SPECIFIC ADHESION OF BIODEGRADABLE
MICROSPHERES TO CYTOKINE ACTIVATED ENDOTHELIAL
UNDER FLOW

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MILIND K. DALAL
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SPECIFIC ADHESION OF BIODEGRADABLE MICROSPHERES
TO CYTOKINE ACTIVATED ENDOTHELUM UNDER FLOW

BY
MILIND K. DALAL

has been approved for
the Department of Chemical Engineering
and the Russ College of Engineering and Technology by

Douglas J. Goetz
Associate Professor of Chemical Engineering

Dennis Irwin
Dean, Russ College of Engineering and Technology
In a variety of disease settings the expression of various endothelial cell adhesion molecules (ECAMs) appears to be increased, making these molecules attractive targets for directed drug delivery schemes. One approach is to use polymeric biodegradable microspheres bearing a mAb for an ECAM whose expression is increased. We used biotin-avidin chemistry to couple a mAb to E-selectin or ICAM-1 to microspheres made from a biotinylated poly (lactic acid) poly (ethylene glycol) (PLA-PEG-biotin) copolymer. We tested the adhesion of the resulting mAb coated biodegradable particles to human umbilical vein endothelial cells (HUVEC). We found that the mAb coated PLA-PEG-biotin microspheres show high specific adhesion to 4 hr. IL-1β activated HUVEC expressing high levels of E-selectin and ICAM-1 relative to the level of adhesion to unactivated HUVEC that express no E-selectin and a low level of ICAM-1. We also found that the adhesion was shear stress dependent. Additionally, we have found that the selectivity of anti-E-selectin coated microspheres to 4 hr. IL-1β activated HUVEC is a strong function of the concentration of anti-E-selectin used to generate the anti-E-selectin coated microspheres. These results suggest that PLA-PEG-biotin particles coated with mAbs to ECAMs could be used to selectively target drugs to endothelium present at sites of inflammation.

Approved: Douglas J. Goetz

Associate Professor of Chemical Engineering
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1 INTRODUCTION

Prevention of adverse side effects by limiting drug activity to the diseased tissue has been the primary objective in the development of new drug delivery systems. Research has also been fuelled by a need to be able to maintain therapeutic drug levels for ever-longer periods of time, use smaller amounts of active agent, fewer doses, less invasive methods of administration, and increased circulation-time of the drug carriers (Starr, 2000). There are several pathways available for targeting drugs to cells and tissues. Of these, the human vasculature is the most convenient since it traverses the entire human body.

1.1 The Endothelium

The inner lining of the human vasculature is a monolayer of cells called endothelial cells or the endothelium. The endothelium expresses on its surface certain protein and carbohydrate moieties called endothelial cell adhesion molecules (ECAMs). These adhesion molecules mediate physical and functional interactions between two cells or between cells and their extracellular matrix. They play an important role in leukocyte recruitment to a site of tissue injury. Leukocyte recruitment involves a multi-step process. It starts with initial attachment of the leukocyte to the endothelium, followed by rolling at a low velocity in the direction of fluid flow, firm adhesion to the endothelium and, finally, extravasation of the leukocyte into the extravascular space (Crutchfield et al., 2000; Shinde Patil et al., 2001). Adhesion molecules on the leukocyte surface (ligands)
are specific for certain adhesion molecules (receptors) on the surface of the endothelium. Receptor–ligand interaction leads to the formation of bonds, and hence, initial attachment of the leukocyte to the endothelium.

The endothelium shows tremendous heterogeneity in the expression of adhesion molecules on its surface (Cybulsky and Gimbrone, 1991; Luscinskas et al., 1994; Pober and Cotran, 1990). Cell adhesion molecules have been classified into four basic families: (a) the selectin family (E-, P- and L-selectin) (b) the integrins (c) the immunoglobulin family [including intercellular adhesion molecule (ICAM-1, -2 and -3); vascular cell adhesion molecule (VCAM-1); platelet-endothelial cell adhesion molecule (PECAM-1)] and mucin-like adhesion molecules [P-selectin glycoprotein ligand (PSGL-1)].

For the purpose of this study, the term “resting” refers to the endothelium lining the vasculature of healthy tissue for in vivo conditions, and unactivated (cultured under normal conditions, i.e. without cytokine stimulation) endothelium for in vitro conditions. Under resting conditions, the endothelium shows a basal level of expression of adhesion molecules on its surface. However, the endothelium can express increased levels and types of adhesion molecules. In a variety of disease settings (e.g., inflammation, plaque development in atherosclerosis, and cancer), the expression of one of the members of the selectin family, E-selectin (CD62 and ELAM-1), has been reported to be increased on the lumenal surface of the endothelium (Duplaa et al., 1996; Fox et al., 1995; Johnson-Tidey et al., 1994; Kansas, 1996; Matsuura, 1997; Mayer et al., 1998; Murakami et al., 2000; Springer, 1994). Another ECAM, ICAM-1, has also been reported to be increased at sites of inflammation (Springer, 1994).
1.2 E-selectin

E-selectin is an ECAM with a structure that consists of an amino terminus, a lectin-like binding domain, an epidermal growth factor-like domain, a membrane-spanning region and a short cytoplasmic region (Bevilacqua, 1993) (Figure 1.1). Under resting conditions, the endothelium expresses very little E-selectin. In vitro, E-selectin expression is well characterized on human umbilical vein endothelial cells (HUVEC). E-selectin expression on HUVEC is upregulated in response to inflammatory stimuli including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), or bacterial lipopolysaccharide (LPS) (Bevilacqua et al., 1989). E-selectin expression requires de novo protein synthesis and occurs two hours after stimulation (Bevilacqua et al., 1989). The expression of E-selectin peaks between 3 to 6 hours after stimulation and decreases thereafter to the basal level within 10-12 hours after stimulation (Bevilacqua, 1993). In vivo, E-selectin is expressed at sites of local inflammation (Kansas, 1996; Springer, 1994). E-selectin plays a role in leukocyte capture and rolling in vivo (Kunkel and Ley, 1996). Ligands specific to E-selectin contain a sialyl Lewis\(^x\) carbohydrate group (Berg et al., 1991; Phillips et al., 1990).

![](image.png)

Figure 1.1: E-selectin Structure. Courtesy of Bevilacqua, 1993.
1.3 Intercellular Adhesion Molecule-1 (ICAM-1)

ICAM-1 is an inducible member of the immunoglobulin superfamily of cellular adhesion molecules. ICAM-1 is a single chain 80-114 kDa protein with 5 immunoglobulin (Ig)-like domains, a single transmembrane region, and a short cytoplasmic domain (Staunton, 1988). Resting endothelial cells have low levels of ICAM-1. Activation with inflammatory cytokines IL-1, or TNF-α in vitro results in increased expression on HUVEC (Dustin, 1991; Mastrobattista, 1999). ICAM-1 functions as a major cell-cell adhesion molecule in inflammation, and is an important molecule involved in the process of leukocyte adhesion and extravasation.

1.4 Targeted Drug Delivery

Broadly speaking, targeted drug delivery means maximizing delivery of drug to the diseased or damaged tissue, and minimizing its delivery to healthy tissue. This would aid in preventing adverse side effects, and also increase the efficacy of the drug. The heterogeneous distribution of ECAMs on the lumenal surface of vascular endothelium provides an opportunity to deliver drugs to select tissues (Bendas et al., 1999; Bendas et al., 1998; Blackwell et al., 2001; Bloemen et al., 1995; Burch et al., 2002; Cybulsky and Gimbrone, 1991; Dickerson et al., 2001; Duplaa et al., 1996; Fox et al., 1995; Irie and Tavassoli, 1986; Johnson-Tidey et al., 1994; Pasqualini, 1999; Ponder, 1983; Spragg et al., 1997; Zhu and Pauli, 1991). The targeting could be achieved by using a polymeric carrier (Gref, 1994) whose outer surface has a ligand for a selectively expressed ECAM
One possible approach for achieving such delivery is to use polymeric biodegradable microspheres bearing a monoclonal antibody (mAb) for E-selectin (or ICAM-1). The carriers would interact with the endothelium in the fluid dynamic environment of the circulation (Goldsmith and Turitto, 1986).

Biodegradable drug carriers have received much attention recently because of their favorable biophysical properties. They degrade over a period of time in the human body, and thus do not have to be surgically removed. Biodegradable drug carriers are also easy to prepare, have a relatively long shelf life, have the ability to carry a therapeutic level of drug, and can be designed to have well-defined drug release rates (Dickerson et al., 2001; Langer, 1998).

Previously Dickerson et al. (2001) passively adsorbed a ligand to E- and P-selectin onto the surface of biodegradable poly (ε-caprolactone) microspheres. They found that the attachment efficiency of the ligand-coated microspheres to cells expressing E-selectin and P-selectin was limited due to the low level of ligand adsorbed to the microspheres. This might be attributed to the presence of a surface stabilizer poly (vinyl alcohol) (PVA), which competes with the ligand for adsorption sites on the microsphere surface (Cannizzaro et al., 1998). To improve on this system it appears that the coupling efficiency of the ligand to the surface of the microsphere would need to be improved.

Another factor to consider for a targeted drug delivery system is the uptake of particles in the blood by the macrophages of the reticuloendothelial system (RES). The RES uses these macrophages to defend the body from bacteria and other foreign particles as well as damaged leukocytes and erythrocytes. Many studies have focused on the
relationship between carrier properties and uptake by RES (Brannonpeppas, 1995; Gref, 1994). These studies suggest that adding polyethylene glycol (PEG) to the surface of the particles will increase the circulation time of the particles.

![Diagram of Normal Endothelium and Endothelium at Site of Disease](image)

**Normal Endothelium**

(Limited expression of ECAMs)

**Endothelium at Site of Disease**

(Increased expression of ECAMs)

Figure 1.2: Targeted Drug Delivery System

1.5 *In Vitro* Flow Assays

*In vitro* experiments have been used extensively by investigators to study processes that occur *in vivo*. In vitro adhesion assays are often employed to study receptor-ligand interactions and their mechanisms. Previously, most assays were conducted under static conditions, wherein the ligand-expressing cells or particles were layered over a receptor-expressing substrate and allowed to settle. The unbound cells or particles were then removed by washing and centrifugation. These static assays were limited in many aspects. Receptor-ligand interaction *in vivo* is a dynamic process and occurs under flow conditions. Particle adhesion under flow is an intricate balance of
forces in which the particle is subjected to adhesive forces mediated by chemical bonds formed between particle and substrate and the disruptive forces exerted by the flowing fluid. Furthermore, the settling time of static assays between two cycles of washing and centrifugation are many times greater than the time of contact between the particle and the substrate under \textit{in vivo} flow conditions. Thus, \textit{in vitro} flow assays more closely model the \textit{in vivo} flow conditions for studying particle-substrate interactions.

1.6 Biophysics of Drug Carrier Adhesion to the Endothelium

The adhesion of the ligand bearing drug carrier to the endothelium is essentially a biophysical process and depends on a number of factors like the carrier size (Cozens-Roberts et al., 1990; Cozens-Roberts et al., 1990; Goldsmith and Turitto, 1986; Hammer and Apte, 1992; Hobbs et al., 1998; Shinde Patil et al., 2001), carrier composition, ligand surface density on carrier (Cozens-Roberts et al., 1990; Cozens-Roberts et al., 1990; Hammer and Apte, 1992), ligand-ECAM interactions (Cozens-Roberts et al., 1990; Cozens-Roberts et al., 1990; Hammer and Apte, 1992) and the fluid shear (Cozens-Roberts et al., 1990; Cozens-Roberts et al., 1990; Goldsmith and Turitto, 1986; Hammer and Apte, 1992). Near the surface of the endothelial cell, the horizontal fluid velocity is almost zero. As the particle nears the cell surface, adhesive forces between the receptor on the substrate and the ligand present on the particle lead to the formation of chemical bonds. Simultaneously, the flowing fluid exerts disruptive forces on the particle. Once the adhesive forces and the disruptive forces balance out or the adhesive force becomes greater, the particle is in mechanical equilibrium and remains adhered to the substrate. In
the event that the disruptive forces overcome the adhesive forces, the particle detaches from the surface.

To optimize the delivery of carriers to discrete segments of the endothelium, the relationship between a subset of the carrier properties and interactions with the endothelium was studied.

1.7 PLA-PEG-Biotin microspheres

The focus of this research was on targeting the inducible ECAMs, E-selectin and ICAM-1, using biodegradable polymers having a ligand specific for the targeted ECAM. The attachment efficiency of biodegradable polymers was investigated using the biotin–avidin coupling system. The biotin–avidin association constant \(K=10^{15}\) is one of the strongest affinities known. The extraordinarily high affinity of avidin for biotin can be attributed to the intricate and elegant composition of the avidin-binding site. Avidin is a tetrameric glycoprotein composed of four identical subunits. Each avidin molecule is capable of binding four biotin molecules. The carrier used for this purpose was a biotinylated poly (lactic acid), poly (ethylene glycol) copolymer (PLA-PEG-biotin) (Brannonpeppas, 1995; Cannizzaro et al., 1998) (Figure 1.3). PLA is the hydrophobic portion of the polymer, while PEG is hydrophilic. When dissolved in water, PEG collects at the water interface, while PLA forms the inner core of the microsphere. Due to the amphiphilic nature of this diblock polymer, the use of surfactants is avoided (Gref, 1994; Shakesheff et al., 1997). Use of surfactants has been known to decrease adsorption of ligand on the microsphere, as it competes with the ligand for adsorption sites on the
microsphere surface (Cannizzaro et al., 1998; Dickerson et al., 2001). PEG also camouflages the microspheres diminishing their uptake by the macrophages of the reticuloendothelial system (RES), and thus increases their circulation-time (Gref, 1994; Shakesheff et al., 1997). Neutravidin, which is the source of avidin, with its carboxylic end removed, acts as a bridge connecting the biotinylated polymer to the biotinylated ligand. Texas red (excitation wavelength – 589 nm; emission wavelength – 615 nm) on the Neutravidin is the fluorescent marker (Figure 1.4).

Figure 1.3: Molecular structure of PLA-PEG-biotin copolymer
Figure 1.4: Schematic representation of conjugation of ligand to PEGylated PLA
2 MATERIALS AND METHODS

Medium 199, Phosphate buffered saline (PBS) without Ca\(^{2+}\) and Mg\(^{2+}\) and Hank’s balanced salt solution (HBSS) with (HBSS+) or without Ca\(^{2+}\) and Mg\(^{2+}\) were obtained from Biowhittaker (Walkersville, MD). FBS was obtained from Hyclone (Urem, UT). Recombinant human interleukin -1 \(\beta\) (IL-1\(\beta\)) was obtained from Calbiochem (San Diego, CA). Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). BSA was added to PBS to make a PBS + 1% BSA buffer, pH 7.40. BSA was added to HBSS+ to make a HBSS + 1% BSA buffer, pH 7.40. This is referred to as blocking buffer. Biotinylated anti-human monoclonal antibody (mAb) CD62E (anti E-selectin) and biotinylated mouse IgG\(_{1}\) were obtained from BD Pharmingen (San Diego, CA). Biotinylated anti-human mAb CD54 (anti ICAM-1) was obtained from Calbiochem-Novabiochem Corporation (San Diego, CA). Neutravidin Texas Red was obtained from Molecular Probes (Eugene, OR). Poly (lactic acid) poly (ethylene glycol) biotin microspheres were generously provided by Dr. Kevin Shakesheff and Dr. Ali Salem (University of Nottingham, Nottingham, UK).

2.1 Cell Culture

Chinese hamster ovary (CHO) cells stably expressing E-selectin and parental CHO cells were a generous gift of Dr. Raymond T. Camphausen (Genetics Institute, Cambridge, MA). The CHO and CHO-E cell lines were cultured in Alpha media containing 10% dialyzed FBS as described previously (Sako et al., 1993). Human
umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA) and maintained in culture as described previously (Goetz et al., 1997). For induction of adhesion molecule expression (E-selectin and ICAM-1), HUVEC were activated by 4 hour treatment with 50 µg/ml IL-1β (Bevilacqua et al., 1987).

2.2 Ligand-Coated Microsphere Preparation

Biodegradable PLA-PEG-biotin microspheres (average size: 8 µm) at a concentration of 6.67 mg/ml were mixed with a 100 µg/ml solution of Neutravidin-Texas red in PBS at a ratio of 1:1 by volume and incubated at 37°C for 20 minutes. Microspheres were washed with PBS + 1% BSA. The wash consisted of spinning the tube for 6 minutes at a maximum force of 16,000 g (1400 rpm). Microspheres were then diluted to a concentration of 5*10^7 microspheres/ml. Microspheres were then incubated at room temperature for 30 minutes with 0.025 mg/ml biotinylated anti-human monoclonal antibody (anti E-selectin or anti ICAM-1) or 0.025 mg/ml human IgG1 diluted in blocking buffer. The microspheres coated with the ligand were washed in PBS + 1% BSA. Prior to use in assays, microspheres were diluted to a concentration of 6 x 10^5 microspheres/ml using HBSS + 1% BSA + 2% heat-treated FBS and incubated for 15 min at 37°C. Microspheres were then passed through the flow chamber. HBSS + 1% BSA was used as the buffer.

For assays with varying mAb (anti E-selectin or anti ICAM-1) concentration, treatment of the PLA-PEG-biotin microspheres with Neutravidin-Texas Red was as described above. Microspheres were then incubated at room temperature for 30 minutes
with biotinylated anti-human monoclonal antibody (anti E-selectin or anti ICAM-1) at the following concentrations of the antibody: 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml, 0.005 mg/ml, 0.0025 mg/ml. The microspheres coated with the ligand were washed in PBS + 1%BSA. Prior to use in assays, microspheres were diluted to 6 x 10⁵ microspheres/ml using HBSS + 1% BSA + 2% heat-treated FBS and incubated for 15 min at 37°C. Microspheres were then passed through the flow chamber. HBSS + 1% BSA was used as the buffer.

2.3 Adhesion of Microspheres to Cellular Substrates under Fluid Dynamic Conditions

The parallel plate flow chamber (Glycotech, Rockville, MD) used for these studies has been described previously (Crutchfield et al., 2000) (Figure 2.1). Cellular monolayers were grown on 35 mm tissue culture dishes and inserted into the flow chamber. The flow field is defined by a gasket, which sits between the flow deck and the 35 mm dish. The shear stress at the bottom surface of the flow chamber is given by \( \tau = \frac{3Q}{\mu} \frac{2wh^2}{2wh} \) where \( Q \) is the volumetric flow rate, \( \mu \) is the viscosity, \( 2h \) is the height (0.2 mm) of the flow field, and \( w \) is the width (0.5 cm) of the flow field. The volumetric flow rate was adjusted to obtain the shear stress ranging between 1.5 dyne/cm² and 5 dyne/cm². The flow chamber was mounted on an inverted microscope (Nikon Eclipse TE300). For the studies with the microspheres, the microscope was equipped with a CCD-330T RC camera (Dage
MTI, Michigan city, IN), which was directly in line with a VCR (SSVO-9500MD, Sony Corp., Japan). The microsphere adhesion assays were carried out as follows. Microspheres (6 x 10^5 microspheres/ml) in blocking buffer (HBSS + 1% BSA) were drawn through the flow chamber at a volumetric flow rate that gave the desired shear stress at the cell monolayer-fluid interface. The entire perfusion period was recorded onto videotape for later off-line analysis. The number of microspheres interacting with the

\[
\tau = \frac{3 \cdot Q \cdot \mu}{2 \cdot w \cdot h^2}
\]
cellular monolayers was determined for eight different fields of view between 2.5 and 3 minutes after initiation of microsphere perfusion. These numbers were averaged and normalized to the area of the field of view to give $n = 1$. Experiments were carried out at $37^\circ C$.

2.4 Statistics

Differences between the means of groups were evaluated using analysis of variance (ANOVA). The Bonferroni Method was used to test the comparisons. The means of the groups were compared with the mean of a particular group and p-values $\leq 0.05$ were considered significant. All error bars represent standard deviations.
3 RESULTS

3.1 Biotinylated Anti-human E-selectin Coated PLA-PEG-Biotin Microspheres Attach to Transfected Cells Stably Expressing E-selectin (CHO-E)

The first step was to determine whether the biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres show specific adhesion to E-selectin. For this purpose, Chinese hamster ovary cells stably expressing E-selectin (CHO-E) were used. Biotinylated anti-human E-selectin (or mouse IgG) coated PLA-PEG-biotin microspheres were perfused over CHO-E (or CHO) cells at 1.5 dynes/cm$^2$. The microspheres were loaded with ligand at a concentration of 25 µg/ml. The anti-E-selectin coated microspheres exhibited significant levels of attachment to the CHO-E cells. The microspheres traveled at the free stream hydrodynamic velocity, abruptly attached to the CHO-E cell monolayer and subsequently remained firmly adhered to the monolayer with no rolling observed. The level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to the CHO-E cells at 1.5 dynes/cm$^2$ was significantly greater when compared to the level of attachment of biotinylated mouse IgG coated PLA-PEG-biotin microspheres to CHO-E cells or the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to untransfected CHO cells (Figure 3.1).
Figure 3.1: Biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres exhibit specific adhesion to CHO-E cells at a shear stress of 1.5 dyne/cm$^2$. The level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to CHO-E cells was significantly greater than the level of attachment of biotinylated mouse IgG coated PLA-PEG-biotin microspheres to CHO-E cells and also significantly greater than the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to CHO cells. Legend: Substrate indicates cell monolayer, CHO-E = cells expressing E-selectin, CHO = untransfected cells; Ligand indicates which molecule was coupled to the PLA-PEG-biotin microspheres, Anti E = biotinylated anti-human E-selectin, IgG = mouse IgG; n=3; * indicates p<0.05 compared to the bar showing anti-E adhesion to CHO-E as per ANOVA test using Bonferroni Method.
3.2 Biotinylated Anti-human E-selectin Coated PLA-PEG-Biotin Microspheres

Attach to 4hr IL-1β Activated HUVEC

After testing the specific attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to E-selectin expressed on CHO-E cells, the next step was to determine their specificity of attachment to cytokine activated endothelium. For this purpose HUVEC were used, since they are arguably the best characterized *in vitro* model of the human endothelium. Previous studies have shown that 4 hr IL-1β activated HUVEC express high levels of E-selectin (Bevilacqua et al., 1987). Biotinylated anti-human E-selectin (or mouse IgG) coated PLA-PEG-biotin microspheres were perfused over 4hr IL-1β activated HUVEC (or unactivated HUVEC) at 1.5 dyne/cm². The microspheres were loaded with ligand at a concentration of 25 µg/ml. The anti-E-selectin coated microspheres exhibited significant levels of attachment to the 4hr IL-1β activated HUVEC. The microspheres traveled at the free stream hydrodynamic velocity, abruptly attached to the 4hr IL-1β activated HUVEC monolayer and subsequently remained firmly adhered to the monolayer with no rolling observed. The level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to the 4hr IL-1β activated HUVEC at 1.5 dyne/cm² was significantly greater when compared to the level of attachment of biotinylated mouse IgG coated PLA-PEG-biotin microspheres to 4hr IL-1β activated HUVEC or the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to unactivated HUVEC (Figure 3.2).
Figure 3.2: Biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres show specific adhesion to 4hr IL-1β activated HUVEC at 1.5 dyne/cm². The level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to 4hr IL-1β activated HUVEC was significantly greater than the level of attachment of biotinylated mouse IgG coated PLA-PEG-biotin microspheres to 4hr IL-1β activated HUVEC and significantly greater than the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to unactivated HUVEC. Legend: Substrate indicates cell monolayer, A HUVEC = 4hr IL-1β activated HUVEC, U HUVEC = unactivated HUVEC; Ligand indicates which molecule was coupled to the PLA-PEG-biotin microspheres, Anti E = biotinylated anti-human E-selectin, IgG = mouse IgG; n>3; * indicates p<0.05 compared to the bar showing anti-E adhesion to activated HUVEC as per ANOVA test using Bonferroni Method.
3.3 Biotinylated Anti-human E-selectin Coated PLA-PEG-Biotin Microspheres Show Decreasing Attachment to E-selectin Expressed on 4h IL-1β Activated HUVEC with Decreasing Concentration of anti-E-selectin used to Coat the Microspheres

The next step was to determine the dependence of ligand coating concentration on microsphere adhesion to cytokine activated HUVEC. For this, biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres were prepared with varying concentrations (50, 25, 10, 5, 2.5 μg/ml) of biotinylated anti-human E-selectin. Biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres were perfused over 4h IL-1β activated HUVEC (or unactivated HUVEC) at 1.5 dynes/cm². The microspheres exhibited significant levels of attachment to the 4h IL-1β activated HUVEC. A Student T-test showed that the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to the 4h IL-1β activated HUVEC at 1.5 dyne/cm² was significantly greater than the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to unactivated HUVEC at all concentrations of mAb. The level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to 4h IL-1β activated HUVEC at 1.5 dyne/cm² was observed to decrease with decreasing concentration of mAb used to prepare the microspheres (Figure 3.3). The level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to unactivated HUVEC was very low and almost constant for all concentrations of the mAb. The selectivity (ratio of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres that bind to 4h IL-1β activated
HUVEC to those that bind to unactivated HUVEC) was thus, a function of concentration of mAb added to the microspheres (Figure 3.4).

Figure 3.3: Biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres show decreasing attachment to 4h IL-1\(\beta\) activated HUVEC with decreasing concentration of mAb used to coat the microspheres. The microspheres were prepared with mAb concentrations of 50, 25, 10, 5, 2.5 µg/ml. The level of attachment of biotinylated anti-human E-selectin PLA-PEG-biotin microspheres to 4h IL-1\(\beta\) activated HUVEC at 1.5 dyne/cm\(^2\) was significantly greater than the level of attachment of biotinylated anti-human E-selectin PLA-PEG-biotin microspheres to unactivated HUVEC at all mAb concentrations (by T-test - not shown). ANOVA indicates that attachment specificity is a function of the concentration of mAb. n>3; * indicates p<0.05 compared to 25 µg/ml bar of activated HUVEC using Bonferroni Method.
Figure 3.4: The selectivity of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres for E-selectin is a function of the concentration of mAb. With decreasing ligand densities, the selectivity decreases. n>3;
3.4 Biotinylated Anti-human E-selectin Coated PLA-PEG-Biotin Microspheres Show Decreasing Attachment to E-selectin Expressed on 4h IL-1β Activated HUVEC with Increasing Shear Stress

The next step was to determine the dependence of shear on attachment of anti-human E-selectin coated PLA-PEG-biotin microspheres to E-selectin expressed on 4h IL-1β activated HUVEC. For this, biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres were prepared at a concentration of 25 µg/ml of the mAb. Biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres were perfused over 4h IL-1β activated HUVEC (or unactivated HUVEC) at shear stresses ranging from 1.5 to 3 dynes/cm². The microspheres exhibited significant levels of attachment to the 4h IL-1β activated HUVEC only at a low shear stress of 1.5 dyne/cm². A Student T-test showed that the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to the 4h IL-1β activated HUVEC was significantly greater than the level of attachment of biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres to unactivated HUVEC at all shear stresses (Figure 3.5).
Figure 3.5: Biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres with a ligand concentration of 25 μg/ml showed significant attachment to 4h IL-1β activated HUVEC only at a low shear stress of 1.5 dyne/cm². These microspheres were perfused over HUVEC monolayers at shear stresses ranging from 1.5 to 3 dyne/cm². The level of attachment of biotinylated anti-human E-selectin PLA-PEG-biotin microspheres to 4h IL-1β activated HUVEC was significantly greater than the level of attachment of biotinylated anti-human E-selectin PLA-PEG-biotin microspheres to unactivated HUVEC at all shear stresses (by T-test – not shown). ANOVA results show that the attachment efficiency of the microspheres to activated HUVEC decreases with increasing shear stress. n>3; * indicates p<0.05 compared to 1.5 dyne/cm² bar of corresponding HUVEC as per Bonferroni Method.
3.5 Biotinylated Anti-human ICAM-1 Coated PLA-PEG-Biotin Microspheres

Attach to ICAM-1 Expressed on 4h IL-1β Activated HUVEC

The next step was to determine whether the biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres show specific adhesion to cytokine activated HUVEC. To test this, HUVEC were used. 4h IL-1β activated HUVEC express high levels of ICAM-1. Biotinylated anti-human ICAM-1 (or mouse IgG) coated PLA-PEG-biotin microspheres were perfused over 4h IL-1β activated HUVEC (or unactivated HUVEC) at 1.5 dynes/cm$^2$. The microspheres were treated with a ligand concentration of 25 µg/ml. The anti-ICAM-1 coated microspheres exhibited significant levels of attachment to the 4h IL-1β activated HUVEC. The microspheres traveled at the free stream hydrodynamic velocity, abruptly attached to the 4h IL-1β activated HUVEC monolayer and subsequently remained firmly adhered to the monolayer with no rolling observed. The level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to the 4h IL-1β activated HUVEC at 1.5 dyne/cm$^2$ was significantly greater when compared to the level of attachment of biotinylated mouse IgG coated PLA-PEG-biotin microspheres to 4h IL-1β activated HUVEC or the level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to unactivated HUVEC, which itself was significant since HUVEC have a basal level of expression of ICAM-1 even when unactivated (Figure 3.6).
Figure 3.6: Biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres show good specific adhesion to 4h IL-1β activated HUVEC at 1.5 dyne/cm². The level of attachment of biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres to 4h IL-1β activated HUVEC was significantly greater than the level of attachment of biotinylated mouse IgG coated PLA-PEG-biotin microspheres attachment to 4h IL-1β activated HUVEC and significantly greater than the level of attachment of biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres to unactivated HUVEC. Legend: Substrate indicates cell monolayer, A HUVEC = activated HUVEC, U HUVEC = unactivated HUVEC; Ligand indicates which molecule was coupled to the PLA-PEG-biotin microspheres, Anti ICAM-1 = biotinylated anti-human ICAM-1, IgG = mouse IgG; n>3; * indicates p<0.05 compared to the bar showing anti-ICAM-1 adhesion to activated HUVEC as per ANOVA test using Bonferroni Method.
3.6 Biotinylated Anti-human ICAM-1 Coated PLA-PEG-Biotin Microspheres Show Slightly Decreasing Attachment to ICAM-1 Expressed on 4h IL-1β Activated HUVEC with Decreasing Concentration of Ligand

The next step was to determine the dependence of concentration of ligand used to coat the microspheres on the specificity of attachment to ICAM-1. For this, biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres were prepared with varying concentrations (50, 25, 10, 5, 2.5 µg/ml) of biotinylated anti-human ICAM-1. Biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres were perfused over 4h IL-1β activated HUVEC (or unactivated HUVEC) at 1.5 dynes/cm². The microspheres exhibited significant levels of attachment to the 4h IL-1β activated HUVEC. A Student T-test showed that the level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to the 4h IL-1β activated HUVEC at 1.5 dyne/cm² was significantly greater than the level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to unactivated HUVEC at all concentrations of mAb. However, ANOVA test showed that the level of attachment was not a function of mAb concentration (Figure 3.7). A significant decrease in attachment to activated HUVEC compared to the attachment to activated HUVEC at an mAb concentration of 25 µg/ml was observed only at 2.5 µg/ml. Compared to the selectivity observed for E-selectin (Figure 3.4), the selectivity for ICAM-1 did not show a dependence on mAb concentration (Figure 3.8).
Figure 3.7: Biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres show slightly decreasing attachment to 4h IL-1β activated HUVEC at 1.5 dyne/cm² with decreasing mAb concentration, mostly at lower concentrations. The microspheres were prepared with mAb concentrations of 50, 25, 10, 5, 2.5 µg/ml. The level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to 4h IL-1β activated HUVEC was significantly greater than the level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to unactivated HUVEC at all mAb concentrations (T-test - not shown). ANOVA test showed that the level of attachment was not a function of mAb concentration. n>3; * indicates p<0.05 compared to 25 µg/ml bar of activated HUVEC by Bonferroni method.
Figure 3.8: The selectivity of attachment (ratio of biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres that bind to 4h IL-1β activated HUVEC to those that bind to unactivated HUVEC) was not a function of the mAb concentrations.
3.7 Biotinylated Anti-human ICAM-1 Coated PLA-PEG-Biotin Microspheres Show Decreasing Attachment to ICAM-1 Expressed on 4h IL-1β activated HUVEC with Increasing Shear Stress

The next step was to determine the dependence of shear on attachment of anti-human ICAM-1 coated beads to ICAM-1 expressed on 4h IL-1β activated HUVEC. For this, biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres were prepared at a concentration of 25 µg/ml of the mAb. These microspheres were perfused over 4h IL-1β activated HUVEC (or unactivated HUVEC) at shear stresses ranging from 1.5 to 5 dynes/cm². The microspheres exhibited significant levels of attachment to the 4h IL-1β activated HUVEC. A Student T-test showed that the level of attachment of biotinylated anti-human ICAM-1 coated PLA-PEG-biotin microspheres to the 4h IL-1β activated HUVEC was significantly greater when compared to their level of attachment to unactivated HUVEC at all shear stresses. The level of attachment of biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres to 4h IL-1β-activated HUVEC was observed to be decreasing with an increase in shear stress, and was thus a function of shear stress, as indicated by the ANOVA test (Figure 3.9).
Figure 3.9: Biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres show decreasing attachment to 4h IL-1β activated HUVEC with increasing shear stress. Biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres with a ligand concentration of 25 µg/ml were perfused over HUVEC monolayers at shear stresses ranging from 1.5 to 5 dyne/cm². The level of biotinylated anti-human ICAM-1 PLA-PEG-biotin microsphere attachment to 4h IL-1β activated HUVEC was significantly higher than the level of biotinylated anti-human ICAM-1 PLA-PEG-biotin microsphere attachment to unactivated HUVEC at all shear stresses (T-test – not shown). The level of attachment of biotinylated anti-human ICAM-1 PLA-PEG-biotin microspheres to 4h IL-1β-activated HUVEC was a function of shear stress, as indicated by the ANOVA test. n=3; * indicates p<0.05 compared to 1.5 dyne/cm² bar of corresponding activated or unactivated HUVEC as per Bonferroni Method.
4 DISCUSSION

The goal of this study was to determine the specificity of attachment of a biotinylated anti-human mAb to E-selectin (or a biotinylated anti-human mAb to ICAM-1) coated biotinylated poly (lactic acid) poly (ethylene glycol) copolymer microspheres to cellular expressed E-selectin or ICAM-1. Neutravidin, coupled with a fluorescent marker Texas Red, was used as a bridge to link the PLA-PEG-biotin microspheres to the biotinylated ligand utilizing the strong affinity between avidin and biotin.

Since drug carrier adhesion to the endothelium in vivo will occur in a fluid dynamic environment, the in vitro adhesion experiments were carried out under fluid dynamic conditions using a parallel-plate flow chamber, which mimics, in part, the flow conditions present in vivo.

Under the fluid dynamic in vitro flow conditions, biotinylated anti-human E-selectin coated PLA-PEG-biotin microspheres exhibited approximately a twenty-fold increase in the level of adhesion to CHO-E cells compared to their level of adhesion to CHO cells as well as compared to the level of adhesion of Mouse IgG coated PLA-PEG-biotin microspheres to both CHO-E and CHO cells. Thus the mAb-coated microspheres did show a high specificity for E-selectin. Therefore the next step was to test their specificity for E-selectin expressed on human endothelium. HUVEC were used for this purpose for reasons described previously. The biotinylated anti-human E-selectin coated microspheres exhibited an approximately 10-fold increase in attachment to 4h IL-1β activated HUVEC compared to their attachment to unactivated HUVEC or compared to the attachment of mouse IgG coated microspheres to both activated and unactivated
HUVEC. Further testing using a mAb to ICAM-1 on the microspheres also showed a 2-fold increase in adhesion to 4h IL-1β activated HUVEC compared to its adhesion to unactivated HUVEC. This decrease in specificity for ICAM-1 can be attributed to the basal level of ICAM-1 expressed by unactivated HUVEC. These results strongly suggest that PLA-PEG-biotin microspheres coated with a specific mAb to a cytokine inducible ECAM show specific augmented adhesion to endothelium expressing the ECAM. These polymeric microspheres can thus be used as potential biodegradable drug carriers for targeted drug delivery.

To put these observations in perspective, the attachment efficiency of the biotinylated anti-human E-selectin coated microspheres over 4h IL-1β activated HUVEC were compared with the attachment efficiency of neutrophils. Based on work of Alon et al. (Alon et al., 1996; Dickerson et al., 2001), it was estimated that 110 neutrophils/mm² would attach directly to a 4h IL-1β activated HUVEC monolayer at 1.8 dyne/cm² over a 2.5 min perfusion period with the concentration of neutrophils in the perfusion buffer being 5*10⁵/ml. Normalizing it to the conditions of the current experiment (3 min of perfusion at a biotinylated anti-human E-selectin coated microsphere concentration of 6*10⁵/ml), this translates to a neutrophil attachment of 158 adherent neutrophils/mm².

Now, the number of biotinylated anti-human E-selectin coated microspheres that attach to 4h IL-1β activated HUVEC at 1.5 dyne/cm² is 32.1 microspheres/mm². Thus, the rate of attachment of the biotinylated anti-human E-selectin coated microspheres is 20% of the rate of neutrophil attachment.
Previously, Dickerson et al. (Dickerson et al., 2001) used microspheres made from the biodegradable polymer, poly (ε-caprolactone) (PCL), using PVA as a stabilizer. HuEP5C7.g2, a mAb to both E- and P-selectin, was adsorbed onto the microsphere surface. These HuEP5C7.g2 coated microspheres showed an attachment rate of just 0.17% of the rate of neutrophil attachment even at a reduced shear stress of 0.3 dyne/cm². Thus, mAb coated PLA-PEG-biotin microspheres show greater attachment efficiency than mAb coated PCL microspheres. This, along with the fact that PEG may increase the circulation time of the particle, makes PLA-PEG-biotin microspheres better candidates as drug carriers for targeted drug delivery.

The next parameter tested was the dose response, i.e., the effect of varying ligand concentration used to prepare the microspheres on specificity of attachment of the biotinylated anti-human E-selectin (or ICAM-1) coated microspheres to E-selectin (or ICAM-1) expressed on endothelial cells. In the case of E-selectin, the number of microspheres that attached was clearly a function of the concentration of the mAb used to coat the microspheres. With decreasing mAb concentration, the number of microspheres that attached decreased. At lower mAb concentrations (5 µg/ml and 2.5 µg/ml) the difference was not as significant as with the higher concentrations (50 µg/ml, 25 µg/ml and 10 µg/ml). The microspheres also showed higher selectivity for E-selectin with increasing mAb concentration as is apparent from Figure 3.4. However, in the case of ICAM-1, there was no significant decrease in the level of attachment of mAb-coated microspheres with decrease in mAb concentration used to coat the microspheres. This could either be due to the fact that microspheres were saturated with mAb to ICAM-1 at a
low mAb concentration of 2.5 µg/ml or that a low concentration of mAb is sufficient for maximum attachment to ICAM-1 present on the endothelium.

Since a drug carrier designed to target the endothelium would interact with the blood in a fluid dynamic environment, adhesive mechanics (Hammer and Apte, 1992) suggests that mAb coated microsphere adhesion to the cellular monolayers would be a function of the level of fluid shear. To investigate this possibility, we perfused biotinylated anti-human E-selectin (or biotinylated anti-human ICAM-1) coated microspheres over IL-1β activated HUVEC at varying shear stresses. For anti-human E-selectin coated microspheres, the adhesion decreased considerably to a very low value with an increase in shear stress even to just 2 dyne/cm², and did not change much thereafter. Thus, significant attachment was observed only at 1.5 dyne/cm² and became negligible at just 3 dyne/cm². In the case of anti-human ICAM-1, the decrease in attachment with an increase in shear stress was more gradual and became negligible only at a high value of 5 dyne/cm². Thus, PLA-PEG-biotin microspheres coated with a mAb to ICAM-1 show significant attachment, and possibly saturation of attachment sites, even at a lower mAb concentration and significantly more attachment at a higher shear stress as compared to microspheres coated with a mAb to E-selectin. However, the drawback with targeting ICAM-1 is that the endothelium expresses a significant level of ICAM-1 even when unactivated, and thus the selectivity of targeting is reduced.

Although the developed biodegradable drug carrier shows good specificity of attachment to targeted receptors in vitro, it clearly has limitations when applied to in vivo conditions. First is the particle size. This is an important issue, since the microspheres
have to be small enough to be able to pass through the capillaries, the approximate diameter of which is 5 µm. Thus, the 8 µm particles we have used cannot be used in vivo. For this purpose, particles of 2 µm or smaller have to be tested for specificity. But during formulation, smaller particle sizes require an increased level of surfactant (Shakesheff et al., 1997). Thus, an optimization would have to be done with regards to particle size and amount of surfactant.

A second issue is related to how well the in vitro flow model simulates the conditions present in vivo. Human blood is a complex mixture of various organelles like erythrocytes, leukocytes, platelets, etc. Blood has a high concentration of erythrocytes, whose presence has shown to significantly alter the adhesion of leukocytes to the endothelium (Melder et al., 1995). Thus, the erythrocytes would also affect the adhesion of the microspheres to the endothelium. Another complexity is the varying flow profile in blood, as compared to the laminar flow in the in vitro model. Blood is actually a non-Newtonian fluid with a complex flow profile, especially within the diseased vasculature. For example, the microvasculature of tumors has been observed to be strikingly different from that of normal tissue (Less et al., 1991). Also, the shear rates in vivo would be dependent on the target location of the drug carriers.

These are just a few examples of the differences between in vitro and in vivo flow conditions. In spite of these, and other shortcomings, the simple shear model devised gives a much more detailed insight into the biophysics of drug carrier adhesion to the endothelium in vivo under flow then what could be achieved by a simple static assay
without consideration of the presence of flow. A way to circumvent this problem would be to test smaller particles in vivo using models described previously (Burch et al., 2002).

In summary, the specificity of adhesion of PLA-PEG-biotin microspheres coated with a mAb to either E-selectin or ICAM-1 has been studied. From the results obtained it can be concluded that anti-E-selectin coated microspheres have a high efficiency of attachment when compared to neutrophils under similar flow conditions. Also, specificity of adhesion is a strong function of concentration in case of E-selectin, while for ICAM-1, decrease in concentration of mAb did not show any significant decrease in the level of attachment. This could be either due to the microspheres being concentrated with the mAb at a low concentration of mAb or a low mAb loading is sufficient for maximum attachment. The results also demonstrate that the fluid shear can significantly influence the interactions of the particle with the endothelium. Thus the microspheres coated with the mAb could be used in a fluid dynamic environment to target endothelial cell monolayers expressing the receptor of interest.
5 REFERENCES


