DEVELOPMENT OF TARGETING NANOPARTICLES MIMICKING THE
ADHESIVE PROPERTIES OF PLATELETS

by

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ABSTRACT

DEVELOPMENT OF NANOPARTICLES MIMICKING THE ADHESIVE PROPERTIES OF PLATELETS

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The development of biodegradable nanoparticles as drug delivery vehicles presents an improved avenue for intracellular targeted drug delivery. Biodegradable nanoparticles have demonstrated an ability to provide controllable, sustained drug release in vitro. However, in vivo studies have shown that nanoparticles are not effective at adhering to vascular walls under shear stress. The purpose of this study was to investigate methods to improve cellular uptake and targeting of nanoparticles in activated or inflamed endothelial cells (ECs) under fluid shear stress and to determine whether the material properties of a biodegradable polymer, poly (lactic-co-glycolic) acid (PLGA), affected cellular uptake. The hypothesis for this project was that by mimicking the binding of platelets with activated ECs (glycoprotein Ibα (GP Ibα)-P-
selectin), GP Ibα-conjugated nanoparticles could exhibit increased targeting and higher cellular uptake in injured or activated endothelial cells under physiological flow conditions. To test this hypothesis, carboxyl polystyrene nanoparticles loaded with green fluorescent dyes were selected as a model particle. Using confocal microscopy, the study found that conjugation of 100 nm polystyrene nanoparticles with GP Ibα significantly increased cellular uptake and targeting under fluid shear stress. To develop therapeutic carriers, biodegradable nanoparticles were developed from PLGA using a standard double emulsion technique. Using microscopy, fluorescent measurement, and protein assays, similar cellular uptake properties were observed for 100 nm PLGA and polystyrene nanoparticles, suggesting that the uptake properties of these nanoparticles in ECs were not strongly affected by their material properties. The study also found that PLGA nanoparticles were able to provide sustained drug release for at least 14 days. Preliminary results from this project demonstrate that our novel platelet-mimicking nanoparticles may be the first step towards developing a targeted, sustained, drug delivery system, with the ability to overcome shear regulated cellular uptake.
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LIST OF ABBREVIATIONS

PCI .......................................................... Percutaneous Coronary Intervention
CABG .................................................. Coronary Artery Bypass Graft
FDA .................................................. Food and Drug Administration
PLA .................................................. Poly Lactic Acid
PGA .................................................. Poly Glycolic Acid
PLGA .............................................. Poly (lactic-co-glycolic) Acid
O .................................................. Oil Phase
W .................................................. Water Phase
W/O/W ............................................. Water-Oil-in-Water
RES ............................................. Reticular Endothelial System
VSMC ........................................ Vascular Smooth Muscle Cell
Caco-2 ........................................ Human Colon Adenocarcinoma Cell Line
EC ................................................ Endothelial Cell
EDC .............................................. 1-ethyl-3-(3-dimethylaminopropyl)

Carbodiimide
NHS ............................................... N-Hydroxysuccinimide
ECAM ............................................. Endothelial Cell Adhesion Molecule
GP ............................................... Glycoprotein
PSGL-1 ........................................... P-Selectin Glycoprotein Ligand-1
mAbs ......................................... Mouse Antibodies
sLe\(^x\) ...................................... Sialyl-Lewis\(^x\)
vWF .......................................... Von Willebrand Factor
ADP .......................................... Adenosine Diphosphate
VCAM ....................................... Vascular Cell Adhesion Molecule
ICAM ....................................... Intercellular Adhesion Molecule
ECM .......................................... Extracellular Matrix
BSA .......................................... Bovine Serum Albumin
PVA .......................................... Polyvinyl Alcohol
LSGS ......................................... Low Serum Growth Supplement
PBS .......................................... Phosphate Buffered Saline
DI ............................................ Deionized
W/V .......................................... Weight by Volume
SEM .......................................... Scanning Electron Microscope
FITC .......................................... Fluorescein Isothiocyanate
RITC .......................................... Rhodamine
MWCO ...................................... Molecular Weight Cut-off
MES .......................................... 2-Morpholinoethanesulfonic Acid
IgG .......................................... Immunoglobulin G
CHAPTER 1
INTRODUCTION

1.1 Cardiovascular Disease

Cardiovascular disease is the leading cause of death for both men and women in the United States today, accounting for almost one million deaths and six million hospitalizations annually, according to the Center for Disease Control and Prevention. The two most common types of cardiovascular disease, heart attack and stroke, account for almost 40% off all annual deaths in the United States [1, 2]. The economic burden associated with those suffering from cardiovascular disease was projected to be over $400 billion for 2006. The depressing aspect of this economic and human devastation is that cardiovascular disease is largely preventable, with most cases associated with unhealthy lifestyles and general ignorance. Mankind’s gradual aggrandizement and industrialization has led to largely sedentary lifestyles accompanied by unhealthy diets, and unless overwhelming social change occurs, no amount of treatment or medicine can reverse the decline of health in society as a whole.

1.1.1 Current Treatments for Cardiovascular Disease

Current treatments for cardiovascular disease consist of drug therapy and surgical procedures, depending on the particular affliction a patient is suffering from. Based on the degree of the arterial occlusion, treatment can involve the use of stents or angioplasty, in which a catheter is guided to the obstruction and then expanded using
hydraulic pressure [3-5]. This type of treatment, known as percutaneous coronary intervention (PCI), is much less invasive than traditional coronary bypass graft (CABG) surgery [6, 7]. CABG involves the removal of a major superficial vein, typically the saphenous vein located in the leg, and relocation of the vein to the site of occlusion, and is still preferred for cases of multivessel coronary artery disease and severe vessel occlusion [8, 9]. Non-surgical treatments for cardiovascular diseases include drug-based therapies consisting of anticoagulants, antiplatelet agents, beta blockers, vasodilators, and statins [10]. Specifically, drugs used to treat cardiovascular inflammation include cyclooxygenase inhibitors, peroxisome proliferator-activated receptors agonists, aspirin, and lipid-lowering agents [11].

1.1.2 Problems with Current Treatments for Cardiovascular Disease

Though current treatments for cardiovascular disease are effective in reducing the risk or major cardiac failure, there are still limitations and obstacles that need to be overcome in order to further improve the current therapies. For example, in angioplasty, balloon expansion at the site of occlusion can lead to vessel wall injury and trigger the inflammatory response. This generates post-treatment issues such as elastic recoil and restenosis, reducing the effectiveness of this procedure [12, 13]. In addition, stenting is performed in over 80% of patients undergoing percutaneous coronary revascularization in the United States, but of these patients, over 15% will have to undergo further coronary intervention due to in-stent restenosis [5, 11]. Recent Food and Drug Administration (FDA) approval of drug eluting stents has shown great promise in reducing the incidence of restenosis, and may prove to be another approach
towards resolving cardiovascular disease [14]. Furthermore, a major issue involved with treatment of cardiovascular diseases using systemic drug delivery is that drugs meant to target the cardiovascular system may instead be delivered to the liver, kidney, or other visceral organs, causing adverse effects such as inadequate dosage for the diseased tissue, or systemic toxicity involving healthy tissue [15]. Development of targeting drug delivery carriers is an appealing strategy to overcome the side effects and increase the effectiveness of drug delivery particulate systems.

1.2 Drug Delivery Particulate Systems

1.2.1 Characteristics of an Ideal Targeted Drug Delivery System

An ideal targeted drug delivery system relies on interaction with specific physiological receptors and physical attachment of the system onto the desired target in order to achieve site-specific drug delivery [16]. The targeted drug delivery system should also be able to deliver drug of high efficacy to the desired location at sufficient dosage, while also maintaining this therapeutic dosage for the desired amount of time [17]. Another quality of an ideal system should be the ability to avoid clearance by the immune system when delivered to the patient via systemic circulation [18]. After drug delivery is completed, the system should be resorbable, leaving the patient free of any residual material.

1.2.2 Nanoparticles for Drug Delivery

Recently, nanoparticles (1-1000 nm) have been proposed for use as potential therapy carriers for various diseases, ranging from cancer to cardiovascular disease. Drug can either be loaded inside the nanoparticles or attached to the surfaces of the
nanoparticles. In addition, materials to formulate nanoparticles may be biodegradable or non-biodegradable, depending on the desired application. When nanoparticles are placed in vivo, they should not generate any kind of immunogenic response, with the same criteria applying to the degradation product of biodegradable nanoparticles. Material selection plays a large role in the properties and characteristics of nanoparticles, and should be considered closely before designing any kind of nanoparticle-based drug delivery system.

1.2.3 Polymers Used to Formulate Nanoparticles

Polymers used in nanoparticle formulation can be divided into two main categories: natural and synthetic polymers. Both types of polymers have various advantages and disadvantages, which will be discussed in the following sections.

1.2.3.1 Natural Polymers

Natural polymers possess material properties similar to those found within substances in the body, thereby provoking less toxicity than some synthetic materials [19]. Examples of these polymers include collagen, gelatin, and chitosan [20]. The advantages of using natural polymers are related to the fact that they are similar to those found in the body, and thus may function biologically on a molecular level, as opposed to a macroscopic level. The primary disadvantage of natural polymers is that their structure is complex, making manipulation of the polymer more difficult.

1.2.3.2 Synthetic Polymers - Biodegradable and Non-biodegradable Polymers

Synthetic polymers encompass a wide range of materials with varying mechanical, degradative, and biocompatible properties. For nanoparticles, polyesters
such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymers poly (lactic-co-glycolic acid) (PLGA), are commonly employed, due to their high mechanical strength and non-toxic degradative properties [21, 22]. They also have the advantage of already being FDA approved for certain applications such as biodegradable sutures, bone pins, and dental implants [23, 24]. Synthetic polymers are advantageous for use in nanoparticles because their material properties are well defined, and can be modified for different applications [22]. The disadvantages of synthetic materials involve foreign body responses due to the body’s innate reaction towards unrecognized substances.

Biodegradable polymers are advantageous because of their ability to be broken down after implantation within the body, resulting in elimination of the material over time. For instance, PLGA undergoes hydrolytic scission of the polymeric backbone and degrades to lactic and glycolic acid, which are then consumed as metabolites via the Kreb’s cycle [21]. For the most part, non-biodegradable polymers are used for modeling purposes because of their stable properties and uniform size distribution, thus non-biodegradable polymers still play an important role in drug delivery research because they provide an “ideal” functional guideline by which to judge an actual drug delivery system. For example, polystyrene was used in nanoparticles for modeling potential drug systems in a number of studies, to prove a proof of concept without concerning the influence of degradation on the particle and drug delivery properties.
1.2.3.3 Advantages of Synthetic Polymers over Natural Polymers

Synthetic polymers are ideally suited for therapeutic carrier applications because their drug delivery properties can be well controlled. Once the drug is loaded or attached to the particle, degradation rate plays a key role in determining the sustainability and amount of drug released. Additionally, synthetic polymers are advantageous over natural polymers because they are more easily modified, and in general, possess less structural complexity than natural substances [19]. Other advantages of synthetic polymers include: 1.) Synthetic polymers are easier to process than natural polymers because of their uniform mechanical properties, 2.) Copolymers can be easily formed using synthetic polymers, resulting in new polymers possessing divergent qualities, and 3.) Synthetic polymers are well characterized due to their wide use in industrial applications, leading to a more complete understanding of polymeric structure and properties [24].

1.2.4 Techniques to Formulate Biodegradable Nanoparticles

There are numerous methods for formulating biodegradable nanoparticles, with the two most commonly applied methods being evaporative emulsion and nanoprecipitation [25-27]. Each method has its advantages and disadvantages, and will be discussed further in the following sections. The key criteria in determining the effectiveness of formulation techniques are particle size and distribution, toxicity of materials used, reproducibility, surface morphology, surface chemistry, surface charge, drug encapsulation efficiency, drug release kinetics, and hemodynamic properties of the particle [17, 28].
1.2.4.1 Evaporative Emulsion for Nanoparticle Formulation [25]

Evaporative emulsion involves the use of a single or double emulsion to formulate nanoparticles. In this technique, synthetic polymer and water-insoluble drugs or fluorescent dyes, are dissolved in an organic solvent (oil phase, o), such as chloroform or dichloromethane, whereas a water-soluble drug is dissolved in water (water phase, w). The two phases are mixed together and then emulsified using sonication. This primary emulsion can then be added to an aqueous surfactant phase, and further sonicated, to form a water-in-oil-in-water emulsion (w/o/w), resulting in the formation of nanoparticles. Any remaining organic solvent is allowed to evaporate overnight or through the use of a dessicator, before finally recovering the nanoparticles through ultracentrifugation or filtration.

1.2.4.2 Nanoprecipitation for Nanoparticle Formulation [26, 27]

Nanoprecipitation utilizes amphiphilic organic solvents to drive spontaneous nanoparticle formation. The amphiphilic solvent containing dissolved polymer and drug is mixed with a continuous aqueous phase containing surfactant. The selection of an amphiphilic solvent results in dissolved polymer within the organic phase spontaneously diffusing into the aqueous phase containing surfactant, causing the rapid formation of dispersed nanodroplets within an aqueous matrix. The precipitated nanoparticles are then solidified within this matrix through solvent evaporation, and particles can be recovered through centrifugation or filtration.
1.2.4.3 Advantages of Evaporative Emulsion for Nanoparticle Formulation

Evaporative emulsion fits all the criteria for selecting an effective nanoparticle formulation method. The method has great flexibility in the choice of solvents and surfactants with lower toxicities needed to form the emulsions [26]. The particles produced using evaporative emulsion, have narrow size distributions, and the process has proved to be easily reproducible [25, 28]. Its primary advantage over nanoprecipitation is the ability to load particles with a variety of different substances, due to the use of sonication. Nanoprecipitation relies on gradient driven diffusion to form nanoparticles, and thus, encapsulation of drug or other substance within the particles relies mostly on water affinity, which may be affected by the polymer used [26]. Most evaporative emulsions use a double emulsion, and through the use of a surfactant in the second emulsion, result in a more stable nanoparticle, with a hydrophilic core, surrounded by a hydrophobic shell. Double emulsions were also found to be effective in formulating nanoparticles for delivery of therapeutic proteins.

1.2.5 Other Drug Delivery Particulate Systems

The growth of bioengineering over the past decade has resulted in the development of numerous systems that can potentially function as drug delivery devices. Liposomes, micelles, and microparticles, are just a few of the recent developments in the ever expanding field of drug delivery [23, 29-32]. Liposomes are colloidal structures with membrane properties similar to those found in cells, i.e., a lipid bilayer surrounding and encapsulating an aqueous core, which can be loaded with drug or other material [32]. Recently, liposomes have been developed with cationic lipids,
pH-sensitive lipids, and other surface modifications in order to increase their effectiveness as drug delivery systems [33]. Micelles are also similar in structure to the lipid bilayer found in cells, but contain only a single lipid layer, consisting of a hydrophobic core, with hydrophilic heads near the surface region [29]. Microparticles (1-1000 μm) are identical to nanoparticles except for the difference in size, which afford microparticles a greater drug loading capacity per particle when compared to smaller sized particles [21, 34-36]. Of these particulate systems, synthetic nanoparticles still remain the best choice for intracellular drug delivery because of numerous reasons, which are discussed in the following section.

1.2.5.1 Advantages of Nanoparticles over Other Drug Delivery Particulate Systems

Biodegradable nanoparticles are better suited for drug delivery than liposomes and micelles because of their small size and ability to remain in circulation for extended periods of time. The small size of nanoparticles allows them to accumulate at diseased tissues such as tumors, which results in an increased amount of drug delivery [20]. Traditional polymeric liposomes and micelles, with surface properties similar to those of the lipid bilayer, are rapidly cleared from circulation through the reticular endothelial system (RES). This greatly reduces their effectiveness as drug delivery devices. Microparticles or microspheres, are composed of the same materials as nanoparticles, and formulated using the same techniques [21]. Their main advantage over nanoparticles with identical material properties deals with an increased drug loading capacity, and ability for longer sustained drug release. However, this advantage also proves to be an Achilles’ heel, because large particles in circulation are rapidly cleared
by the RES. *In vitro* studies conducted using various cell types (vascular smooth muscle cells, VSMC), human colon adenocarcinoma cell line (Caco-2), and endothelial cells (ECs) have also shown that cellular uptake is optimum for particles in the range of less than 200 nm [37, 38]. Larger particle sizes also affect the cellular uptake mechanism and intracellular trafficking of particles. In essence, even if microparticles avoid detection and clearance by the RES, they are still less effective than nanoparticles in providing targeted, sustained drug delivery due to intracellular trafficking and reduced cellular uptake because cells prefer to uptake smaller size particles. Other advantages of nanoparticles over microparticles include: 1.) Nanoparticles do not trigger local inflammation, while microparticles can produce a pronounced inflammatory response followed by fibrotic encapsulation and 2.) Nanoparticles present no risk for forming arterial occlusions because of their small size [25, 39-41]. Biodegradable nanoparticles, as discussed earlier, also have the ability to degrade within the body, thus providing a significant advantage over micelles and liposomes in sustaining drug delivery [21].

**1.2.6 Strategies for Developing Targeting Nanoparticles**

The most common strategies for developing targeting nanoparticles involve conjugation with either a ligand or antibody. In most cases, ligands are preferable, because they are more stable when subjected to experimental parameters (e.g., light, heat, pH, etc.), less immunogenic, and not as prone to batch-to-batch variations like antibodies [17, 18, 42, 43]. The most successful method for conjugating any ligand or surface molecule onto nanoparticles involves carbodiimide chemistry and/or avidin-
biotin affinity. The carbodiimide technique is especially effective when used on PLGA nanoparticles because of the carboxylic acid groups of PLGA. The carbodiimide used in most nanoparticle conjugations is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), because of its high solubility in water and ability to activate carboxyl groups to form bonds with different functional groups. Depending on the functionality of the ligand used, it may be able to bind directly to EDC via an amide linkage (Figure 1.1). Avidin-biotin affinity is another ligand incorporation technique that utilizes the specific binding of avidin to biotin for ligand binding onto nanoparticle surfaces. Avidin, a protein isolated from egg whites, is a tetramer containing four identical subunits. These subunits contain a high-affinity binding site for biotin, with a dissociation constant of $10^{-15} M$ [44]. Biotin, also known as vitamin H, may be used to modify a protein or carbohydrate, through activation of its carboxylic group with a NHS ester. The stable biotinylated ligand is attached to avidin, resulting in four biotinylated conjugates per mole of avidin. This highly selective form of carbodiimide chemistry has had a great effect on the development of targeted nanoparticle therapy.
1.2.7 Problems with Using Nanoparticles for Drug Delivery

Nanoparticles have shown great promise in providing an effective method for targeted, sustained drug delivery \textit{in vitro}. However, during conditions of high shear stress, such as those found \textit{in vivo}, nanoparticles have been unable to effectively arrest on vascular surfaces, reducing their ability to target cardiovascular diseases. Goetz et al have shown that increasing shear rates above 300 s\textsuperscript{-1} can cause a significant decrease in the adhesion of particles onto endothelium, and preliminary studies conducted in our lab have confirmed this observation. Cellular uptake and subsequent drug delivery using nanoparticles is dependent upon efficient adhesion onto targeted surfaces because cells need to have enough time to uptake these nanoparticles after the particle adherence. Overcoming the effects of shear regulated cellular uptake, especially under high shear
conditions, is an essential step in the development of any effective, nanoparticle-based, drug delivery device for targeting diseased endothelium.

1.3 Endothelial Cells

1.3.1 Functions of Endothelial Cells

The normal composition of vascular vessels consists of three layers: the outer adventitia, medial layer, and inner intima [45]. Endothelial cells are responsible for lining the inner wall of all blood vessels and form a non-thrombogenic barrier to prevent blood cells circulating in the lumen from interacting with the vessel wall [46]. ECs are also actively involved in many processes dealing with vascular function including, inflammation, vasodilation and vasoconstriction, hemostasis, and thrombosis [46]. Normal function of endothelium is regulated by release of various endothelium-derived factors such as prostacyclin, nitric oxide, thromboxane A2, and growth factors [47]. Dysfunction of the endothelium can lead to cardiovascular diseases such as atherosclerosis, through increased adhesion of platelets and other blood cells onto activated endothelium.

1.3.2 Endothelial Cell Adhesion Molecule Expression in Damaged or Inflamed ECs

Endothelial cell dysfunction leads to an increased expression of endothelial cell adhesion molecules (ECAMs) on the surface of the cells. These ECAMs stimulate the adhesion of blood cells such as platelets and leukocytes onto the injured endothelium as shown in various in vitro and in vivo studies. ECAM expression can be triggered by PCI or by exposure to histamine or thrombin, which increases platelet adhesion through selectin-mediated processes [23]. P-selectin is the first ECAM expressed in activated
EC and is translocated from storage bodies onto the plasma membranes within seconds [48]. P-selectin interacts with platelets and leukocytes through two ligands, glycoprotein (GP) Ibα and P-selectin glycoprotein ligand-1 (PSGL-1), respectively, and results in rolling adhesion of these cell types onto activated endothelium [48]. Another selectin expressed in activated ECs is E-selectin, which also mediates platelet and leukocyte adhesion [23].

1.3.3 Strategies to Target Injured ECs

Targeted drug therapy represents a paradigm shift in the approach of drug delivery. The ability to target diseased tissues and/or cells instead of relying on an outdated concept of systemic drug delivery is an emerging research area in bioengineering. In particular, targeting inflamed endothelium with therapeutic agents has been studied extensively within the past few years. These studies have focused on increased expression of ECAMs in diseased endothelium. One approach has been the use of immunoliposomes conjugated with Ab H18y7, an antibody for E-selectin [33]. This approach utilized the increased expression of E-selectin in activated endothelial cells as a target for drug delivery. The study found that liposomes conjugated with Ab H18y7 showed a 275 fold increase in cellular adhesion onto activated ECs, as compared to unactivated ECs [33]. These liposomes, when loaded with the cytotoxic agent doxorubicin, significantly decreased cell survival rates of activated ECs, but did not affect the survival rates of unactivated ECs [33]. Another approach to targeting inflamed endothelium has been the use of ligands conjugated with microparticles.
Microparticles, conjugated with ligands or humanized monoclonal antibodies (mAbs), have been used in various studies to target expressed P-selectin and E-selectin [23, 30, 34, 35, 49-53]. Two ligands used in the various studies were Sialyl-Lewis\textsuperscript{x} (sLe\textsuperscript{x}), a carbohydrate found on neutrophils, and PSGL-1, present on the surface of leukocytes [23, 35, 52]. Microparticles conjugated with sLe\textsuperscript{x} were found to mimic the adhesive behavior of leukocytes, on surfaces coated with either P- or E-selectin, displaying rolling adhesion that was dependent on ligand surface density [23]. Similarly, in vivo studies using mice models conducted with PSGL-1 coated microparticles, found an increase in microparticle adhesion via P-selectin expressed on activated endothelium [52]. These results from “leukocyte-inspired particles” are promising developments in the field of targeted drug delivery, and represent the nascent stages in a new approach to improving site-specific therapy. Though platelets, a blood cell type, demonstrate higher adherence to activated endothelium under high shear conditions than leukocytes, there is no current strategy to mimic platelet binding to target activated endothelium for drug delivery applications.

1.4 Platelets

1.4.1 Role of Platelets in Cardiovascular Disease

Platelets are known to play a large role in the early stages of inflammation, a key trigger for cardiovascular disease. In normal blood flow, leukocytes and other cells flow freely within the blood vessels and do not adhere to the endothelium lining the lumen of the vessels. However, certain conditions such as hypertension, hyperglycemia, obesity, smoking, and diets high in saturated-fat, can lead to endothelial
cell activation and inflammation [54, 55]. The activation of ECs leads to increased
ECAM expression, which initiates the adherence of platelets and leukocytes.
Activation of ECs can also be triggered by surgical cardiovascular intervention such as
stenting or angioplasty, resulting in damage to the endothelium. Another frequent
trigger of platelet attachment onto vessel walls is the rupture of atherosclerotic plaque,
which leads to the exposure of the underlying ECM, and platelet adhesion mediated by
von Willebrand Factor (vWF) and collagen [56]. Arterial occlusion can also lead to
platelet attachment due to the increased shear stress generated in the occluded vessel,
resulting in thrombus formation through attachment to vWF found in plasma [56].
After attachment to activated or injured endothelium, platelets function by releasing
proinflammatory mediators and expressing proinflammatory surface molecules, that
further stimulate the interactions of leukocytes and endothelium [57, 58]. Monocytes
triggered by the release of proinflammatory signals play a large role in the development
d of atherosclerotic plaque, which are a precursor of cardiovascular disease [59]. The
platelet-mediated inflammatory reactions can also lead to vasoconstriction, thrombus
formation, and the development of myocardial infarction [4].

1.4.2 Platelet Interactions with the Vascular Wall and Damaged ECs

Platelets play an important role in cardiovascular disease by interacting with
vessel walls and triggering adhesion of circulating leukocytes. Platelets are able to
adhere to activated endothelium or the subendothelial matrix using different binding
ligands present on the platelet surface. Once platelets have successfully adhered onto
injured or activated vascular walls, they provide a highly reactive surface that mediates
recruitment of additional platelets from the bloodstream. Adhered platelets release a series of chemical signals, including adenosine diphosphate (ADP), and thromboxane A2, which leads to activation of platelets and subsequent platelet aggregation. Activation of platelets results in conformational changes and stickiness, which allows platelets to form aggregates through interaction with plasma proteins [60]. Platelet activation also leads to recruitment of circulating leukocytes to the growing platelet aggregate, which triggers the inflammatory response.

1.4.3 Role of GP Ibα in Platelet Adhesion

Initial platelet adhesion onto injured endothelium or subendothelium is triggered by GP Ib-IX-V, an adhesion receptors located on the platelet surface, which is responsible for binding to vWF and P-selectin [56]. The GP Ib-IX-V complex consists of four glycoproteins, GP Ibα, GP Ibβ, GP IX, and GP V [61-63]. The GP Ibα portion of the complex contains the major binding ligands responsible for interaction with vWF, Mac-1, α-thrombin, and P-selectin [56]. The GP Ib-IX-V complex is one of the receptors responsible for the initial adhesion or platelet tethering that comprises the first contact between platelets and injured endothelium under conditions of high shear stress [4, 54]. Exposure of subendothelial collagen fibrils to blood allows circulating vWF to attach with the exposed collagen, providing a substrate for GP Ib-IX-V attachment [4]. Activation of endothelial cells results in the expression of adhesion cell molecules (ECAMs such as P-selectin, E-selectin, VCAM-1, and ICAM-1), which mediate platelet attachment through GP Ibα [59, 64]. As shown in Figure 1.2, the GP Ibα ligand in platelets interacts with vWF in the extracellular matrix (ECM) and P-selectin expressed
Figure 1.2 Roles of Platelet Glycoprotein Ib in Adhering to Activated or Injured Endothelium [56]

on activated endothelium. The role of GP Ibα as a vWF counterreceptor has been well documented in various studies; however, the role of P-selectin in triggering platelet adhesion under conditions of high shear stress has received far less discussion [31, 56, 63, 65-70].

The N-terminal domain of GP Ibα, containing approximately 300 amino acids, includes the major ligand-binding regions responsible for adhesion to vWF and P-selectin, as shown in Figure 1.3 [56, 71]. Previous studies have shown that unactivated platelets are able to display rolling adhesion on the surfaces of activated endothelium in a GP Ibα/P-selectin dependent process [4, 72]. The adhesion process is mediated by GP Ibα interaction with P-selectin expressed on the surfaces of activated endothelium, or collagen bound vWF [56]. Under normal physiological conditions, platelets are able to adhere to vessel walls at shear stresses of up to 60 dyne/cm² and for pathological conditions, such as vessel wall injury, adhesion can occur at over 100 dyne/cm² [63, 69]. It is clear that P-selectin mediated attachment of platelets on activated endothelium occurs at much lower shear stresses than vWF mediated attachment; however, this
selectin-mediated attachment of platelets can still occur at much higher shear stresses than what is found in normal venous circulation (~5-6 dyne/cm²) [63, 65, 69, 73]. Platelet adhesion mediated by P-selectin has also been shown to occur at much higher shear stresses than selectin-mediated leukocyte adhesion (>7 dyne/cm²), indicating that the GP Ibα ligand is highly effective at adhering platelets onto endothelial and subendothelial surfaces [69].

1.4.4 Rationale for Developing Platelet Mimicking Nanoparticles

Targeting nanoparticles to activated endothelium by mimicking platelet (GP Ibα) adhesive properties may provide broader capture than via leukocytes. Platelet physiology suggests that in addition to targeting activated endothelial cells, these nanoparticles may permit controlled drug delivery to thrombosis zone sites as well as the leaky endothelium seen with many tumors. However, GP Ibα nanoparticles may not

Figure 1.3 Structural Diagram of GP Ibα [74]
enter into tumors or wounds, which is a beneficial feature of leukocyte-based targeting. Also, platelet mimicking nanoparticles may be inappropriate in coagulopathies unless anti-coagulants are also delivered. Further research on the GP Ibα ligand is necessary to determine its full range of interactions. Evaluation of these interactions may help in predicting the behavior of GP Ibα-conjugated nanoparticles \textit{in vivo}.

1.5 Overview of Research Project

1.5.1 Goals/Objectives

The overall objective of this project was to increase cellular adhesion and subsequent uptake of nanoparticles by endothelial cells under conditions of high shear stress. Utilizing knowledge of platelet physiology, GP Ibα was selected as an ideal ligand for conjugating onto the surface of nanoparticles in order to increase their adhesiveness under high shear. GP Ibα can also serve as a targeting ligand that binds specifically to P-selectin expressed in activated ECs. These “platelet-mimicking nanoparticles” should specifically adhere onto damaged or activated ECs under conditions of high shear stress, inducing increased cellular uptake and retention of nanoparticles. Figure 1.4 represents a schematic of the proposed project and Figure 1.5 displays the chemical reactions used in attaching GP Ibα onto the surfaces of the nanoparticles.
Figure 1.4 Platelet-mimicking Nanoparticles for Targeting Dysfunctional Endothelium

Figure 1.5 Chemical Reactions Used in Attaching GP Ibα onto Carboxylated Nanoparticles

1.5.2 General Hypothesis

GP Ibα conjugated onto nanoparticles (~100 nm) can increase shear regulated uptake in damaged or activated endothelium by selectively binding to P-selectin or vWF, and provide a sustained, targeted drug delivery system. This hypothesis is based on the following observations:

- GP Ibα is responsible for the initial adhesion of platelets onto damaged or activated endothelium through its interaction with P-selectin (in activated
endothelial cells) and wWF (deposited on damaged endothelium), under conditions of high shear stress [56, 67, 71, 75, 76].

- GP Ibα-mediated platelet adhesion onto endothelium and sub-endothelium can proceed under much higher shear stresses than other vascular adhesion processes, such as selectin-mediated leukocyte adhesion [69].

- *In vitro* studies have shown that cellular uptake for ECs is optimum for particles with an approximate diameter of 100 nm [37, 38].

1.5.3 Specific Aims

In order to prove our hypothesis, three specific aims, listed below, will be pursued.

- **Aim 1** – Determine whether the material properties of polymers (polystyrene versus PLGA) will affect cellular uptake in ECs.
- **Aim 2** – Evaluate the effectiveness of PLGA nanoparticles in providing sustained drug release.
- **Aim 3** – Conjugate polystyrene nanoparticles with GP Ibα and determine the effects on adhesion under shear stress.

1.5.4 Innovative Aspects

Developing nanoparticles with the adhesive properties of platelets is the first step in developing an endothelium-specific drug delivery system, with the ability to overcome high shear conditions found in physiological circulation. GP Ibα-conjugated nanoparticles not only have the potential to target both activated and damaged
endothelium, but may also be able to function as a competitive inhibitor to platelet-
endothelial binding in vivo.

1.5.5 Successful Outcome of the Project

A successful outcome for this project would provide a method to deliver therapeutic agents directly to activated or injured endothelial cells, thereby preventing subsequent recruitment of blood and immune cells to the injured site. Nanoparticles based on this project could either be developed as drug delivery devices or used as models for investigating the mechanisms of platelet-endothelial cell interaction and its role in cardiovascular disease.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

Poly (DL-lactide-co-glycolide) (MW ~40,000, copolymer ratio 50:50) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). 100 nm Fluoresbrite® YG Carboxylate Microspheres made of polystyrene and 6-coumarin were purchased from Polysciences, Inc. (Warrington, PA). Chemicals, if not specified were purchased from Sigma-Aldrich (St. Louis, MO), including dichloromethane, Triton® X-100, bovine serum albumin (BSA), polyvinyl alcohol (PVA), and histamine. Human aortic endothelial cells (ECs) and low serum growth supplement (LSGS, 2% fetal bovine serum, hydrocortisone (1 µg/ml), human epidermal growth factor (10 ng/ml), basic fibroblast growth factor (3 ng/ml), and heparin (10 µg/ml)), were purchased from Cascade Biologics (Portland, OR). Cell culture media, supplements, and buffers, including Medium 199, 1X trypsin-EDTA, penicillin-streptomycin, and phosphate buffered saline (PBS) were purchased from Invitrogen Corp. (Carlsbad, CA). Antibodies used in the study were purchased from Abcam Inc. (Cambridge, MA) and R&D Systems Inc. (Minneapolis, MN). Purified GP Ibα was obtained from our collaborator on the project, Dr. Jing-Fei Dong, from Baylor College of Medicine (Houston, TX).
2.2 Methods

2.2.1 Characterization of Cellular Uptake

Cellular uptake of nanoparticles in ECs was characterized using PLGA and polystyrene nanoparticles. Studies were conducted to determine optimal incubation time and nanoparticle concentration for both types of polymers.

2.2.1.1 PLGA Nanoparticle Formulation

Nanoparticle formulation was performed using a standard double emulsion process consisting of a water-in-oil-in-water emulsion. PVA was dissolved in 60 ml of deionized (DI) water and 25 µl of dichloromethane in order to obtain a 2% w/v solution. The solution was then centrifuged at 1000 rpm for five minutes or until a pellet was produced and filtered using 0.2 µm Corning® syringe filters (Corning Inc., Corning, NY). 170 µg of 6-coumarin and 300 mg of PLGA were dissolved in 10 ml of dichloromethane to produce a 3% (w/v) solution of PLGA. One ml of 10% BSA in deionized water was added to the PLGA solution in order to produce BSA-loaded fluorescent nanoparticles. This mixture was then emulsified by sonicating at 55 W (300 V/T Ultrasonic Homogenizer, Biologics, Inc., Manassas, VA) for one minute, producing the primary oil-water emulsion. The emulsion was added drop wise to the PVA solution prepared earlier and the resulting suspension was sonicated for two minutes, to produce a double emulsion. This emulsion was then stirred overnight to allow dichloromethane to evaporate and placed in a vacuum dessicator for one more hour to facilitate solvent evaporation. After desiccating, the solution was centrifuged at 27,000 rpm for 20 minutes at 4 °C and the pellet was resuspended in deionized water by
sonicating at 55 W for 30 s. This washing procedure was repeated two times to remove any PVA and unencapsulated BSA left over in the suspension. The supernatant from the washing steps was stored and used to determine BSA loading efficiency, by evaluating the amount of BSA not loaded into the nanoparticles with the total amount of BSA used. After the last centrifugation step, the nanoparticles were resuspended in 7 ml of deionized water by sonicating at 55 W for 30 seconds in an ice bath. This suspension was centrifuged at 1000 rpm for 10 min at 4 °C to remove any large aggregates. Supernatant containing the nanoparticles was then frozen at -80 °C for 45 minutes before being lyophilized for two days. The lyophilized nanoparticles were stored at -20 °C until used in experiments.

2.2.1.2 Particle Size Analysis

Particle size analysis was done using a ZEISS Supra 55 VP scanning electron microscope (SEM). Nanoparticles were resuspended in water at a concentration of 500 µg/ml by sonication at 55 W for 20 s. Small drops of the nanoparticle solution were placed on the SEM disks and allowed to dry, and the disks were sputter coated for 10 minutes with carbon. Particle size distribution and zeta potential were performed by Particle Technology Labs Ltd. (Downers Grove, IL) using a Beckman Coulter RapidVue and Malvern Zetasizer Nano, respectively.

2.2.1.3 Cell Culture

ECs were grown in complete M199, supplemented with LSGS and 1% penicillin-streptomycin, following the company protocol. Upon reaching confluence,
cells were passaged or used for experiments. All cells used for the study were between passage 8 and 9.

2.2.1.4 Nanoparticle Uptake Study

To determine cellular uptake of nanoparticles, cells were seeded onto 24-well plates (Falcon®, Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 30,000 cells/well and allowed to grow for two days. Stock solutions of either PLGA or polystyrene were prepared in low serum growth medium at a concentration of 2 mg/ml. PLGA nanoparticles used for the uptake study were loaded only with 6-coumarin and not BSA, in order to evaluate cell protein content discussed below. Nanoparticle suspensions were dispersed by using a water bath sonicator (Branson 2200, Branson Ultrasonics Corp., Danbury, CT) for 10 minutes.

Two studies were performed in parallel to determine the effect of concentration and time on nanoparticle uptake. In order to determine “optimum” nanoparticle concentration, 50-300 µg/ml nanoparticle solutions were prepared in low serum growth medium using the stock nanoparticle solutions. Medium from the 24-well plate was aspirated and replaced with the various nanoparticle solutions and then incubated for one hour (Forma Scientific water-jacketed incubator, Thermo Electron Corp., Waltham, MA). Cells grown in media without nanoparticles served as controls.

The second study, for evaluating the effect of incubation time on cellular uptake, was performed using the same stock nanoparticle solutions. As in the first study, medium was removed from the 24-well plate and replaced with a 50 µg/ml nanoparticle solution in low serum growth medium. Cells were incubated for different
times ranging from 30 minutes to six hours. Cells without nanoparticle solutions served as controls.

After incubation, the nanoparticle medium was removed from the wells, and the remaining cell monolayer was washed two times with cold PBS to remove any remaining nanoparticles. One ml of 1% Triton® X-100 was added to each cell sample and incubated for one hour in order to lyse the cells. 25 µl of cell lysate from each well was used in order to determine total cell protein content using the Pierce BCA protein assay (Fisher Scientific, Hampton, NH). Total protein concentration was calculated using a BSA standard curve and normalized with particle concentrations in the cell lysis samples for nanoparticle uptake studies.

A fluorometer (VersaFluor™, Bio-Rad Laboratories, Hercules, CA) was used in order to quantify nanoparticle uptake by analyzing the fluorescent intensities in the cell lysates. 100 µl of the lysate from each well was added to a polystyrene cuvet (Fisherbrand®, Fisher Scientific) containing 900 µl of 1% Triton® X-100 as the diluent. Samples were read at EM 480 nm/EX 510 nm for the PLGA and polystyrene nanoparticles. A standard curve was obtained by serial dilution of nanoparticles in 1% Triton® X-100, and the fluorescent intensities of standard samples were measured using the same filters as above. The uptake of the nanoparticles by ECs was calculated by normalizing the particle concentration in each sample with total cell protein, which correlates to the number of cells in the sample.
2.2.1.5 Evaluation of nanoparticle uptake using confocal microscopy

ECs were seeded on glass cover slips (Fisherbrand®, Fisher Scientific) at a concentration of $10^6$ cells/cm$^2$ and then placed inside Corning® 60 mm sterile polystyrene tissue culture dishes (Fisher Scientific). Media on the glass cover slips was replaced after four hours to allow for sufficient cell attachment and then samples were incubated overnight. After incubation, the media from the cover slips was replaced with a nanoparticle suspension containing 50 µg/ml of PLGA nanoparticles for one set of cover slips and 50 µg/ml of polystyrene nanoparticles for the other set, followed by an additional hour of incubation. The suspensions were then removed and the cover slips were washed with cold PBS two times, followed by addition of cold FM® 4-64 FX (Texas-Red® dye, 5 µg/ml) (Invitrogen) in PBS for 10 minutes in order to stain the cell plasma membranes. The glass cover slips were imaged using a confocal laser scanning microscope (Carl Zeiss LSM Meta 510, Goettingen, Germany) equipped with the FITC and RITC filters (Ex($\lambda$) 488 nm, Em($\lambda$) 543 nm), while still in the membrane staining solution. The images were analyzed using Carl Zeiss LSM Image Browser (version 3.5).

2.2.2 Characterization of PLGA Nanoparticles as Drug Delivery Devices

BSA was used a model drug to characterize release properties from PLGA nanoparticles. Drug loading efficiency for BSA-loaded PLGA nanoparticles was also evaluated.
2.2.2.1 Release Profile for BSA-loaded PLGA Nanoparticles

PLGA nanoparticles loaded with BSA, following the protocol listed in Section 2.2.1.1, were used to model drug release characteristics. A nanoparticle stock solution containing 150 µg/ml of BSA-loaded nanoparticles was prepared in 0.1 M PBS. The solution was dispersed using a water bath sonicator for 10 minutes. Two ml of stock solution was placed inside 3 ml dialysis bags (Spectra/Por® Biotech CE Membrane, Spectrum Laboratories Inc., Rancho Dominguez, CA) with a molecular weight cutoff (MWCO) of 100,000. Samples were dialyzed against 25 ml of PBS inside a water bath shaker (Gyrotory® Water Bath Shaker, Model G76, New Brunswick Scientific, Edison, NJ) heated to 37 °C for 14 days. At designated time intervals, 1 ml of dialysate was removed from each sample and stored at -20 °C for later analysis. Dialysate volume was reconstituted by adding 1 ml of fresh PBS to each sample. After 14 days, dialysate samples were analyzed using a protein assay to determine the amount of BSA released into the dialysate.

2.2.2.2 BSA Loading Efficiency

BSA loading efficiency was determined using the supernatant from the nanoparticle formulation protocol discussed in section 2.2.1.1. The supernatant was analyzed using a protein assay to determine the amount of BSA unencapsulated within the nanoparticles. This value was then compared with the total amount of BSA used in the nanoparticle formulation protocol to determine the BSA loading efficiency of the particles.
2.2.3 Characterization of Nanoparticle Adhesive Properties

Polystyrene nanoparticles were conjugated with GP Ibα in order to increase adhesion onto P-selectin surfaces. Nanoparticle solutions were then used in a parallel flow chamber system to generate the necessary shear stress.

2.2.3.1 Biotinylation Protocol

Carboxylated nanoparticles, 100 nm in size, were conjugated using carbodiimide chemistry and avidin-biotin affinity. Protein ligands were first biotinylated using the Biotin-X-NHS Kit (EMD Biosciences, Inc., San Diego, CA). Briefly, 10 mg of protein was dissolved in 0.9 ml of DI water, and then added to 0.1 ml of sodium bicarbonate. A 0.1 M solution of biotin/NHS was prepared using 11 mg of biotin/NHS dissolved in 0.25 ml of dimethylformamide, and added to the protein solution. The protein/biotin solution was then incubated at room temperature for one hour. Gentle agitation was applied to the solution using a rocker (Gyrotory® Water Bath Shaker) at low speeds, to avoid foaming. After one hour of incubation, the solution was dialyzed against 0.1 M PBS for one hour to remove any unreacted biotin/NHS. When dialysis was completed, the solution was stored at 4 °C until used.

2.2.3.2 Nanoparticle Conjugation Protocol

Approximately, $10^7$ polystyrene nanoparticles were measured out and added to 1 ml of DI water. This nanoparticle solution was then added to a 15 mg/ml EDC solution in 0.1 M MES buffer, pH 4.75. The solution was reacted at room temperature for four hours to ensure carboxyl group activation and EDC attachment. After four hours, 500 µg of avidin (EMD Biosciences, Inc.) was added to the nanoparticle solution
and allowed to interact overnight in 0.1 M sodium bicarbonate, pH 8.5 solution. The un-reacted avidin was removed using dialysis. Once avidin attachment was completed, 120 µl of 50 µg/ml biotinylated protein dissolved in 0.1 M PBS was added to the solution and reacted at room temperature under gentle agitation, for one hour. After the reaction was completed, the nanoparticle solution was dialyzed against 0.1 M PBS for three hours to remove any unreacted material, and then stored at 4 °C until used. Great care was taken to avoid exposure of the nanoparticles to light throughout the entire procedure.

2.2.3.3 Nanoparticle Conjugation with Antibody

To confirm the conjugation of ligands onto nanoparticles, 100 µl of 30 µg/ml primary mouse antibody monoclonal to BSA or GP Ibα, was added to approximately $10^5$ ligand-conjugated nanoparticles in PBS. The nanoparticles were either conjugated with BSA or GP Ibα as the ligand. This solution was then dialyzed for one hour against PBS, before the addition of a fluorescent secondary antibody (Rabbit polyclonal to Mouse IgG (Texas Red®)). 100 µl of 30 µg/ml secondary antibody was added to the nanoparticle solution and incubated for one hour followed by one hour of dialysis against PBS. The nanoparticle solution was then stored at 4 °C and protected from light before being analyzed using confocal microscopy.

2.2.3.4 Surface Preparation for P-selectin Coated Slides

Glass slides (Fisherfinest®, Fisher Scientific) were cut to 24mm x 56mm and incubated with 500 µl of 20 µg/ml P-selectin (R&D Systems) for four hours at 37 °C, followed by one hour of incubation with a 1% BSA solution in PBS to block any
nonspecific binding sites. Half the slides were then further coated with P-selectin antibody for one hour at room temperature in order to serve as a negative control. The slides were then washed gently with a 0.9% NaCl solution to remove any unbound P-selectin or antibody. Slides were used immediately after preparation.

2.2.3.5 Surface Preparation for EC Coated Slides

Glass slides were etched with a NaOH solution in order to increase cellular adhesion of ECs on the slides after seeding. Briefly, slides were placed in 0.5 N NaOH for two hours at room temperature, rinsed several times with DI, and dried in an oven for one hour. Slides were then autoclaved before being used. Cell suspensions were obtained in similar fashion to the protocol used in section 2.2.1.5, and then seeded onto pre-etched glass slides at a density of $10^5$ cells/cm$^2$. Cells were allowed to attach overnight, and grown for two days before being used in experiments. After two days, cells were treated with 500 µl of 25 µM histamine, for 12 minutes at room temperature, in order to induce P-selectin expression on ECs. Stimulated cells were then used immediately in flow chamber experiments. After the flow chamber studies were completed, the cells membranes were then stained to produce a contrast between the intercellular and extracellular regions of the cells, as previously discussed in Section 2.2.1.5.

2.2.3.6 Parallel Flow Chamber

Three parallel plate flow chambers were run simultaneously in order to study nanoparticle adhesion onto coated glass cover slides. The flow chambers consisted of a polycarbonate slab, with a silicon gasket, and placement of the glass cover slides on top
of the gasket as shown in Figure 2.1. The system was held together using a vacuum pump (Gast Manufacturing, Inc., Benton Harbor, MI), and shear stress was generated using a syringe pump (KD Scientific, Holliston, MA). Wall shear stress (\(\tau_w\)) for the flow chamber was calculated according to the following equation:

\[
\tau = \frac{6Q\mu}{bh^2}
\]

where \(Q\) is the volumetric flow rate, \(\mu\) is the fluid viscosity, \(b\) is the channel width, and \(h\) is the channel height.

Figure 2.1 Schematic of a Parallel Flow Chamber. The chamber consists of (A) a polycarbonate slab, (B) a silicone gasket, and (C) a glass coverslip held together by a vacuum. A vacuum pump was connected to the vacuum port and a syringe pump was connected to the inlet port in order to generate shear stress. The glass coverslip was either coated with P-selectin, P-selectin/anti-P-selectin, or seeded with activated ECs.

Slides either coated with P-selectin, P-selectin/anti-P-selectin, or containing an EC monolayer were assembled into the \textit{in vitro} parallel plate flow system. The chamber and syringe pump were configured to produce 5 dyne/cm\(^2\) of shear stress. The nanoparticle solutions were diluted in PBS for P-selectin/anti-P-selectin surfaces studies.
or in low serum media for cell studies to produce an approximate concentration of $10^6$ nanoparticles/ml. Nanoparticle solutions were collected before and after the flow chamber to quantify the amount of nanoparticles bound to the glass cover slides. The glass slides were also observed using confocal microscopy to determine the difference in nanoparticle adhesion. Experiments performed using the flow chamber systems are summarized in Table 2.1.

Table 2.1 Summary of Flow Chamber Experiments (Total = 36 experiments)

<table>
<thead>
<tr>
<th>Nanoparticle Samples</th>
<th>P-Selectin Coated Slides</th>
<th>P-selectin/Anti-P-selectin Coated Slides</th>
<th>Number of Experiments</th>
</tr>
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<td>(1) Control Nanoparticles</td>
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<td>6</td>
<td>12</td>
</tr>
<tr>
<td>(2) GP Ibα-conjugated Nanoparticles</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>(3) GP Ibα-conjugated Nanoparticles + GP Ibα mAbs</td>
<td>6</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>
CHAPTER 3

RESULTS

3.1 Cellular Uptake Study

3.1.1 Nanoparticle Characterization

PLGA nanoparticles, loaded with BSA as a model therapeutic protein and 6-coumarin as a fluorescent dye, were produced using the double emulsion technique. These nanoparticles were within a size range of 50-150 nm (Figure 3.1a). Average hydrodynamic radius was found to be 100 nm, with a negative zeta potential of -2.54 mV. In addition, polystyrene nanoparticles were used as a model particle. Size distribution for the polystyrene nanoparticles was narrower than for PLGA nanoparticles, with a mean hydrodynamic radius of 100 nm (Figure 3.1b).

Figure 3.1 SEM Images of Nanoparticles. (A) Represents PLGA and (B) Polystyrene Nanoparticles.
3.1.2 Nanoparticle Uptake

Nanoparticle uptake for both types of particles displayed similar trends for both the concentration and time studies. For the concentration study, the PLGA and polystyrene nanoparticles displayed an almost linear uptake at concentrations up to 200 µg/ml, before reaching saturation (Figures 3.2 and 3.3). The nanoparticle uptake, with respect to time, reached saturation in less then 30 minutes for the PLGA samples, while the polystyrene nanoparticles took one hour to reach saturation (Figures 3.4 and 3.5). Both types of particles displayed a saturated uptake rate after two hours of incubation. Confocal microscopy was used to confirm the uptake of the particles inside the cells. Images of ECs were taken after one hour of incubation with the nanoparticles solutions (50 µg/ml) using a fast scan option to obtain varying slice thickness of the cells (Figure 3.6). Slice thickness was set at one µm, with an average of 18 slices taken per image, depending on cell thickness. All images were analyzed at the middle point of the cell and confirmed that the fluorescence present was localized within the cell membranes, and not present on the surface. This was the case for both types of nanoparticles used in the study.
Figure 3.2 Effect of Concentration on PLGA Nanoparticle Uptake in ECs. Values were obtained after one hour of incubation with nanoparticles solutions and represent mean ± SD (n=6).

Figure 3.3 Effect of Concentration on Polystyrene Nanoparticle Uptake in ECs. Values were obtained after one hour of incubation with nanoparticles solutions and represent mean ± SD (n=6).
Figure 3.4 Effect of Time on PLGA Nanoparticle Uptake in ECs. Samples were incubated with 50 µg/ml of nanoparticle solution. Values represent mean ± SD (n=6).

Figure 3.5 Effect of Time on Polystyrene Nanoparticle Uptake in ECs. Samples were incubated with 50 µg/ml of nanoparticle solution. Values represent mean ± SD (n=6).
3.2 PLGA Nanoparticles as Drug Delivery Devices

3.2.1 BSA Release Profile

Dialysate samples were taken from the BSA-loaded nanoparticles, placed in dialysis tubing, at varying time intervals. For the first 24 hours, samples were taken after 30 minutes, one hour, and two hours. Samples were then taken every two hours for the remainder of the first day. For the next 13 days, samples were taken exactly 24 hours after the end of the first day, resulting in total of 22 data points. BSA-loaded nanoparticles displayed a burst effect, releasing almost 55% of the protein after 40 hours, followed by a much slower release in the following days. The particles were able to provide sustained release of protein throughout the 14 day experiment (Figure 3.7).
3.2.2 BSA Loading Efficiency

BSA loading efficiency in the particles was approximately 85%, indicating that almost all of the BSA added to the PLGA was incorporated inside the nanoparticles. Loading efficiency was calculated according to the following formula:

\[
\% \text{ Loading Efficiency} = \frac{(\text{total amount of BSA used in nanoparticle formulation} - \text{amount of BSA present in supernatant})}{\text{total amount of BSA used}}
\]

3.3 Nanoparticle Adhesive Properties

3.3.1 Nanoparticle Conjugation

Nanoparticles conjugated with fluorescent secondary antibodies were imaged using confocal microscopy to determine the extent of ligand conjugation. Figure 3.8 shows polystyrene nanoparticles conjugated with BSA as the ligand. The first two images show the individual fluorescences of the nanoparticles and Texas Red® antibody, respectively. The third image overlays the first two images and clearly shows
an overlapping of the antibodies with the nanoparticles. The images were taken at the same slice thickness to eliminate the possibility that the fluorescent antibodies were either above or below the nanoparticles. Overlaying the first two images produces an almost entirely yellow image, indicating that the green fluorescence attributed to the nanoparticles and the red fluorescence representing the antibodies, encompassed the same area.

![Confocal Images](image)

Figure 3.8 Confocal Images of BSA Conjugation onto Polystyrene Nanoparticles - (A) FITC filter displaying fluorescent nanoparticles, (B) RITC filter displaying Texas Red® secondary antibody attached to BSA primary antibody, (C) Overlay of images A and B, displaying overlapping fluorescent intensities. Images were taken at Ex(λ) 488 nm, Em(λ) 543 nm.

3.3.2 Parallel Flow Chamber

Samples prepared according to Table 2.1 were run on the parallel flow chamber system configured to produce a wall shear stress of 5 dyne/cm². This value was chosen because it is approximately the same value as shear stress found in normal venous circulation [63].

3.3.2.1 P-selectin and Anti-P-Selectin Coated Slides

Specific interaction between P-selectin coated surfaces and GP Ibα-conjugated nanoparticles was observed when nanoparticle solutions before and after the flow
system were analyzed (Figure 3.9). GP Ibα nanoparticles displayed a 60% adhesion rate on P-selectin coated surfaces, while control nanoparticles and nanoparticles samples with pre-incubation of anti-GP Ibα ligands to GP Ibα-conjugated nanoparticles displayed adhesion rates of 19% and 22%, respectively. Surfaces coated with anti-P-selectin displayed no specific interactions with any of the nanoparticles, with nanoparticle adhesion ranging from 2.5% to 24%. Confocal microscopy analysis of the various slides confirmed this observation, with the highest amount of fluorescence detected on P-selectin coated slides subjected to GP Ibα-conjugated nanoparticles.

![Figure 3.9 Nanoparticle Adhesion onto P-selectin and Anti-P-selectin Coated Surfaces Using Different Nanoparticle Samples. Values represent mean ± SD (n=6).](image)

Figure 3.9 Nanoparticle Adhesion onto P-selectin and Anti-P-selectin Coated Surfaces Using Different Nanoparticle Samples. Values represent mean ± SD (n=6).
3.3.2.2 Endothelial Cell Seeded Slides

Glass slides seeded with activated ECs were run on the flow chamber under the same parameters as the P-selectin/anti-P-selectin coated slides. Confocal images of the ECs showed significant cellular uptake when GP Ibα nanoparticles were used, while other nanoparticles samples displayed insignificant cellular uptake. GP Ibα nanoparticles were observed localized within cellular membranes, with a very low concentration of nanoparticles present in extracellular spaces, as shown in Figure 3.10a. Samples were observed at the middle point of each cell using the stack imaging option, with slice thickness set at 1 µm. In contrast, control and anti-GP Ibα nanoparticles were present in extracellular spaces at very high concentrations, with minimal nanoparticles observed within the cells, as seen in Figures 3.10b and 3.10c, respectively. Control nanoparticles were observed to have the worst cellular uptake under exposure to shear stress, with almost no nanoparticles seen within the cells. Control samples were observed using the stack imaging option, and most nanoparticles were localized on the surface of the glass slides. Negative control samples, conjugated with anti-GP Ibα, had a slightly higher number of nanoparticles observed within the cells, but the majority of particles were seen in extracellular spaces.
Figure 3.10 Confocal Images of Cellular Uptake of Polystyrene Nanoparticles in Activated ECs Under Shear Stress. (A) Represents GP Ibα Nanoparticles, (B) control, and (C) anti-GP Ibα-conjugated nanoparticles. Plasma membranes were dyed using Texas Red® and imaged using a RITC filter. Fluorescent nanoparticles were imaged using a FITC filter. The third image (far right) represents an overlay of RITC and FITC filters. All images were taken at Ex(λ) 488 nm, Em(λ) 543 nm.
CHAPTER 4
DISCUSSION

4.1 Cellular Uptake Study

PLGA and polystyrene nanoparticles were investigated in order to determine the effect of material properties on cellular uptake. PLGA nanoparticles were chosen for the study because they are well suited for drug delivery due to their biodegradability via hydrolysis and also, are already approved by the FDA for certain applications, as previously discussed [23]. Polystyrene particles were chosen because they are well known for their narrow size distribution, uniform composition, and stabilization.

Preliminary studies conducted in our lab have suggested that cellular uptake in VSMCs and ECs decreases as the size of the nanoparticle increases, with optimal uptake occurring at 100 nm. Similar to our observation, other studies done using a human colon adenocarcinoma cell line (Caco-2) have shown that optimal cellular uptake occurred when using 100-200 nm PLGA nanoparticles [37, 38]. Moreover, previous studies have shown that nanoparticles below 200 nm are usually internalized in endothelial cells through receptor-mediated endocytosis, while larger nanoparticles are internalized through phagocytosis [36, 77, 78]. The mechanism of cellular uptake plays a large role in intracellular trafficking, thus greatly modulating the effectiveness of any internalized drug [79]. Internalization of drugs or particles in endothelial cells through phagocytosis usually leads to intracellular lysosomal delivery, which results in
compartmentalization of the drug within lysosomes followed by degradation through acidic hydrolysis [79]. Depending on the therapy required, certain drugs may either be targeted towards the nucleus or other intracellular targets.

The uptake of nanoparticles in ECs is nearly linear with dosage at low concentrations (<200 µg/ml). Two separate studies conducted using similarly sized polystyrene and PLGA nanoparticles (100 nm) in Caco-2 cells, also displayed cellular uptake proportional to dosage at higher particle concentrations (500 µg/ml) [37, 38]. These results suggest that controlled amounts of drug can be delivered using biodegradable nanoparticles with particle concentration-based dosages.

The uptake of nanoparticles with respect to time displayed an initial rapid uptake of nanoparticles, followed by a plateau effect, suggesting that saturation of the cellular uptake pathway was reached. Other studies involving Caco-2 cells displayed a similar trend in cellular uptake, however, the uptake pathway was not saturated until after two hours of incubation. In contrast, our study found that the uptake of nanoparticles in endothelial cells was saturated after one hour of incubation [37, 38]. The difference in saturation time may be due to particle compositions, sizes, and dosage kinetics, as well as different cell types used. Our studies were investigated with ECs whereas others studied Caco-2, an epithelial cell line. These results imply that the cellular uptake pathway for endothelial cells is more active at lower incubation times, and reaches the saturation limit more rapidly than for epithelial cells.

The PLGA particles produced were near the optimal size found in most published studies, and displayed similar uptake properties when compared to identically
sized non-biodegradable (carboxyl polystyrene) particles, indicating that nanoparticle uptake in endothelial cells was not affected by these two types of materials used in the study. Other studies have shown that certain cell types (Caco-2) do not uptake polystyrene nanoparticles as efficiently as PLGA nanoparticles, possibly due to increased surface hydrophobicity or charge [37, 78]. Polystyrene surfaces are much more hydrophobic than certain biodegradable aliphatic polyester polymers such as PLGA; this could affect the uptake pathway in certain cell types. This bears further investigation as it suggests that cellular uptake of nanoparticles can vary greatly when considering different cell types and particle surface properties.

Fluorescent quantification of PLGA nanoparticles was performed under the assumption that 6-coumarin loaded within the nanoparticles did not leach out, and any fluorescence detected consisted of only nanoparticles. This assumption was based on previous studies, which have shown that fluorescent markers loaded into PLGA nanoparticles remain incorporated within the polymer matrix, without leaching out [25, 38, 80]. Dye leakage from nanoparticles could contribute to false fluorescent readings, increasing the observed cellular uptake due to the increased fluorescence associated with free dye. Another factor that may affect nanoparticle uptake in ECs involves concentration of serum in media used. Studies have shown that media with high serum content may result in excessive cell exocytosis, due to the energy dependent nature of the process [80]. Exocytosis of nanoparticles would contribute to incorrect assessment of cellular uptake in our study. To avoid this issue, low serum media was used in all cellular uptake studies.
Based on results of this study, optimal nanoparticle concentration for both PLGA and polystyrene nanoparticles in ECs was found to be 200 µg/ml. With respect to time, the optimal incubation period was found to be 30 minutes for PLGA nanoparticles and 1 hour for polystyrene nanoparticles. The optimal size for nanoparticle uptake was approximately 100 nm. Both types of nanoparticles displayed similar cellular uptake properties, suggesting that either material could be used in formulation of nanoparticles for uptake in endothelial cells.

4.2 PLGA Nanoparticles as Drug Delivery Devices

BSA (MW ~66,000) was chosen as a model therapeutic protein for incorporation within PLGA nanoparticles because of its high solubility in water, relatively large size, and its ability to be easily quantified. During the nanoparticle formulation process, BSA was encapsulated within the aqueous core of the PLGA nanoparticles at ~85% efficiency. This encapsulation efficiency was very high, and indicated that almost all of the protein used in nanoparticle formulation was entrapped within the PLGA nanoparticles. Studies conducted with similar PLGA nanoparticles displayed drug loading efficiencies ranging from 70-95%, indicating that our data was consistent with previous observations [25, 81, 82]. Studies have also shown that anti-inflammatory agents, for treatment of activated endothelium, can be loaded into PLGA nanoparticles at very high efficiencies [81].

Drug release from nanoparticles is often described as a biphasic shift, with an initial burst effect followed by sustained release [82, 83]. This burst affect should be avoided, because it can result in drug delivery outside of the therapeutic range. The
release characteristics of BSA from our nanoparticles showed a burst effect after one day, followed by sustained release for the remaining 13 days. This burst effect released approximately 55% of total protein loaded within the nanoparticles. Other studies have shown similar results, with burst effects ranging from 20% to 60% of total entrapped protein released after one day [84]. Reducing the burst release effect seen in PLGA nanoparticles will result in increased time for sustained drug release. PLGA polymer properties such as molecular weight and ratio of acidic moieties, play a large role in determining the degree of burst release, and can also affect sustained drug release. Selecting a polymer with a different molecular weight may be a method to reduce the burst effect. The molecular weight of the PLGA used to formulate the nanoparticles was ~40,000 Da. Using a higher molecular weight polymer will increase the viscosity of the polymer solution, thus reducing the diffusion of protein from the PLGA emulsion into the surrounding aqueous phase and conserving residual drug within the PLGA matrix [82].

Our PLGA nanoparticles demonstrated sustained BSA release for at least 14 days. Diffusion plays a role in the release mechanics immediately following the burst phase, because the PLGA nanoparticles have not yet begun to degrade. Sustained release is also due to the mosaic structure of the BSA surface, with both hydrophilic and hydrophobic patches. Diffusion enables the molecule to adapt to the local environment; possibly enhancing release into the aqueous environment. This would be less likely with a more hydrophilic, small molecule drug, such as curcumin or paclitaxel. Degradation of PLGA nanoparticles involves numerous factors, but typically occurs
after 2 to 4 weeks of placement within an aqueous medium [23, 85]. Surface analysis of PLGA nanoparticles has shown the presence of micro-pores and micro-caves, also supporting the notion that diffusion is a key mechanism in the release of BSA from our nanoparticles [86]. Similar studies conducted using PLGA, and modified PLGA polymers, detected sustained release of progesterone and paclitaxel from loaded nanoparticles for over three weeks [82, 87]. This confirms that PLGA nanoparticles can be loaded with various hydrophilic and hydrophobic drugs, and provide sustained release for several weeks. Hydrophobic drugs may be loaded within particles using a single emulsion process, while hydrophilic drugs require the use of a double emulsion. However, as previously mentioned, a large burst effect can reduce the length of sustained release. Copolymer ratio of PLGA is another factor that can affect the sustained release rate of selected drugs. It has been well documented that increasing the ratio of glycolic acid present within a polymer leads to faster degradation and increased polymer swelling [88, 89]. Both these factors will increase the release rate of drug from PLGA nanoparticles. Lactic acid makes PLGA less susceptible to hydrolysis because of steric hindrance due to the presence of a methyl group, leading to less water accessibility [88, 89]. Increasing water accessibility would translate into shorter sustained release of drug, which may be positive or negative depending on the particular application desired. The polymer used in our nanoparticles had a 50:50 ratio of lactic to glycolic acid, which produced a sustained release of drug for at least 14 days. Future work on this project may include altering the ratio of acidic moieties comprising PLGA in order to increase or decrease drug release properties.
Many factors involved with the release properties from PLGA nanoparticles have to be considered when formulating nanoparticles, as evidenced by the previous discussion. The PLGA used in formulating our nanoparticles was well suited for drug release because of its material properties (i.e. copolymer ratio, molecular weight, carboxylation), presenting a good baseline by which other nanoparticles and materials can be judged. Knowledge of the role these factors play in effecting sustained release has given us all the tools necessary to successfully formulate drug releasing nanoparticles with controlled, sustained release properties. Having successfully characterized the release properties from PLGA nanoparticles, the next aim used to test our hypothesis was the development of targeting nanoparticles.

4.3 Nanoparticle Adhesive Properties

P-selectin coated surfaces were exposed to various nanoparticle solutions using a parallel flow system to determine the adhesiveness of nanoparticles under fluid shear stress. The parallel flow chamber was chosen over other flow systems because of easy operation and ability to produce constant shear stress. The control nanoparticles, showed very low adhesion onto both P-selectin and P-selectin/anti-P-selectin surfaces, indicating that non-selective binding under shear stress was minimal. The negative control sample involving anti-GP Ibα-conjugated nanoparticles also showed insignificant levels of adhesion onto P-selectin coated surfaces. GP Ibα-conjugated nanoparticles displayed approximately 60% adhesion of nanoparticles onto the surface which, when compared with the control samples, proved to be statistically significant. In a parallel series of experiments involving surfaces coated with anti-P-selectin, none
of the nanoparticle samples showed significant adhesion, indicating that P-selectin was a necessary substrate for nanoparticle adhesion under these shear stress conditions. Combining the results from the two experiments leads to a clear conclusion, that GP Ibα-conjugated nanoparticles display selective adhesion onto P-selectin coated surfaces under these conditions of shear stress.

Observation of P-selectin and anti-P-selectin coated slides using confocal microscopy was used to quantify the amounts of adherent nanoparticles. These observations confirmed the findings from the first experiments measuring nanoparticle adhesion percentage (using a fluorometer). P-selectin coated slides, in conjunction with control and negative control nanoparticles, displayed very little fluorescence, with randomly dispersed adhesion of nanoparticles onto the surfaces. P-selectin coated slides treated with GP Ibα conjugated nanoparticles displayed evenly dispersed fluorescence covering the majority of the slide. These results suggest that P-selectin/GP Ibα binding plays an important role in enhancing the adhesion of nanoparticles on activated EC surfaces.

Nanoparticles conjugated with GP Ibα displayed much higher uptake in ECs activated with histamine when compared with unactivated EC controls. Also, using confocal microscopy, GP Ibα conjugated nanoparticles were observed within the cytoplasm of activated ECs, while control nanoparticles were mainly localized in the extracellular spaces of activated or unactivated ECs. Only a small percentage of GP Ibα-conjugated nanoparticles were observed outside cellular membranes. This observation confirms that GP Ibα adhesion with P-selectin expressed in activated ECs is
necessary for cellular uptake under conditions of fluid shear stress. Control nanoparticles without the presence of a binding ligand showed very poor cellular uptake when observed under the microscope. These particles were unable to attach to cellular membranes under fluid shear stress, and consequently, were not taken up by the cells. Control nanoparticles were found at very high concentrations in the extracellular space, with almost no nanoparticles localized within the cells. GP Ibα-conjugated nanoparticles pre-incubated with anti-GP Ibα antibodies were also unable to attach to the cellular membranes under fluid shear stress, indicating that GP Ibα is necessary for mediating nanoparticle adhesion under these conditions. Blocking the GP Ibα ligand with an antibody resulted in minimal cellular uptake and nanoparticle targeting abilities, with the majority of nanoparticles dispersed in extracellular spaces, indicating once again, the specific interaction between GP Ibα and P-selectin.

Previous studies involving the development of “leukocyte-inspired” particles, discussed in section 1.3.5.1, displayed similar adhesion results under shear stress. Microparticles conjugated with sLe^x and an antibody to intracellular adhesion molecule-1 (ICAM-1), were found to adhere to surfaces coated with P-selectin and ICAM-1. Particle adhesion studies however, were conducted at lower fluid shear stresses than our study, due to the lower binding strength of their ligands. Our platelet-mimicking nanoparticles were able to adhere onto P-selectin coated surfaces at higher rates of fluid shear. In addition, the “leukocyte-inspired” particles were several micrometers in size, which proves to be a disadvantage for drug delivery systems in vivo, especially for intracellular drug delivery.
PLGA microparticles based on leukocyte binding ligands were also studied to determine the effect of degradation on binding efficiency. The study found that targeting ability was limited by polymer degradation, and not by degradation of the binding ligand. The conjugation method used in the study was similar to our method for attaching GP Ibα onto our nanoparticles, and provides evidence that conjugating ligands on PLGA nanoparticles is possible using carbodiimide chemistry.

One final note on the GP Ibα ligand concerns its role in mediating adhesion onto vWF. Previous discussions have defined the role of GP Ibα in targeting platelets towards vWF in vivo. Our current study did not include vWF; however, its binding action with GP Ibα can occur under much higher fluid shear stress than found in P-selectin mediated adhesion. Other studies have successfully characterized this vWF mediated adhesion under shear stress, while less work has been done on the role of P-selectin. Future work on this project will include studies involving vWF coated surfaces and our GP Ibα-conjugated nanoparticles.
CHAPTER 5

CONCLUSIONS

The successful development of platelet-mimicking nanoparticles supports our hypothesis that the GP Ibα ligand can increase nanoparticle targeting abilities and endothelial cell uptake under conditions of fluid shear stress. Our results have shown that nanoparticles conjugated with the GP Ibα ligand significantly increased adhesion to P-selectin coated surfaces over control nanoparticle adhesion. Cellular uptake studies conducted under fluid shear stress also displayed increased nanoparticle uptake and targeting ability when ECs were activated with histamine to express P-selectin.

Other results involving formulation of PLGA nanoparticles have shown that they possess favorable qualities for a drug delivery system. We were able to successfully load PLGA nanoparticles with BSA, a theoretical drug, with high efficiency. PLGA nanoparticles provided sustained BSA delivery for two weeks, and displayed similar EC uptake properties compared with polystyrene nanoparticles. Optimal nanoparticle dosage was found to be 200 µg/ml for both types of nanoparticles, while optimal time was found to be 30 minutes and one hour, for PLGA and polystyrene nanoparticles, respectively. Our endothelial cell uptake results and characterization of PLGA drug delivery properties agreed with existing studies, suggesting that nanoparticle based systems may have a promising future as drug delivery devices.
CHAPTER 6

LIMITATIONS AND FUTURE WORK

Though the novel GP Ibα-conjugated nanoparticles developed in this project demonstrate that by mimicking platelets, the adhesion and uptake of nanoparticles in activated ECs can be induced, the applicability to targeted drug delivery of these nanoparticles may have some limitations:

- Amount of GP Ibα bound onto the nanoparticle surfaces may be small due to the limited number of exposed carboxyl groups in PLGA. To overcome this limitation, deposition of more carboxyl groups onto the surfaces of polymeric nanoparticles can be performed using a pulse plasma polymerization process.

- GP Ibα nanoparticles may induce other side-effects by interacting with blood cells, such as platelets and leukocytes, resulting in production of aggregates. Experiments using human blood containing these particles need to be performed to see whether this is the case. An alternative strategy for this limitation is to incorporate the peptide (sequence of GP Ibα) that is shear-stress regulated and binds only to the regions of P-selectin expressed on activated endothelium, instead of glycocalicin (the whole extracellular part of GP Ibα), onto nanoparticles.

- Evaluation of material properties in EC uptake was limited to PLGA and polystyrene. Other materials may have different cellular uptake properties depending on hydrophobic and hydrophilic interactions, surface charge, and
• degradation properties. To overcome this limitation, future studies may be conducted using different polymers, such as PLGA-PEG.

• ECs used in fluid shear stress studies were not grown to confluence due to slow cell proliferation. To overcome this limitation, cell culture parameters such as media content and cell seeding density may be adjusted to provide faster cell proliferation.

Future work on this project will involve transitioning the system from a modeling basis into more of a drug delivery device. Suggestions for future work are listed below:

• Conjugation of GP Ibα with PLGA nanoparticles will need to be performed, as will loading of particles with an anti-inflammatory drug.

• Flow cytometry will need to be performed on the conjugated nanoparticles to determine ligand surface density.

• Careful examination of PLGA parameters will need to be performed in order to reduce the burst release effect and increase sustained drug delivery. The degradation of PLGA will also play a factor in the targeting and adhering properties of GP Ibα.

• Nanoparticle adhesion studies will need to be performed on vWF treated surfaces under higher shear stresses, and cellular uptake will need to be quantified using PLGA nanoparticles.

• Cellular uptake studies can also be performed using different cell types under static and shear conditions to quantify the effectiveness of GP Ibα nanoparticles.
Delivery of drug-loaded nanoparticles such as dexamethasone-loaded GPIbα nanoparticles in selected segments of the endothelium using animal models in the future is clearly a necessary step to completely understand and evaluate our novel biodegradable platelet-mimicking nanoparticles.
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BIOGRAPHICAL INFORMATION

Arthur Lin completed his Bachelor of Science Degree in Chemical Engineering at The University of Texas at Austin in August 2002. He was born in Kaohsiung, Taiwan on May 12, 1979, and moved to Texas with his family at the age of two. After receiving his degree, he worked for two years as a production engineer at Formosa Plastics Corporation, before pursuing a Master of Science Degree at the University of Texas at Arlington beginning in the Fall 2004. His research interests include drug delivery applications and tissue engineering, with a focus on nanoparticle-based systems.