelial cells, the cells were treated with TNF-α for 2 h. The nanoparticles at a concentration of $1.3 \times 10^{11}$ particles/mL were infused into the microchannels with a Harvard syringe pump at a flow rate of 50 µL/min. Image acquisition was performed using an Olympus IX-71 inverted fluorescent microscope (Olympus). All acquired images were 12-bit grayscale and taken at 20X magnification. Fluorescent images of the channel were acquired before and every 5 min after the introduction of nanoparticles. Fluorescence signal was quantified by determining the average pixel value of an ROI drawn around one segment in the center of the microchannel (as shown in Figure 5.2b). We confirmed that negligible photobleaching of the fluorescently tagged nanoparticles occurred with the acquisition parameters of the microscope.

5.2.3 Mouse tumor model

All animal procedures were conducted under a protocol approved by the CWRU IACUC. We used an orthotopic 4T1 breast tumor model in mice. The 4T1 cell line
was engineered to stable express green fluorescent protein (GFP) to allow tracking and quantification of the cells in vivo and histologically. Briefly, we inoculated 0.5 x 10⁶ 4T1 cells orthotopically in a no. 9 mammary fat pad of female BALB/c mice that was surgically exposed while the mice were anesthetized. The animals were used in the in vivo studies at week 2 (only primary tumor) or week 5 (primary and metastatic tumors). Based on our prior experience, we chose these time points, since they represent different stages of angiogenesis, necrosis, invasion, and metastasis and are informative and relevant to the human disease.

5.2.4 Fluorescence Molecular Tomography

We performed fluorescence imaging on the 4T1 mammary model in mice (at week 2 or 5) using the FMT 2500 Quantitative Tomography In Vivo Imaging System (Perkin Elmer). Phantoms for each nanoparticle formulation were used to calibrate the FMT to take quantitative deposition measurements. We then intravenously injected each of the four formulations at a dose of 1.3 x 10¹⁴ particles per kg b.w. The animals were imaged before and after IV injection of the formulations at multiple time points (15, 30, 45 min and 3, 6, 24 h).

5.3 Results

5.3.1 Fabrication and characterization of the integrin-targeted nanoparticle

Fabrication of the integrin-targeted nanoparticle (termed RGD-NC) was based on the nanochain technology,[75] which is a two-step approach using solid-phase chemistry. In the first step, amine-functionalized IO nanospheres were attached on a solid support via a crosslinker containing a disulfide bridge. Liberation of the nanosphere using
thiolytic cleavage created thiols on the portion of the particle’s surface that interacted with the solid support resulting in a particle with two faces, one displaying only amines and the other only thiols. Therefore, we were able to topologically control the conversion of amines on the surface of the IO nanospheres into thiols, resulting in a particle with asymmetric surface chemistry. In the second step, employing solid-phase chemistry and step-by-step addition of particles, the two unique faces on the same IO nanosphere served as fittings to assemble them into linear nanochains (Figure 5.1b). The nanochains were analyzed via visual inspection of multiple TEM images. As shown in Figure 5.1c, the nanochains were synthesized in a highly controlled manner. Most of the nanochains are linear and consist of 4 IO spheres with the overall geometrical dimensions of the particle being about 100 x 20 nm (length x width). To evaluate the robustness of the nanochain synthesis, the number of IO nanospheres per nanochain was measured in TEM images. While 6% of the total particles in the suspension were the parent (unbound) IO spheres, the majority of the particles (72%) comprised of nanochains with 4 IO spheres (12 and 10% were nanochains with 3 or 5 IO spheres, respectively). As shown in Figure 5.1d, the hydrodynamic size of the particle and its constituent IO spheres, as measured by dynamic light scattering (DLS), verified the TEM images. It should be noted that DLS measured the effective hydrodynamic diameter based on the diffusion of the particles. Since the hydrodynamic diameter measured by DLS does not correspond to the geometrical size of non-spherical particles, we relied on visual analysis of TEM images to measure the exact dimensions of the nanochain. Detailed characterization of the nanochain particles is reported in a previous publication.[75]
Figure 5.1. Characterization of the RGD-NC nanoparticles. (a) Illustration of the models for the successful delivery of RGD-NC nanoparticles to metastasis via vascular targeting. (b) Diagram of the RGD-NC nanoparticle and its constituent components. (c) TEM image of RGD-NC nanoparticles predominantly composed of four IO spheres. (d) Size distribution of the parent IO nanospheres and RGD-NC nanoparticles obtained by DLS measurements.

The cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC) was conjugated onto the distal end of the PEG-NH₂ on the particle’s surface. In addition to conjugation of the peptide, the nanochain particles were labeled with an NIR fluorophore (VivoTag 680) to be detectable by fluorescence imaging. To evaluate the effect of the geometry on the magnetization, we compared the r2 relaxivity of the RGD-NC particle to that of its parent IO nanospheres by measuring the transverse \( R_2 \) relaxation rates at 1.4 Tesla. The r2 value of the RGD-NC particle was 121 s\(^{-1}\) mM\(^{-1}\), which was 2.1-fold higher than that of its constituent IO spheres. Detection of metastasis via receptor-mediated targeting depends on the generation of signal from each nanoparticle. Thus, we calculated the T2 relaxivity on a per nanoparticle basis, which was 8.4 times higher for the RGD-NC particle compared to its constituent IO nanospheres.
5.3.2. Evaluation of RGD-NC margination and targeting ability \textit{in vitro}

Targeting of the RGD-NC nanoparticles to integrin-expressing endothelial cells was evaluated \textit{in vitro} under static and flow conditions. Bovine aortic endothelial cells (BAEC) were treated with TNF-\(\alpha\) to induce expression of \(\alpha_\beta_3\) integrins\cite{204} and then incubated with an excess of the RGD-NC nanoparticles for different periods of time. As shown in Figure 5.2a, the time course of the nanoparticle uptake by the cells showed that the binding of the nanoparticles occurs rapidly during the first 30 min of incubation. In a similar manner, we evaluated the cellular uptake by 4T1 cells indicating that the integrin-targeting RGD-NC nanoparticles were also able to target the cancer cells (Figure 5.2b). This is significant, because the metastatic 4T1 cells colonize the endothelium as we show later in the histological evaluation.

Successful vascular targeting requires that a nanoparticle can escape the blood flow and drift towards the blood vessel walls (e.g. high margination), followed by strong attachment to the targeting site offsetting the blood flow forces that tend to detach the particle (e.g. high avidity). Since both margination and avidity of nanoparticles in circulation strongly depends on the geometry of the nanoparticle,\cite{19, 29-31, 35} we measured the margination rates and avidity of the nanochains in microvasculature constructs under flow conditions using our previously established \textit{in vitro} method.\cite{19} The experiments were conducted in a microfluidic flow network setup (Figure 5.2c), because channel dimensions and infusion rates can be accurately controlled removing the complexity of \textit{in vivo} studies. Firstly, TEM images of the nanochain suspension in cell culture media were obtained, after the nanoparticles were flowed in the microchannel for
20 min at 50 µL/min, indicating that the particles maintain their structural integrity under flow conditions (Figure 5.2c). To separate margination from targeting avidity, we initially evaluated the margination of non-targeted nanochains. To avoid undesirable specific binding events, the channel was coated with fibronectin, which captures marginating particles in a broad non-specific manner.[19] At a flow rate of 50 µL/min, which is in the range of expected blood flow in tumor microcirculation,[158] the nanochain exhibited 2.3-fold higher margination than the IO sphere (Figure 5.2d). Targeting avidity of the RGD-NC particle was also assessed under flow using the microfluidic device coated with TNF-α-treated BAEC cells. RGD-NC nanoparticles and RGD-targeted nanospheres displayed a biphasic behavior comprising of an initial rapid attachment phase followed by a slower attachment rate (Figure 5.2e). Importantly, after 5 and 20 min, the RGD-NC nanoparticles achieved 9.5 and 2.9-fold higher attachment compared to their spherical counterparts. We should note that the ligand density on the surface of nanospheres or nanochains was the same being about 25 RGD peptides per sphere. Thus, the total number of RGD peptides on a nanochain was about 100.
Figure 5.2. *In vitro* targeting of integrin-expressing endothelial cells under static and flow conditions. (a) After treatment of BAEC cells with TNF-α, the cells were incubated with an excess of RGD-NC nanoparticles for 15, 30, 45, 60 and 180 min (n=3 for each time point). The cells were fixed and stained for iron using Prussian blue. Representative bright field images are shown. Quantification of the signal in fluorescence and bright field images provided the time course of RGD-NC targeting. (b) Comparison of the cellular uptake of RGD-NC nanoparticles by BAEC and 4T1 cells after 3 h of incubation with the nanoparticles. (c) Schematic of the microfluidic experimental setup. The TEM image shows the nanochain suspension in cell culture media after the particles were flowed at 50 µL/min for 20 min. (d) The margination of the
nanospheres and nanochains was compared in a fibronectin-coated microchannel. At t<0, the microchannel was filled with PBS. At t=2.15 x 10^{12} nanoparticles/mL were flowed through the microchannel at 50 µL/min. At t=10 min, the lumen was flushed with PBS. The nanoparticles deposited in the microchannel were collected by an ethanol flush. Those collections were quantified with a fluorescence reader which was the basis of the quantitative analysis. (e) The targeting avidity of the nanospheres and nanochains was evaluated under flow conditions in microchannel seeded with BAEC cells. A series of 12-bit images were captured using a fluorescence microscope which enabled the dynamic quantitative analysis showed in the plot. At t<0, the microchannel was filled with the cell culture media. RGD-NC nanoparticles or RGD-targeted nanosphere with the same concentration of particle/mL were flowed through the microchannel at 50 µL/min (n=4 per conditions).

5.3.3 Targeting metastases in vivo with FMT

To evaluate the ability of the RGD-NC particles to target metastasis, we used mice with a late stage 4T1 tumor (week 5 after tumor inoculation). The efficacy of the RGD-NC nanoparticles to target metastatic tumors was quantitatively evaluated in a group of mice harboring metastatic 4T1 tumors (n=6) using FMT imaging. Figure 5.3a shows representative images of a normal mouse (top row) and a mouse with metastases (bottom row) imaged with the FMT system at t=30 min after injection of RGD-NC. The relatively low signal in the lungs of normal animals (n=6) suggested the presence of the agent primarily in the bloodstream. Since RGD-NC nanoparticles are primarily cleared by liver Kupffer cells (and splenic macrophages), the liver of the same animals exhibited relatively appreciable signal compared to the lungs. On the other hand, FMT imaging of mice with late stage 4T1 tumors showed significant accumulation of RGD-NC primarily in regions of the liver and lungs. Using the designated ROIs for each organ (as shown in
**Figure 5.3b**, we measured the concentration of RGD-NC in locations of those organs displaying significantly enhanced signal (*i.e.* hot spots). In each ‘metastatic’ animal, we identified 1-3 hot spots in the liver and lungs designated as ROI-1 and ROI-2 in **Figure 5.3a**, respectively. The quantitative analysis shown in Figure 4b revealed a significant concentration of the agent in these hot spots. More importantly, these hot spots displayed a 15 and 7.2-fold increase of signal compared to the background signal in healthy liver and lungs.

**Figure 5.3.** Evaluation of the ability of the RGD-NC nanoparticles to target metastatic 4T1 tumors (late-stage tumor model: week 5 after tumor inoculation). (a) Representative FMT images show the accumulation of the RGD-NC particles in the liver and lungs of healthy and metastasis-bearing mice at 30 min post-injection. In the animal with metastases, hot spots with a significantly elevated concentration of the particles are indicated in the liver and spleen as ROI-1 and ROI-2, respectively. (b) Quantification of the fluorescence signal obtained from the FMT
images of a group of healthy mice and a group of metastatic mice 30 min after injection of RGD-NC particles (data presented as mean ± standard deviation). The signal of the hot spots in the lungs and liver of the metastatic group was compared to the average signal of these organs in the healthy group (n = 6 animals per group).

5.4 Conclusions

A solid phase chemistry approach facilitated the generation of a nano-chain consisting of 4 linearly assembled iron oxide nanospheres. The nano-chain’s oblong shape enabled heightened margination behavior and increased binding avidity to endothelial cells in comparison to its spherical counterpart. Accordingly, the targeted nano-chain was able to accumulate in metastatic lesions in the liver and the lungs of mice with metastatic breast cancer. While imaging of micrometastasis can enable early detection of metastatic disease that is currently unfeasible, the promising results of this vascular targeting strategy further motivate the testing of the nanochain as a delivery vehicle for cytotoxic agents. In the next chapter, the nanochain will be coupled to a liposome encapsulating doxorubicin. Then, animals with metastatic breast cancer will be treated with the targeted nanochains. In addition, a radiofrequency triggered drug release mechanism will be evaluated for its ability to improve the therapeutic effect of the targeted nanochains.
CHAPTER 6

Treatment of Cancer Micrometastasis Using a Multicomponent Chain-like Nanoparticle

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and

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6.1 Introduction

The vast majority of breast cancer mortality is due to metastatic disease [205]. For example, the 5-year survival rate of breast cancer patients sharply decreases from 98% in cases with localized primary lesions to 23% in cases of distant metastases [205]. Although oncologists have potent small molecule chemotherapeutics such as anthracyclines (e.g. doxorubicin; abbreviated as DOX), the dose of these agents is constrained by their toxicity to normal tissue, because they are distributed within cancer and healthy tissues in a non-specific manner [37].

Due to the unique structures and material properties that appear at the nano-scale, nanoparticles provide new opportunities to address the complexity of cancer metastasis. To date, though, applications of nanotechnology have mainly focused on primary tumors. Nanoparticles have been developed to exploit the leaky vasculature of primary tumors to enhance the intratumoral drug delivery due to the EPR effect [134, 165, 206-208]. While the EPR strategy may be effective in well-vascularized tumors larger than 100 mm$^3$, it is ineffective in micrometastatic disease, which presents small clusters of malignant cells within variable tissue types [38, 39]. However, targeting an occult lesion hidden within a large population of normal cells presents a unique challenge [38].

To overcome these limitations of current drugs in their molecular or nanoparticle form, we designed a multicomponent nanochain (termed nChain), which is comprised of three iron oxide (IO) nanospheres and one drug-loaded liposome chemically linked into a linear, chain-like assembly (Fig. 6.1a,b). The multicomponent nature and shape of the nChain particle result in two unique features that facilitate enhanced treatment of difficult-to-reach cancer sites. The micrometastasis-specific features of the nChain-based
therapy are illustrated in Fig. 6.1c. First, the nChain particle is capable of transporting a large drug cargo to metastases via vascular targeting of the endothelium associated with micrometastasis. The nChain utilizes a cyclic RGD peptide as a ligand to target the $\alpha_v\beta_3$ integrin receptor, which has a well-established role in the development of breast cancer metastasis [209-215]. While initial adhesion of circulating tumor cells onto the endothelium involves cell rolling, the metastatic site quickly transitions to firm attachment that is mediated by $\alpha_v\beta_3$ integrin [209, 211-214]. Although $\alpha_v\beta_3$ integrin mediates the adhesion of cells to a large number of extracellular matrix proteins, it is minimally expressed on normal blood vessels [76, 216, 217]. Thus, $\alpha_v\beta_3$ integrin-mediated vascular targeting can be highly specific towards blood vessels associated with metastatic disease, particularly since extravascular metastases are preceded by metastatic cancer cells residing inside the lumen of blood vessels [209, 218, 219]. Furthermore, the size, shape and flexibility of nChain particles substantially increase their probability of homing to micrometastases. The structure of the nChain particle increases both the lateral drift and margination of nanoparticles towards the blood vessel walls in microcirculation (i.e. continuous scavenging of vascular walls), and targeting avidity of nanoparticles (i.e. latching on vascular target) due to geometrically enhanced multivalent attachment on the vascular target [36].
Fig. 6.1. Illustration of the nChain particle and its therapeutic effect on micrometastasis. (a) Schematic of a linear nChain particle composed of three IO nanospheres and one drug-loaded liposome. (b) TEM image of nChain particles. (c) Illustration of the successful delivery of nanochain-based drug to metastasis via vascular targeting and RF-triggered drug release.

However, even after successful targeting of a nanoparticle to micrometastasis, the overall effectiveness of this event primarily reflects the biological activity of “free” drug against neighboring cancer cells. While nanoparticles typically release their content
slowly, drug release from nChain particles can be remotely triggered due to mechanically induced defects of the liposomal membrane caused by the oscillation of the IO portion of the nChain in the presence of a mild radiofrequency (RF) field [76]. For these analyses, we chose the drug DOX, which can rapidly diffuse through cellular membranes and reach nuclear DNA, which functions as a sink for DOX [220-222]. Application of an RF field rapidly liberated DOX molecules from nChain particles resulting in widespread anticancer activity throughout micrometastatic sites.

6.2. Materials and methods

6.2.1. Materials

The 4T1-GFP-luc cell line was received as a gift from Dr. Ruth Keri (Case Western Reserve University, Cleveland, OH). Female Balb/c mice were purchased from Charles Rivers (Wilmington, MA). The primary antibody for the specific endothelial antigen CD31 was purchased from BD Biosciences Pharmingen (San Diego, CA). Secondary antibodies and cell culture media were obtained from Invitrogen (Carlsbed, CA). The TUNEL assay kit was obtained from purchased from Roche Diagnostics (Indianapolis, IN). Cross-Linked Ethoxylate Acrylate Resin (CLEAR) resin, reaction vessels, other accessories for solid-phase chemistry and the cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC) peptide were purchased from Peptides International Inc (Louisville, KY). The crosslinkers 3,3’-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) and sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC), and the cleaving agent Tris[2-carboxyethyl] phosphine (TCEP) were obtained from Thermo Fisher Scientific (Cleveland, OH). Polyethylene glycol (PEG) conjugates were purchased
from Laysan Bio (Arab, AL). General solvents and chemicals were obtained from Thermo Fisher Scientific (Cleveland, OH). Doxorubicin (DOX) was obtained from Sigma (Saint Louis, MO).

6.2.2. Synthesis and characterization of nanoparticles

To fabricate the multicomponent nChain particles, we employed a stepwise solid-phase chemistry approach to assemble the particles following a modification of a previously published method [75, 76]. In the first step, solid-phase chemistry was used to partially modify the surface functionality of IO nanospheres with a hydrodynamic diameter of 27 nm (the size of the IO core is 10 nm) (Fig. 6.2a). Amine-PEG-functionalized IO nanospheres were conjugated onto amine-functionalized CLEAR resin via a homobifunctional crosslinker (DTSSP) reactive towards amines containing a cleavable disulfide bridge. The IO nanospheres were allowed to bind to the solid support and then cleaved off using a reducing agent (TCEP). The thiolysis cleavage liberated the IO nanosphere from the solid support converting the amines to a different chemical functionality (thiol group) on the portion of the nanosphere’s surface that was linked to the resin. In the second step, by defining the topology of two different functional groups on the surface of the parent nanospheres, the two unique faces on the parent IO nanosphere served as fittings to chemically assemble them into nanochains using solid-phase chemistry (Fig. 6.2b). The same type of resin was used and the modified nanospheres were introduced in a step-by-step manner using a heterobifunctional crosslinker with NHS-MAL functionality (sulfo-SMCC). It should be noted that each step included multiple washing/drying cycles to remove any unbound nanospheres and
excess reagents from the nanoparticle-resin complex. As a final component, an amine functionalized DOX-loaded liposome with a hydrodynamic diameter of 35 nm was added. The liposomes were prepared by sequential extrusion through a 50 nm filter followed by sonication for 30 min at 30°C. DOX was remotely loaded to the liposomes against an ammonium sulfate gradient [223]. The thiol of the cysteine residue of the c(RGDfC) peptide was used to conjugate the targeting ligand to the remaining amine-terminated PEG on the surface of nChain particles. Finally, the $\alpha_v \beta_3$ integrin-targeting nChain particles were cleaved off the resin and recovered.

The nanoparticles were characterized in terms of their size using a ZetaPALS dynamic light scattering system (DLS; Brookhaven Instruments, Holtsville, NY). TEM images were obtained using a Tecnai F30 instrument (FEI, Hillsboro, OR) operated at 300 kV. The concentration of iron was determined via ICP-OES (Optima 7000 DV; Perkin-Elmer, Waltham, MA). Due to the simplified purification procedure and easy handling of multiple reaction vessels, the solid-phase-based synthesis enabled us to manufacture large amounts of nChain particles that exhibited a high degree of uniformity with the majority of the particles (73%) comprised of three IO nanospheres and one liposome [36, 76]. Due to the high intraliposomal space available for drug encapsulation and the efficient remote loading technique (entrapment efficiency was $\sim$95% of the initial amount of DOX added to the liposomal suspension) [223], the drug cargo was high ($6.8 \times 10^{-5}$ ng DOX per nChain particle). More detailed characterization of the nanochain particles can be found in previous publications [75, 76].
Fig. 6.2. Reaction scheme of the controlled assembly of multicomponent nChain particles using solid-phase chemistry. (a) In the first step, chemical bifunctionality on the surface of parent IO nanospheres is topologically controlled resulting in nanospheres with two faces, one displaying only amines and the other only thiols. (b) In the second step, the two unique faces on the parent nanosphere serve as fittings to chemically assemble them into nanochains.

In order to assess the in vivo performance of the nChain particles, we compared them to other control formulations using different therapeutic protocols, which are summarized in Table 1.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Formulation</th>
<th>Therapeutic protocol</th>
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<tbody>
<tr>
<td>nChain</td>
<td>$\alpha_\beta_3$ integrin-targeting nChain nanochains</td>
<td>no radiofrequency (RF) application</td>
</tr>
<tr>
<td>nChain/RF</td>
<td>$\alpha_\beta_3$ integrin-targeting nChain nanochains</td>
<td>45 min after injection of the formulation, the RF field was applied for 60 min</td>
</tr>
<tr>
<td>NT-nChain</td>
<td>non-targeted nanochains</td>
<td>no RF application</td>
</tr>
<tr>
<td>Lip</td>
<td>$\alpha_\beta_3$ integrin-targeting 35-nm liposome</td>
<td>no RF application</td>
</tr>
<tr>
<td>Lip/RF</td>
<td>$\alpha_\beta_3$ integrin-targeting 35-nm liposome</td>
<td>45 min after injection of the formulation, the RF field was applied for 60 min</td>
</tr>
<tr>
<td>NT-Lip</td>
<td>non-targeted 35-nm liposome</td>
<td>no RF application</td>
</tr>
<tr>
<td>DOX/RF</td>
<td>free doxorubicin</td>
<td>45 min after injection of the formulation, the RF field was applied for 60 min</td>
</tr>
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*Table 6.1. Summary of different formulations and therapeutic protocols and their abbreviations*
6.2.3 In vitro RF-triggered drug release

The nChain suspension was exposed to an RF field using a custom-made solenoid (10 kHz frequency at a power of 2-30 Watts into the samples, solenoid's resistance ~5 Ohms). Triggered release from the nChain particles was measured using the fluorescence properties of DOX (λ<sub>ex/em</sub>=485/590 nm). The cytotoxicity of released drug from the nChain particles was compared to the liposomal drug and free drug. Briefly, cytotoxicity studies were performed by seeding 13762 MAT BIII cells at a density of 10<sup>5</sup> cells/well in a 6-well plate 24 h before incubation with the formulations. Prior to incubation, cells were washed three times with fresh medium and then incubated with the treatment for 180 minutes at a concentration of 150 µM doxorubicin per well. The cells were washed three times with fresh medium and incubated for 48 h at 37°C and 5% CO<sub>2</sub> in a humidified environment. The number of viable cells was determined using a formazan-based cell counting assay (CCK-8). Untreated cells were served as live controls for normalization of the data.

6.2.4. Tumor model

All animal procedures were conducted under a protocol approved by the CWRU IACUC. We used the orthotopic 4T1-luc-GFP breast tumor model in mice [36, 76]. Using flow cytometry and antibodies specific to the α<sub>v</sub> and β<sub>3</sub> subunits, previous studies have shown the overexpression of α<sub>v</sub>β<sub>3</sub> integrin in the 4T1 cell line [224]. The 4T1-luc-GFP cell line was engineered to stable express firefly luciferase and green fluorescent protein (GFP). Briefly, we inoculated 0.5×10<sup>6</sup> 4T1-luc-GFP cells orthotopically in a no. 9 mammary fat pad of female BALB/c mice that was surgically exposed while mice were
anesthetized. We have previously established that disseminated metastases are developed within 14 days of 4T1 inoculation. At that point, the primary tumor was resected using established methods [225]. The animals were used 5 days after surgery to allow the animals to recover.

**6.2.5. Biophotonic in vivo imaging**

Starting at 10 days after tumor inoculation, 200 μl of D-luciferin (10 mg/ml) were intraperitoneally administered and imaged after 10 min using Bioluminescence Imaging (BLI) with a preclinical In Vivo Imaging System (IVIS, Perkin Elmer, Waltham, MA). The animals were imaged every 3-5 days until the terminal point of the study. At the endpoint, organs were extracted and processed for histological analysis. The time-course of cancer progression was obtained by quantification of the whole body BLI signal, which was normalized to the background signal of healthy animals.

**6.2.6. Survival study**

The animals were injected with nChain via tail vein at a dose of 0.5 mg/kg DOX 19 days after tumor inoculation. A 60-min application of the RF field (amplitude B=2 mT, frequency f=10 kHz, RF power=3-5 Watts) was employed 45 min after injection, using a custom-made solenoid (N=105 turns, Inner Diameter=2.8 cm) [76]. The RF coil was positioned 1 cm from the animal and oriented such that the magnetic field was directed toward the abdominal and thoracic region. During this procedure the animals were anesthetized through the administration of inhalant isoflurane. Three subsequent treatments were applied at time intervals of three days. Following the same dose and schedule, control groups included untreated animals and animals treated with DOX.
followed by RF, targeting liposomes followed by RF, and nChain. In addition to BLI imaging, the tumor growth was allowed to progress until the animals showed changes in grooming, weight, behaviors, at which point animals were euthanized in a CO₂ chamber. Time of death was determined to be the following day.

6.2.7. Fluorescence in vivo imaging

This study was performed at 25 days after tumor inoculation. Following identification of lung metastasis using BLI imaging, Fluorescence Molecular Tomography (FMT) imaging was performed at multiple time points after injection (t=0, 30 min and 2, 5, 24 hours) of nChain, targeting liposomes and non-targeting liposomes at a dose of 0.5 mg/kg DOX. Each nanoparticle formulation was labeled with an NIR fluorescent dye (Vivotag 680). Phantoms for each formulation were used to calibrate the FMT to take quantitative deposition measurements of regions of interest containing lung metastasis. Organs from animals injected with saline were also imaged to determine background fluorescence at all excitation wavelengths. To verify the findings of the in vivo imaging, organs were imaged ex vivo using a CRi Maestro fluorescence imaging system.

6.2.8. Histological evaluation

Animals were injected with nChain and targeting liposomes at 25 days after tumor inoculation. Animals were euthanized at either 30 min, 120 min or 48 h post-injection. Briefly, the animals were anesthetized with an IP injection of ketamine/xylazine and transcardially perfused with heparinized PBS followed by 4% paraformaldehyde in PBS.
Organs were explanted and processed for cryosectioning. Serial tissue sections of 12-μm thickness were stained for the specific endothelial antigen CD31 and with the nuclear stain DAPI. The tissue sections were imaged at 5, 10 and 20x magnification on the Zeiss Axio Observer Z1 motorized FL inverted microscope. To obtain an image of an entire large section of an organ (e.g. liver lobe), a montage of each section was made using the automated tiling function of the microscope. Direct fluorescence (red) imaging was used for DOX. Direct fluorescence of GFP (green) imaging was performed for imaging the location of metastatic lesions. Apoptotic cells were identified based on TUNEL-stained nuclei.

6.2.9. Statistical analysis

Means were determined for each variable in this study and the resulting values from each experiment were subjected to one-way analysis of variance with post hoc Bonferroni test (SPSS 15, Chicago, IL). A P value of less than 0.05 was used to confirm significant differences. Normality of each data set was confirmed using the Anderson-Darling test.

6.3. Results

6.3.1. In vivo evaluation of nChain’s ability to target metastasis

To evaluate the nChain’s efficiency at seeking micrometastasis, we compared the nChain particles to αvβ3 integrin-targeting 30-nm liposomes (termed Lip) and their non-targeting variant (termed NT-Lip) in their ability to target micrometastasis in lungs. In these studies, we used the murine 4T1 breast cancer cells, which represent a model of
triple-negative breast cancer (TNBC). The 4T1 cells are syngeneic with Balb/C mice, thereby facilitating studies of TNBC development in immunocompetent mice, which ultimately develop metastases at organ sites reminiscent of those observed in human patients [225-230]. In order to more carefully recapitulate clinical breast cancer settings, we surgically resected primary 4T1 tumors, developed in the mammary fat pad of mice, whose micrometastatic foci remained intact. The stable expression of firefly luciferase of the 4T1-luc-GFP cells enabled longitudinal bioluminescence imaging (BLI) of the location and progression of metastases, which showed that the primary tumor of untreated animals grew rapidly within 12 days after 4T1 cell engraftment (Fig. 6.3a). While surgical resection of the primary tumor at day 14 resulted in elimination of BLI signal, metastasis quickly became detectable in the thoracic region and primary site followed by rapid spread to the rest of the body.

Following BLI imaging to identify the location of lung metastasis, FMT was used to noninvasively and quantitatively monitor the accumulation of nanoparticles labeled with an NIR fluorophore in lung metastasis (Fig. 6.3b). The nChain particles displayed maximum fluorescence signal in metastases within 2 h post-injection (Fig. 6.3c). At that time point, vascular targeting of nChain resulted in ~6% of the administered dose being localized in lung metastasis, which was 7.5-fold higher than targeting liposomes. As expected, NT-Lip exhibited negligible accumulation at metastatic sites in the lungs. The in vivo FMT imaging-based measurements of nChain concentration were validated by direct measurement of iron concentration in lung tissues of animals using inductively coupled plasma optical emission spectroscopy (ICP-OES). Furthermore, to confirm the localization of nChain in metastasis, lungs were imaged ex vivo indicating the
colocalization of nChain particles and cancer cells (Fig. 6.3d). Similar to lungs, *ex vivo* imaging of liver also indicated that nChain particles exhibited highly specific deposition at liver metastasis (Fig. 6.4b) in addition to the typical clearance of nChain particles by macrophages (Fig. 6.5).

**Fig. 6.3. Evaluation of the ability of nChain particles to target metastasis *in vivo*.** (a) The timeline of surgery and BLI imaging are shown with respect to implantation of 4T1 cancer cells into the mammary fat pad of female BALB/c mice. Representative BLI images of an untreated animal indicate the progression of metastatic disease. (b) Representative FMT images of the same mouse show the accumulation of nChain particles in lung metastasis at 2 h post-injection. The nChain particles were injected 11 days after surgical removal of primary tumor (25 days after tumor inoculation), which was the time point of early onset of lung metastasis. (c) Using an NIR fluorophore as a label, the time-course of nanoparticle accumulation in lung metastases was obtained by quantification of the fluorescence signal in the FMT images of mice injected with
nChain, integrin-targeting liposome (Lip) and non-targeting liposome (NT-Lip) at a DOX dose of 0.5 mg/kg b.w. (n=4 mice in each group; * P<0.02 by Student’s t-test). The lungs of the animals injected with nChain were excised and digested 5 h after administration and the iron concentration was measured using ICP-OES (data point indicated as ICP in the graph). Control animals were used to correct for background levels of endogenous iron. (d) Using a CRi Maestro fluorescence imaging system, ex vivo imaging of lungs 5 h after injection indicated the colocalization of nChain particles and 4T1 metastatic cells expressing GFP.

Fig. 6.4. Multimodal whole body *in vivo* imaging and organ *ex vivo* imaging. At 11 days after surgical removal of primary tumor (25 days after tumor inoculation), a mouse was IV injected with nChain particles labeled with an NIR fluorophore at a dose of 0.5 mg/kg DOX. The same mouse was imaged with FMT, BLI and micro-CT. The location of lung metastasis and time-course of nChain biodistribution and deposition in metastasis are shown in the representative
images of Fig. 3. High-resolution angiogram (99 μm) was obtained using a Siemens Inveon micro-CT system and an iodinated liposomal contrast agent following a previously established method [21]. (a) Using the visualization software Amira, 3D-rendered volumes of the micro-CT and FMT images were co-registered. As indicated by the ROI (in yellow), only a portion of the lung area from the FMT image was used to indicate the localization of nChain particle in lung metastasis. (b) At 5 h post-injection, the liver of the same mouse was imaged ex vivo using a CRi Maestro fluorescence imaging system.

Fig. 6.5. Organ distribution of nChain particles in healthy mice. At 24 h after intravenous injection of nChain particles at a dose of 0.5 mg/kg DOX to the tails of the rat tumor model, animals were perfused and organs were then excised, washed, blotted dry, and weighed. Each organ was divided into two halves, which were used to measure either DOX or iron content. DOX was extracted following an established protocol [171]. Fluorescent readings of the samples were obtained to detect DOX (λ_ex/λ_em = 485/590). For measuring iron, after digestion of tissue, the iron concentration was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES). Organs from control animals were used to correct for background levels of fluorescence or endogenous iron (n=5 mice).
6.3.2 *In vitro* on-command triggered drug release using RF

Through their interaction with magnetic fields, the magnetic component (IO spheres) of the nChain particle efficiently converts magnetic energy to mechanical energy, which is dependent on the strength and frequency of the magnetic field, as well as the configuration of the IO spheres in the nanochain.[231] Thus, drug release can be remotely triggered due to defects of the liposomal membrane caused by the oscillation of the magnetic ‘tail’ of the nChain particle in the presence of an RF field (Fig. 6.6a). Magnetic field generation was accomplished using an RF source and a solenoidal coil that was size-matched to the sample, which was placed inside the coil. Fig. 2b shows that the release of DOX can be triggered in a controlled manner under the RF field (10 kHz frequency, 1-50 W dissipated power) at a very low concentration of nChain particles expected to deposit in tumor tissues during *in vivo* applications. Notably, the release rate could be modulated by adjusting the operating parameters of the RF field. We should emphasize that no temperature increase occurred in the nChain suspension under the ‘mild’ RF field (experiment was performed at room temperature).
Fig. 6.6 *In vitro* evaluation of the RF-triggered release profile of DOX from nChain particles. (a) Illustration of the defects on the liposome caused by ‘vibration’ of the IO spheres under an RF field. (b) Triggered release from nChain particles using an RF field at 10 kHz and different energy outputs (the sample was located 1 cm away from the RF coil). The samples were exposed to the RF field for the entire duration of the experiment. Besides nChain particles, the RF field (30 W) was applied to mixtures of liposomes with IO nanospheres or IO nanochains at a ratio of 1:3 (liposome: IO spheres). (c) Effect of temperature on the drug release from nChain particles (incubation time was 60 min). (d) Drug release from nChain particles at different particle concentration under an RF field at 10 kHz/30W (the sample was located 1 cm away from
the RF coil). (e) Drug release from nChain particles at different distance from the RF source (RF field: 10 kHz/30W). (f) Amplitude of the magnetic field at different distances from the RF source (RF field: 10 kHz/30W). (g) Cytotoxicity of nChain (with or without RF) on 13762 MAT B III cells. Control treatments included black nanochains, free DOX, and liposomal DOX. The two data points marked with asterisks are statistically different compared to the other conditions ($P<0.01$).

The DOX release profile from nChain particles in the absence of RF is shown in Fig. 6.6c. To investigate the effect of temperature on the release rate, the nChain suspension was incubated at different temperatures for 60 min. A 5% release of the DOX cargo was released at 37 °C, which is consistent with the behavior of the parent liposome. The liposome component of the nChain particle is composed of the phospholipid DPPC, which does not result in thermosensitive liposomes. While DPPC membranes have a transition temperature of 41 °C, the addition of cholesterol to the liposomal membrane has a significant stabilizing effect.[232, 233] Due to the increased transition temperature of the DPPC/cholesterol membrane ($T_m>50$ °C) [151] and the stable entrapment of the precipitated DOX in the liposome [234], a minor increase in release of DOX is expected at elevated temperature due to increase of DOX solubility [233]. Thus, the time course of the release profiles showed an initial burst in the first 5 min followed by a plateau (data not shown). A temperature of 50 °C was required to cause a significant release in 60 min, which is significantly higher than the observed temperature of the release experiment under the RF field.

To further investigate whether mechanical vibration is the release mechanism, we measured the release from suspensions of dramatically different concentrations of nChain
under the same RF field. As shown in Fig. 6.6d, the same release rate per nChain particle is achieved from low concentrations as well as very high concentration of the particles. So far the *in vitro* studies excluded bulk heating of the nChain suspensions under the RF field. However, significant local heating can be generated around nanoparticles [235, 236]. To explore the possibility of local heating, a fluorophore linked on the surface of the nChain particle was used as a thermometer based on an established method [236]. As shown in Fig. 6.7, there is no significant heat generation around the nChain particles. Thus, contrary to heat-induced drug release, we can conclude that the triggered release mechanism of nChain is concentration-independent and is probably based on mechanical forces that occur on the single particle scale. We then investigated the dependence of the release rate on the distance of the nChain suspension from the RF source. Fig. 6.6e shows that the release rate is significantly lower at 4 cm away from the RF coil after a 90 min exposure ($P<0.01$), which is consistent with the relationship of the magnetic field strength to distance (Fig. 2f). Furthermore, Fig. 6.6g shows the cytotoxic effect on mammary adenocarcinoma cells (13762 MAT BIII) of released DOX from nChain, non-released nChain, empty nanochains (no DOX), and free DOX. The empty nanochain had no effect on cancer cells. While nChain had moderate cytotoxicity, the RF-triggered release of DOX from nChain had significantly higher cytotoxic effects ($P<0.01$) due to release of free DOX. Notably, the released drug is 100% bioavailable.
Fig. 6.7 Dependence of fluorescence intensity to temperature. (a) Alexa 488 fluorophore was conjugated on the surface of nanochain particles without DOX (empty liposome). A 96-well plate with multiple samples of the nanoparticle suspension was slowly heated using the built-in temperature control of the plate reader (Biotek Synergy HT). The samples were allowed to equilibrate at each new temperature for 20 min before the measurement of the fluorescence intensity. Starting at 37 °C (ΔT=0), the relationship of ΔT to the change in fluorescence intensity was recorded and plotted. (b) The change in fluorescence intensity of the nanochain suspension in response to the RF field (3 W/10 kHz) indicates that no local increase of temperature occurs around the nanoparticles.

6.3.3. In vivo evaluation of nChain’s therapeutic efficacy

All the animals selected for this study exhibited comparable progression of metastatic disease in terms of location, size and BLI signal intensity. At 5 days post-surgery, the animals were intravenously injected with nChain. A 60-min application of the RF was employed 45 min after injection (nChain/RF group), which corresponds to the time window of maximum nChain deposition in metastases. Control treatments included free DOX followed by RF (DOX/RF), αvβ3 integrin-targeting 30-nm liposomal DOX followed by RF (Lip/RF), and nChain without RF (nChain). The treatments were
administered four times (**Fig. 6.8a**), each at a dose of 0.5 mg DOX per kg of body weight, which is ~10-fold lower than the typical clinical dosage of liposomal DOX [169, 237]. As shown in representative BLI images (**Fig. 6.8b**), the DOX/RF treatment had negligible therapeutic benefits, while nChain (without RF) exhibited a moderate effect. Most importantly, the whole body BLI signal of animals treated with nChain/RF completely disappeared within a few days after initiation of treatment.

As a metric of the response of metastasis to the various treatments (n=7 in each group), quantification of BLI signal was used (**Fig. 6.8c**). While the nChain therapy (without RF) decelerated metastatic growth for a few days compared to the DOX/RF and Lip/RF treatments, the growth of metastasis quickly became similar to the untreated group. Importantly, animals treated with nChain/RF exhibited a dramatic decrease of BLI signals that quickly approached baseline levels. The entire nChain/RF group (n=7) displayed no BLI signal until day 54, at which point metastatic disease recurred in a subset of animals (3/7). While 100% of the mice in other groups died within 50 days, 57% of the nChain/RF-treated group was still alive at 150 days (**Fig. 6.8d**). The other 43% of the nChain/RF-treated group exhibited an increase in mean survival (68 days) compared to the groups treated with nChain without RF (44.1 days), Lip/RF-treated (35.2 days), DOX/RF (28.5 days) and the untreated group (30.1 days).
Fig. 6.8. Treatment of breast cancer metastasis using the 4T1 mammary model in mice. (a) The timeline of surgery and schedule of treatments are shown with respect to tumor inoculation. (b) The response of cancer metastasis to treatment was monitored using longitudinal BLI imaging. Representative images are shown for animals treated with DOX/RF, Lip/RF, nChain and nChain/RF. In the case of treatments combined with the RF field, 45 min post-injection, animals were exposed for 60 min to an RF field (amplitude B=6.3 mT, frequency f=10 kHz) using a custom-made solenoid coil. All nanoparticle formulations were administered at 0.5 mg/kg DOX, while free DOX was injected at 5 mg/kg. (c) Quantification of the whole body BLI light emission is shown for the nChain/RF treatment and control treatments including nChain, DOX/RF and Lip/RF. The inset shows the same plot with the y-axis being in logarithmic scale (n=7 mice in each group; * P<0.03 by Student’s t-test). (d) The survival time of the animals treated with nChain/RF, nChain, DOX/RF and Lip/RF is compared to that of the untreated group.
6.3.3. Histological evaluation

To assess the localization of nChain in micrometastasis and the degree and topology of DOX delivery, histological analysis was performed on separate groups of mice 30 min, 120 min and 48 h after injection (n=3 in each group). A representative image of a liver lobe is shown in Fig. 6.9a displaying the presence of clusters of metastatic cells dispersed in the liver. Notably, 30 min post-injection, nChain accumulated almost exclusively at metastatic sites. Imaging at higher magnification showed that metastatic cancer cells were localized primarily on the endothelial walls (Fig. 6.9b). Importantly, nChain particles were predominantly distributed around those same blood vessels colonized by 4T1 cells (Fig. 6.9c). Application of the RF field 120 min after injection resulted in widespread delivery of DOX at distant cells away from nChain deposits (Fig. 6.9d). Imaging at higher magnification shows that RF-triggered release resulted in delivery of DOX to cell nuclei (Fig. 6.9e), while no spread of DOX was observed in the case of nChain-treated animals that were not exposed to RF, since the fluorescence signal of intraliposomal DOX is quenched (Fig. 6.9f). Most importantly, 48 h after the nChain/RF treatment, DOX was found in the vast majority of cancer cells (Fig. 6.9g), while most of the cancer cells were apoptotic as gauged by TUNEL staining (inset of Fig. 6.9g). Contrary to the nChain/RF group, the nChain and Lip/RF treatments exhibited very low DOX delivery to cancer cells and negligible anticancer effect (Fig. 6.9h,i). Application of the RF field alone had no effect on cancer cells. Similar to liver, histological analysis of lungs showed that the nChain/RF treatment resulted in localization of nanoparticles and widespread delivery of DOX in micrometastatic sites (Fig. 6.10). The in vivo anticancer effects of the nChain/RF treatment observed in
histology are consistent to cell cytotoxicity studies (Fig. 6.11). While 30-nm liposomal DOX, 100-nm liposomal DOX and nChain without RF exhibited low cytotoxicity (less than 10% relative cytotoxicity), RF-triggered release of DOX from nChain had significant cytotoxic effects (18%), which was similar to the effect of free DOX.

Fig. 6.9. Histological evaluation of the anticancer effect of the nChain particle on micrometastasis in the liver. (a) Fluorescence imaging of an entire histological section of a lobe of liver 30 min after systemic administration of nChain at 0.5 mg DOX/kg b.w. Nuclei were stained with DAPI (5x magnification). Images of entire histological sections of the organ were
obtained using the automated tiling function of the microscope. At 30 min post-injection, the location of metastatic cancer cells is shown with respect to the location of endothelial cells (b), nChain particles and DOX (c) in the same histological section (10x magnification; insets: 20x magnification). (d) At 120 min post-injection, fluorescence imaging of an entire histological section shows the widespread distribution of DOX molecules after a 60-min application of RF employed at 45 min post-injection (5x magnification). At 120 min post-injection, higher magnification imaging shows the distribution of DOX molecules with (e) or without RF (f) with respect to the location of cancer cells and nChain particles (10x magnification; inset: 20x magnification). At 48 h post-injection, fluorescence imaging of entire histological sections shows the distribution of DOX molecules with respect to cancer cells for the nChain/RF (g), nChain (h) and Lip/RF treatment (i). Insets show apoptotic cells in a small portion of the same images. Apoptotic cell nuclei were stained with TUNEL.
Fig. 6.10. Histological evaluation of the anticancer effect of the nChain particle on micrometastasis in the lungs. (a) Fluorescence imaging of an entire histological section at 30 min after systemic administration of nChain at 0.5 mg DOX/kg b.w. Nuclei were stained with DAPI (5x magnification). Images of entire histological sections of the organ were obtained using the automated tiling function of the microscope. (b) At 30 min post-injection, the location of metastatic cancer cells is shown with respect to the location of endothelial cells, nChain particles and DOX in the same histological section (20x magnification). At 48 h post-injection, fluorescence imaging of entire histological sections shows the distribution of DOX molecules.
with respect to cancer cells for the nChain/RF (c), nChain (d) and Lip/RF treatment (e) (5x magnification).

**Fig. 6.11. Cytotoxicity of nChain particles on 4T1-GFP-luc cells.** Cell viability was used as an indication of the cytotoxic effects of nChain (with or without RF) on 4T1 cancer cells at 100 μM DOX. Control treatments included free DOX, and 30-nm and 100-nm liposomal DOX. The 4T1 cells were seeded at a density of $10^5$ cells/well in a 6-well plate 24 h before incubation with the formulations. Cells were incubated with the treatment for 180 minutes at a concentration of 100 μM DOX per well. The cells were washed three times with fresh medium and incubated for 48 h at 37°C and 5% CO$_2$ in a humidified environment. The number of viable cells was determined using a formazan-based cell counting assay (CCK-8). Untreated cells were served as live controls for normalization of the data. The data points marked with asterisks are statistically different compared to the other conditions (*$P<0.01$).
6.4. Discussion

Breast cancer was selected for this study due to the potential for rapid clinical translation, since it is the most prevalent cancer among women in the US [205]. While systemic chemotherapy, in its adjuvant mode, prevails as the standard treatment for breast cancer, these potent agents are toxic to both normal and cancer tissues leading to use of suboptimal doses for eradication of metastatic disease [37]. Here, we focused on evaluating the therapeutic efficacy of the nChain treatment in the adjuvant mode in an animal model of TNBC. Among the subtypes of breast cancer [238], TNBC is an extremely aggressive, metastatic and difficult-to-treat subtype. Interestingly, although TNBC comprise 15-25% of all invasive breast cancers, this unique breast cancer subtype displays an unusually aggressive metastatic phenotype that results in their disproportional mortality among breast cancer patients [239-241]. This is due to the fact that drugs cannot easily reach micrometastases, which represent small clusters of cancer cells hidden within a large population of normal cells. However, metastases upregulate specific cell-surface molecules that differ from the rest of its host organ. Specifically, following intravasation, αvβ3 integrin expression on the circulating tumor cells plays central role in the formation of metastases at a distal site, which makes this integrin a suitable candidate for targeting micrometastasis [209-215, 242-244]. Indeed, in this work, the in vivo studies indicate that vascular targeting of αvβ3 integrin associated with micrometastasis provides enhanced selectivity. While various receptors have been exploited to target nanoparticles (e.g. folate, EGF, HER2, integrin receptors), the preclinical development of these systems has focused on targeting tumors at the primary
site. Attention of the field has recently shifted towards metastasis, but there are still few examples of therapeutics for metastases [38, 245].

In order to further enhance the selectivity of vascular targeting, we exploited the engineerability of nanotechnology to control the geometrical shape of nanoparticles. In fact, in the last decade, the field of nanomedicine has recognized that the particle shape governs the navigation of circulating nanoparticles through different biological processes, including intravascular and transvascular transport, and ultimately targeting of difficult-to-reach cancer sites. One of the pivotal steps dictating the transport of flowing nanoparticles is their margination (i.e. radial drift) towards the blood vessel walls. In the case of vascular targeting, near-the-wall margination is not just desirable, but it is required for a nanoparticle to interact with the tumor vascular bed and have subsequent meaningful interactions. Nanoparticle margination is dictated by forces, which influence particle translational and rotational motion, including buoyancy, gravity, drag, van der Waals interactions, electrostatic double layer interactions, and steric repulsive interactions. Under a balance of these forces, spherical nanoparticles tend to follow blood flow [96, 97]. Contrary to spherical nanoparticles, oblong-shaped nanoparticles are subjected to torques resulting in tumbling and rotation, which increase the lateral drift of nanoparticles towards the blood vessel walls in microcirculation [29-31]. Furthermore, the particle shape also governs the specificity of targeting nanoparticles using receptor-ligand systems. Compared to nanospheres, oblong-shaped nanoparticles exhibit substantially enhanced targeting avidity due to geometrically enhanced multivalent docking, which can effectively offset hemodynamic forces that tend to detach the nanoparticle from the endothelium [35, 36]. Not surprisingly, our in vivo studies showed
that an outstanding 6% of the administered nChain particles accumulated in micrometastases within 2 h after injection. Furthermore, similar to any type of nanoparticles carrying a cytotoxic agent, the biodistribution of the nChain particles to the major organs is important to assess. The accumulation of nChain in the heart, lungs, brain and kidney 24 h after administration was about 3.5, 3, 2 and 6% of the injected dose, respectively (Fig. 6.5). As expected, the majority of nChain particles was found in the reticuloendothelial organs (liver and spleen). In a previous study [76], we showed that the overall biodistribution of nanochains was comparable to the behavior of the 100-nm liposomal DOX.

Even with that enhanced deposition in metastases, the nChain treatment (without RF) provided only modest benefits. This is primarily related to the drug release profile from nanoparticles. While free drug in its molecular form quickly spreads within the tumor interstitium [220-222], nanoparticles release their content at a relatively slow rate, once they deposit at the target site. This slow release generates a low temporal and spatial concentration gradient of the drug, resulting in non-cytotoxic levels of the drug distal from the particle [222]. Although the slow release of drug from nanoparticles does not favor cytotoxic effects, it improves the drug’s safety profile during the particle’s circulation in the blood. Thus, our objective was to couple the high selectivity of vascular targeting to a unique triggered drug release mechanism to deliver and spread the drug cargo at cytotoxic levels throughout the hard-to-reach micrometastatic sites. Contrary to heat-induced drug release achieved by other nanoparticle designs (e.g. thermosensitive nanoparticles) [246], the release mechanism of nChain particles is concentration-independent resulting in efficient drug release even from very low concentration of
nanoparticles [76]. In a previous publication [76], we showed that through their interaction with magnetic fields, the IO component of the nChain particle efficiently converts magnetic energy to mechanical energy resulting in “mechanical” disruption of the liposomal membrane. When magnetic nanoparticles are subjected to an external, oscillating magnetic field, there are two relaxation mechanisms (Brownian and Néel relaxation) that govern their magnetization response in an effort to align with the applied field. Brownian relaxation is typically the dominant relaxation mechanism for nanoparticles larger than about 25 nm. In the case of nChain, Brownian relaxation is restricted by the bonds between the constituent nanospheres, such that Brownian motion may be observed as a mechanical “vibration” of the chain, rather than true rotational motion. Regarding Néel relaxation, to reorient its magnetic moment with an applied field, the nanoparticle must overcome an energy barrier, which results in dissipation of excess heat, which has been exploited for hyperthermia applications. However, our studies showed that no local heating is generated around the nChain particle as a response to the 10 kHz field and that mechanical vibration at the selected frequency is the primary mechanism of drug release.

6.5 Conclusions

Indeed, the application of RF on nChain-treated animals significantly impacted the progression of metastasis and, in most cases, resulted in eradication of metastatic disease. Due to the highly selective deposition of the nChain particles at metastases and subsequently efficient spreading of drug, this significant therapeutic outcome was achieved at a very low dose (i.e. 0.5 mg/kg), which is 10-20-fold lower than the typical
clinical regimens of liposomal DOX [169, 237]. Thus, both the particle shape and the multicomponent nature of the nChain played an essential role in its therapeutic efficacy.

Historically, attempts to improve nanoparticle homing to tumors have relied on the EPR effect and targeting of various receptors to direct drugs to the primary site. In this study, we show that an integrin-targeting nChain coupled with a vascular targeting strategy and RF-triggered drug release provides an increased likelihood of highly effective treatment of micrometastasis using a low dose of chemotherapy. We envision that successful clinical translation of the nChain particle will provide a powerful chemotherapeutic agent that can be used in the standard adjuvant setting for the majority of breast cancer patients. Considering that expression of $\alpha_v\beta_3$ integrin plays central role in the development and progression of metastasis in melanomas, prostate, pancreatic and cervical cancers [215], vascular targeting of $\alpha_v\beta_3$ integrin appears to be possible for other types of cancer. Taking into consideration that RF can penetrate deep into tissues, the nChain therapeutic could be employed not only for breast cancer but for diverse types of cancer that display aggressive and metastatic phenotypes including cancers of the gastrointestinal tract, lung, prostate, brain.
CHAPTER 7

Conclusions and Future Directions
7.1. Summary of work

The work in this dissertation firmly establishes that the engineering of a nanoparticle’s size and shape is critical for specific tumor targeting. In this context, multimodal imaging defined design rules to target different classes of nanoparticles to tumor regions with unique blood flows. The multimodal imaging study also demonstrated that a single nanoparticle design is not always adequate to completely treat a tumor. In application to future work, the multimodal imaging protocol can easily be adapted to evaluate the targeting performance of new classes of nanoparticles. Furthermore, taking under consideration of the metastatic microenvironment, design rules derived from the multimodal imaging study and the in vitro microfluidic experiments enabled the rationale engineering of a new class of multi-component nanoparticles, termed nanochains. The nanochains exhibited a remarkable targeting avidity to micrometastatic lesions – 6% of the injected dose of nanochains was localized specifically in the tumor region. Most importantly, the high localization of the targeted nanochain led to remarkable therapeutic efficacy in a metastatic breast tumor model. A subset of tumor-bearing animals treated with the nanochains and exposed to a RF field that triggered drug release were fully cured of the metastatic disease, whereas standard drugs currently used in the clinic did not achieve any benefits. These results represent a significant breakthrough in the use of nanotechnology to treat aggressive and invasive cancers.

7.2. Individualization of nanoparticle-based tumor-specific therapies

The findings from this work strongly suggest that a single nanoparticle design cannot sufficiently treat all tumors. In particular, our multimodal imaging study
highlighted the necessity to treat tumors not as a single entity, but as an integrated collection of variable microenvironments within the same tumor. From the maps generated from our multimodal imaging protocol, it can be seen that a single tumor has regions with radically different blood flows, vascular permeabilities, and expression of biomarkers. Because of this heterogeneity in tumor hemodynamics and phenotype, a different nanoparticle may be best suited for different microenvironments even within the same tumor. For instance, the multimodal imaging study in Chapter 4 clearly demonstrated that 30 nm, 65 nm, and 100 nm nanoparticles deposit preferentially in different tumor regions based on the mean regional blood flow. The class of 65 nm liposomes deposited most in the regions of low blood flow, while the class of 100 nm liposomes deposited most in the regions of high blood flow. These results imply that more than one class of nanoparticle will be necessary for even distribution in tumors with a large range of unique blood flows. Therefore, an approach to increase therapeutic efficacy could be to treat patients with a cocktail of nanoparticles of different sizes. To facilitate this approach, hemodynamic maps obtained through functional imaging could guide selection of components of the nanoparticle cocktail. The nanoparticle cocktail’s composition can be dictated by the output of a compartmental model that predicts nanoparticle deposition based on blood flow. Other parameters (e.g. vascular permeability) could be mapped and correlated to the deposition of different classes of nanoparticle to determine the best prognosticator of nanoparticle tumor therapy.

If we are to increase the robustness of this approach, it would be necessary to thoroughly investigate nanoparticle design parameters beyond size. Our work highlights the importance of shape in the definition of intravascular transport. Future studies should
investigate the relation of blood flow to the intratumoral deposition of nanoparticles of different shapes. In addition to deep interstitial targeting, the effect of blood flow on nanoparticles designed for vascular targeting should also be investigated. Then, it could be truly understood what shape and aspect ratio is appropriate to target metastatic lesions in regions and organs with different blood flows. The relationship between nanoparticle size, nanoparticle shape, and blood flow should also be evaluated with different targeting ligands, which have unique binding kinetics and are expressed at different levels in different tumors. Very interestingly, the multimodal imaging studies demonstrated that receptor targeting benefited nanoparticles of a small size, while it had no effect on nanoparticles of a larger size. It is likely that the critical size in which targeting does not produce a tangible benefit depends on the shape of the particle. If the effects of both size and shape on transport are analyzed in a comprehensive manner, then we will be able to develop rationally designed nanocarriers that can target lesions with high efficacy in any microenvironment.

7.3. Clinical translation of the nanochain platform

With the therapeutic success described in this work, it is important to develop a well-organized plan to expedite the translation of the nanochain platform technology. Our studies have highlighted the importance of optimizing both a nanoparticle’s biochemical and biophysical interactions with the tumor microenvironment. More specifically, a vascular targeting strategy enabled the high co-localization of nanochains with metastatic lesions. It was the RF triggered drug release mechanism, however, which enabled the nanochains to treat metastatic lesions with high therapeutic efficacy. It should be emphasized that therapeutic efficacy was achieved with 10% of the dose used
for standard chemotherapy in the clinic. Clearly, the size, shape, and surface chemistry of the nanochains all play a role in the effectiveness of the therapeutic strategy. The question is how to improve the nanochain further so it is adaptable to treat multiple forms of cancer. For clinical translation, it is important to examine how to optimize both targeting and the drug release mechanism of the nanochain by consideration of the two paramount properties required for a FDA approved translational technology – safety and efficacy.

7.4. Evaluation of the safety of the nanochain platform

The nanochain used in our studies encapsulates doxorubicin, which is a highly cytotoxic drug that leads to severe side effects that include cardiotoxicity in its free form. Although liposomes have been demonstrated to stably encapsulate the drug, and moreover, the dose in our studies was one tenth of the dose used with free doxorubicin, it is still important to verify the safety profile of the nanochains. In particular, the biodistribution of the nanochains must be evaluated more carefully. A remarkable 6% of the injected dose of RGD targeted nanochains co-localized specifically at the site of metastasis, which is significantly higher than the co-localization of both non-targeted and RGD-targeted liposomes (both < 1%) at the site of metastasis. Still, a significant amount of the nanochain accumulates in other regions of the body. More specifically, high, non-specific accumulation of the nanochain is often observed in the liver and the spleen. Fortunately, off-target effects of nanochain delivery can be reduced through improved design of the RF triggered drug release mechanism. Fine tuning of the RF field enables
control of the spatiotemporal distribution of the drug. Our *in vitro* evaluation of the RF triggered drug release mechanism identified that drug release is dependent on the distance from the center of the coil. With a new multi-channel coil design, it may be possible to design an RF coil, which triggers the release of drug at a very specific location. The ability to control drug release is extremely important, since the triggering of drug release in healthy tissues could be highly detrimental. Treatment plans involving multiple rounds of chemotherapy would exacerbate these undesired side effects. Because we only evaluated biodistribution after a single dose of therapy, long term studies will be needed to assess the impact of multiple nanochain doses in organs with high nanoparticle accumulation. These results will be particularly important in organs such as the liver, where metastatic lesions and healthy tissue are in close proximity to each other.

### 7.5. Enhancement of nanochain site-specific delivery

After the safety of the nanochain platform has been evaluated, it will then be essential to optimize and validate the efficacy of the nanochains in larger animals and in humans. The delivery of nanochains to metastatic lesions employed a vascular targeting strategy, which relies on the use of two targeting mechanisms. First, an appropriate surface ligand was used to functionalize the nanochain so it could specifically target overexpressed receptors in the vascular bed of a metastasis. Our specific target choice was the $\alpha_v\beta_3$ integrin, which is overexpressed on cancer cells to facilitate binding to intracellular adhesion molecules and vascular cellular adhesion molecules in the metastatic niche. In addition, we applied the knowledge that oblate shaped nanoparticles had a higher tendency to escape the blood flow and move towards the vessel wall. This potent combination facilitated the high localization of nanochains at the site of metastasis.
With respect to this targeting strategy, there are two major directions in which nanochain targeting can be further improved. One direction is to optimize the surface functionalization of the nanoparticle. Our current assumption is that the nanochains are all captured by metastatic cells nested on the endothelial wall of the blood vessel. This binding and subsequent internalization is a result of receptor-ligand interactions between the cyclic RGD motif on the nanochains and the expression of $\alpha_v\beta_3$ integrins on the cancer cells. In addition to $\alpha_v\beta_3$ integrin, there are other cancer cell receptors (e.g. p-selectin, ICAM1), which may be tested as other potential targets for the nanochain. Another approach would be to choose receptors that are overexpressed in pre-metastatic niches, which are areas predisposed to the development of metastasis. Implementation of this strategy would lead to the elimination of potential sites of metastasis before circulating tumor cells can colonize in these locations. Conveniently, there is an abundance of receptors overexpressed at the pre-metastatic niche, such as p-selectin, e-selectin, l-selectin, and $\alpha_v\beta_3$ integrin, which could be conveniently targeted with the nanochain. To improve the selectivity of the nanochain, a novel approach that incorporates multiple types of ligands onto a single nanochain could be used. Because tumor cells are notorious for expressing biomarkers in a variable manner, a multiple ligand targeting approach would also help guarantee a more complete treatment of the disease. It is also important to note that the expression of metastatic biomarkers changes as the disease progresses. Therefore, the targeting of multiple types of ligands would increase the probability that lesions in different stages will be detected with a single formulation. Validation of nanochain targeting and biodistribution will then need to be pursued in human metastatic tumor xenografts.
As emphasized throughout this work, it is important to consider nanoparticle size and shape along with surface chemistry to maximizing tumor therapeutic efficacy. We designed the nanochain with the knowledge that an oblate shape increases its ability to escape the blood flow and move towards the blood vessel wall. In addition, the use of an oblate shape provided a significant benefit in terms of binding avidity to overexpressed wall receptors. The effect of nanochain geometry on targeting is highlighted by the data which show that the RGD functionalization of doxorubicin liposomes only marginally increased deposition at the metastatic tumor site; addition of the iron oxide chain was necessary to produce a significant increase in targeting. Only one nanochain geometry (27 nm x 110 nm hydrodynamic size), however, was evaluated in the treatment of metastasis. Given the flexibility to produce nanochains with different aspect ratios, it would be prudent to confirm that nanochain geometry is optimized for tumor delivery. Different aspect ratios can be evaluated through modification of the size of constituent nanospheres in the nanochain. In addition, nanochain aspect ratio can be adjusted by altering the number of spheres in the chain. Throughout optimization, nanoparticle size should be constrained to prevent an undesired decrease in pharmacokinetic performance. Nanoparticles of a larger size tend to be more easily recognized by macrophages, which leads to higher uptake and off-target delivery. In addition, it may be beneficial to test assemblies of different shaped nanoparticles (e.g., nanochains of nanocubes in lieu of nanospheres). For example, nanocubes will have a different surface presentation of ligands than nanospheres, which may alter the binding avidity of the chain as a whole. Another key parameter in nanochain design to consider is the stiffness, which may be varied by modifying the number and length of linkers between constituent nanospheres.
Stiff nanochains would have more inelastic collisions with the vascular wall, which would increase the rate of margination from one side of the blood vessel to the other. On the other hand, stiff nanochains with a high rate of margination may not be able to slow down easily to initiate binding with receptors on the vessel wall. Flexible nanochains, however, have improved binding avidity due to the particle’s ability to wrap more tightly over receptor clusters on the cell surface.

Although the nanochains were designed with a vascular targeting strategy in mind, it is important to realize that they will also be needed to treat larger, angiogenic tumors. It may continue to be common for patients to be diagnosed with a combination of micrometastatic and metastatic lesions. In comparison to a micrometastasis, the treatment of a large metastatic lesion will require drug delivery further away from the blood vessel. To treat a large, angiogenic metastatic lesion, the principles used to design nanoparticles to penetrate primary tumors will apply. In order to maximize therapeutic efficacy, the size of the nanochain should be optimized to reduce the effect of interstitial pressures on delivery. Therefore, the nanochain could travel through the blood vessel’s endothelial gaps into the interstitium of the metastatic lesion. Admittedly, one of the greatest challenges is to deliver nanochains to lesions which are larger than micrometastases of a few cells, but smaller than metastatic lesions which have become angiogenic. For this situation, another agent may need to be administered to disrupt the vascular endothelial wall, thus enhancing transcellular permeability at the site of the metastasis. This would provide the nanochain with a route to travel into the deep interstitial space, where it could target metastatic cells which have extravasated out of the blood vessel. In summary, success of the nanochain strategy relies on the choice of target in the metastatic tumor.
microenvironment and the choice of geometry that will maximize nanochain margination and accumulation at a specific metastatic site.

7.6. Optimization of nanochain therapeutic potency

Nanochain performance can also be enhanced through the encapsulation of more contemporary chemotherapeutics. In the nanochain therapeutic efficacy studies, doxorubicin was chosen so the nanochain could be compared in performance with Doxil, the liposomal chemotherapeutic used in the clinic. Anthracycline chemotherapeutics, while highly potent, are also known for their particular adverse side effects. A more important issue to consider in the development of a treatment protocol, however, is the development of chemoresistance. This problem is especially prevalent in a therapy regimen that requires multiple doses of chemotherapy. If the initial dose is not lethal to the cell, the cell may develop increasing resistance to the chemotherapeutic. As a result, the nanochains would become less effective in the second and subsequent rounds of chemotherapy.

One way to reduce the effects of chemoresistance is to encapsulate more potent therapeutics into the nanochain. Due to the presence of both hydrophilic and hydrophobic compartments in a liposome, a large variety of drugs can be encapsulated. Docetaxel, for example, has been shown to improve the prognosis of metastatic cancer patients. Beyond cytotoxic agents, the entire family of tyrosine kinase inhibitors (TKI) can benefit from site-specific targeting and widespread delivery only at the target site. Sorafenib is a TKI that could be incorporated into the nanochains to treat primary kidney cancer. Another example of a TKI is Erlotinib, which targets the epidermal growth factor receptor and
targets non-small cell lung cancer and pancreatic cancer, both diseases with a very high mortality rate. Trametinib is a mitogen-activated protein kinase inhibitor which has been demonstrated to effectively treat metastatic melanoma. Dabrafenib, a B-Raf inhibitor, also is used to treat metastatic melanoma. Cocktails of different chemotherapeutics could also be used to simultaneously deliver multiple drugs to the same cancer cells, which reduces the possibility of tumor survival through drug resistance mechanisms. For example, a combination of trametinib and dabrafenib could be used in the nanochain as a more effective means to treat metastatic melanoma. The best chemotherapeutic or set of chemotherapeutic agents should certainly be selected for encapsulation into the nanochain to maximize therapeutic potential.

We assert that the advantage of the triggered RF mechanism is its ability to release a high dose of drug in a short time, which overwhelms drug transporters that pump chemotherapeutics out of the cancer cells. Increasing this rate of drug release would be another approach to tackle the problem of chemoresistant cells. One unexplored parameter is the effect of the RF wave frequency on drug release. A different frequency may change the oscillation behavior of the iron oxide component of the chain and subsequently affect the rate of drug release. It is also important to consider the design of the nanoparticle in relation to the rate of drug release. While the length of the nanochain affects its targeting to metastatic lesions, it may also affect the mode of vibration and subsequently affect the rate of drug release. It is important to realize that the nanochain with the optimal drug release kinetics may not have the optimal targeting performance or pharmacokinetics. Therefore, a compromise must be made between these
criteria, where the nanochain for clinical translation is selected based on what is most effective therapeutically in the patient.

An alternative therapeutic approach would be to use the nanochain platform to ferry agents for immunotherapy to tumors. Immunotherapy is a growing field in which therapeutics are designed to enhance the body’s natural defenses to destroy tumor cells. The expression of integrins by the circulating tumor cells also facilitates binding between the cancer cells and platelets and leukocytes [247]. Both platelets and leukocytes can enhance binding with the endothelial wall through an increased number of binding interactions between integrins and wall adhesion molecules. Activated platelets, in cooperation with fibrinogen, are also known to protect cancer cells from uptake by natural killer cells. A nanochain encapsulating agents which counteract these immune protection mechanisms could promote a natural response that leads to the elimination of tumor cells. In addition, the nanochain has the ability to deliver these agents with high site specificity. Off-target immunotherapy causes more adverse side effects than off-target chemotherapy, so it is especially important to use a delivery platform with high specificity to the targeted disease.

7.7. Manufacturing and other considerations for clinical implementation

With the optimal nanochain formulation in hand, it is then necessary to consider challenges to nanochain manufacturing and clinical adoption. While our current laboratory methods provide sufficient nanochains for small animal studies, significant scale-up is necessary to conduct testing in large animals and, eventually, humans. The process must be capable of producing high quantities of nanochains of reproducible sizes
and shapes. In addition, the process must follow both Good Laboratory Practices (GLP) and Good Manufacturing Processes (GMP) for approval by the FDA. The nanochains should also be stable in terms of size and have a reasonably long shelf life. When moving towards clinical implementation, the RF coil would also need to be redesigned for a human patient. In contrast to mice, which have a height of ~1 cm, human patients are much larger. As a result, a higher penetration depth will be required to treat metastases in the thoracic cavity (e.g., lungs and liver). Accordingly, the power of the RF may need to be adjusted in order to achieve sufficient therapeutic effect at sites deep below the skin. Depending on the site of the disease, selection of the RF parameters may also need to take into account attenuation through different tissues (e.g. bone).

Another issue to address is how to initially detect sites of metastasis to direct targeting of the RF field. The adaptability of the nanochain’s components, however, facilitate its use also as a contrast agent to detect metastatic lesions. For instance, if the nanochain were labeled with a radiotracer, positron emission tomography (PET) or single photon emission computed tomography (SPECT) imaging could be used to verify the presence of metastases in the patient. Because both of these imaging modalities have extremely sensitivity, it will be easy to identify nanochain binding to very small lesions. In addition, few radiotracers would need to be conjugated on an individual nanoparticle, which preserves the majority of the surface for functionalization with targeting ligands. Once the metastases are located, the acquired PET or SPECT images could be used as maps to direct the localization of the RF field to the nanochains.

Overall, the nanochain is a platform technology that has the potential to enhance the delivery of a wide variety of drugs to diseases beyond metastatic breast cancer. The
goal is to expand the platform to treat other cancers with a high mortality rate, such as brain cancer. In order to achieve this goal using nanoparticle therapy, it is essential to study and address each of these cancers’ unique challenges. Brain cancer, for example, has the challenge of the low permeability of the blood brain barrier. The ability to tailor the nanochain’s biophysics and ability to biochemically interact with its environment, however, provides the opportunity to treat these types of diseases. With its advantages of improved targeting and rapid, triggered drug release, the nanochain platform technology has the potential to significantly improve outcomes for patients with metastatic cancer.
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