TARGETED DRUG DELIVERY TO HUMAN AORTIC SMOOTH MUSCLE CELLS USING BIODEGRADABLE NANOPARTICLES

by

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ABSTRACT

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Restenosis is a common problem that can occur in angioplasty patients. In order to reduce the restentosis rate, various treatments such as glucocorticoids and drug eluting stents have been used for angioplasty patients. However, their limitations include the inefficient delivery of drugs to inhibit smooth muscle cell proliferation, a major cause of restenosis. Therefore, we propose the use of biodegradable nanoparticles as a drug delivery system for transporting a therapeutic agent to human aortic smooth muscle cells (HASMC) to prevent their proliferation. In this study the glucocorticoid dexamethasone (DEX) was chosen as the therapeutic agent. A biodegradable polymer, poly ($_{D,L}$lactide-co-glycolide) (PLGA) was used to formulate the nanoparticles through the evaporative emulsion technique. The mean nanoparticle diameter was found to be approximately 130 nm with a range of 88-190 nm. Various uptake studies were conducted to determine the optimal nanoparticle incubation time, dosage, and biocompatibility. Based on the results it was concluded that PLGA nanoparticles were biocompatible to HASMC, and the optimal time and concentration for cellular uptake were

4 hours and 800 μg/ml, respectively. Drug release studies indicated PLGA nanoparticles produced a sustained release of DEX. Over a period of 3 weeks about 12 % of DEX was released from the nanoparticles. HASMC proliferation studies conducted using DEX indicated that cellular proliferation is significantly reduce when using a DEX concentration of 100 nM or higher. Additional studies were performed to compare our DEX encapsulated nanoparticles against non-encapsulated DEX. The comparative studies suggested that PLGA nanoparticles encapsulated with DEX were more sufficient than free DEX (DEX added directly in the cell media) to inhibit HASMC proliferation. Lastly, the nanoparticles were ligand conjugated to PDGF-BB and compared against non-targeted nanoparticles. It was evident that the cellular uptake on non-targeted nanoparticles was significantly less than that of PDGF-BB targeted particles. Our results suggest that PDGF-BB peptide conjugated PLGA nanoparticles can be used as a targeted and controlled drug delivery vehicle to reduce the restenosis rate for patients who are undergoing cardiovascular interventions.

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LIST OF ABBREVIATIONS

PTC	Percutaneous Transluminal Coronary
PTCA	Percutaneous Transluminal Coronary Angioplasty
DES	Drug Eluting Stent
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
SMC	Smooth Muscle Cell
VSMC	Vascular Smooth Muscle Cell
HASMC	Human Aortic Smooth Muscle Cell
EDC	1-ethyl-3-(3-dimethylaminopropyl) Carbodiimide
DEX	Dexamethasone
PDGF	Platelet Derived Growth Factor
NP	Nanoparticle
PVA	Polyvinyl Alcohol
PBS	Phosphate Buffered Saline
DI	Deionized

W/V..... Weight by Volume

TEM..... Transmission Electron Microscope

MES..... 2-Morpholinoethanesulfonic Acid

CHAPTER 1

INTRODUCTION

1.1 Cardiovascular Disease

Heart disease is the leading cause of death in the United States, accounting for almost 700,000 deaths per year (1). The most common type of heart disease is coronary heart disease, killing more than seven million people each year worldwide according to the Center for Disease Control. Economically, over $250 billion was estimated to be spent in 2006 for health care for heart diseases. It is unfortunate that cardiovascular disease is the number one killer amongst men and women of most ethnicity when it could be prevented. There are six risk factors of cardiovascular disease: high blood pressure, high cholesterol, diabetes, smoking, lack of physical activity, and obesity (1). The majority of these factors can be prevented with proper diet and exercise. However, with the unhealthy sedentary lifestyles led by an overwhelming part of society, heart disease will continue to be a problem.

Angioplasty is a common procedure used to dilate blocked arteries for treatment of patients with cardiovascular diseases. This procedure calls for a shunt, consisting of a short tube, to be inserted into an artery followed by the insertion of a balloon catheter. By using x-ray imaging on a monitor, the balloon catheter is guided to the blocked artery, and then inflated to widen the artery wall in order to restore the blood flow to the heart. After the balloon is deflated, a stent can be placed at the site to support the arterial wall and to prevent renarrowing of the artery. There were an estimated one million percutaneous transluminal coronary (PTC) procedures performed in the United States in 2000, of those procedures 465,000 required the use of intracoronary stents (2). Stenting is becoming popular due to its advantages over having angioplasty alone. Stents act as a mechanical scaffold providing strength to prevent the collapse and the renarrowing of the artery.

A problem with the angioplasty/stenting procedure is that the endothelial cells lining the artery wall can be damaged, causing neointimal hyperplasia, the underlying vascular smooth muscles cells (SMCs) migrate and proliferate at the injured vessel wall (2-4). Initially the arterial injury is caused by the stent and the balloon inflation (2, 4). The injury is heightened as cells migrate to the injured site. Macrophages and neutrophils migrate to the damaged site where they release chemokines (2-4). The release chemokines encourage the migration of smooth muscle cells and the remodeling of the extracellular matrix. The smooth muscle cell migration and proliferation can lead to a reoccurrence of the arterial blockage. Restenosis, the renarrowing of the artery, is an associated response to the vascular injury (5). According to published data, about 20-39% of all the percutaneous transluminal coronary angioplasty (PTCA) results in restenosis (2). One of the major risk factors associated with restenosis include other diseases such as diabetes. Additional factors are the length of the stent and the size of the vessel. It has been demonstrated that the small vessels tend to have a higher restenosis rate (6). Other contributing factors are the amount of damage caused by the procedure itself and the stent design (7, 8). Another problem with stenting is the arterial branching and bifurcations as stent implementation may not be possible.

1.2 Current Treatment Options

Restenosis is an ongoing problem with balloon angioplasty and stenting. Various methods such as the systemically administration of therapeutic agents, drug eluting stents, and radiation have been investigated as a treatment option for the prevention of restenosis. Therapeutic agents such as antithrombotic agents and glucocorticoids have been administered to decrease the inflammation and neointimal hyperplasia, which in turn would reduce the high restenosis rate (9). Given systemically these agents have typically failed to deliver sufficient therapeutic concentrations to the injured areas (9). When drugs are given systematically the therapeutic effect is not localized to the injured area but instead it may be delivered to other

organs. Non-localized administration of drugs results in adverse side effects, inadequate dosing, and possible toxicity if taken at high doses. The concentrations of medicines at the site of infection in the coronary artery may not have been sufficient as there may have been a limited amount of systemic administration due to the side effects (2). To date there has been no consistent reduction of restenosis using systemic drug therapy.

Drug-eluting stents (DES) are also being used to prevent in-stent restenosis (9). The purpose of drug-eluting stents is to allow the therapeutic agent to elute into the tissue surrounding the stent (9). The advantage of drug-eluting stents compared to systematic drug treatment is that only the localized area is targeted. However, studies indicate that the polymer coating itself can cause complications. In animal studies it was noted that the polymers increase the inflammation and neointimal hyperplasia compared to bare metal stents (10.11). Aside from ensuring that the polymer is biocompatible, it should be suitable for sterilization. Although DES has shown great promise to successfully reduce restenosis many researchers are looking for a more effective method for local drug delivery to prevent the proliferation of smooth muscle cells. We propose the use of targeted biodegradable nanoparticles as a drug delivery system. The localized drug delivery will prevent the loss of therapeutic agent, resulting in a more effective therapy.

1.3 Nanoparticles

Nanoparticles (NP) have shown great promise as delivery vesicles for various disorders. When determining the necessary properties of materials to form the nanoparticles the desired application must be taken into consideration. The polymer chosen to formulate the nanoparticles can be a natural or synthetic polymer provided that it is biocompatible (no immunogenic reaction should occur when placed inside the human body). The materials required to create the particles may also be biodegradable or non-biodegradable depending on the application. The advantage of natural polymers such as collagen or gelatin is that they

encompass properties similar to the body (12). However, they are not as functional as synthetic polymers. Synthetic polymers are favorable due to their ability to be manipulated. The mechanical strength, degradation, and biocompatibility can by manipulated to attain the desired properties (13, 14). Copolymers can be easily formulated, resulting in a polymer with different qualities. The most common example of this is poly ($_{D,L}$ lactic-co-glycolic acid) (PLGA) which is a copolymer of poly (lactic acid) (PLA) and poly (glycolic acid) (PGA). Also, synthetic polymers are less complex than natural polymers; resulting in an increased understanding of synthetic polymers as compared to natural polymers (15, 17). Natural polymers vary in purity and mechanical strength. The disadvantage is that synthetic materials can initiate an immune response.

It is also advantageous if the polymers are biodegradable. Biodegradability allows for the polymer to be broken down following implantation and eventually there will be no remaining materials. PLGA is an example of a biodegradable polymer that degrades into lactic and glycolic acid through autocatalytic hydrolysis of the ester bonds (16). The bi-products of PLGA are further degraded through the Kreb's cycle (13). Although it is desired to have a biodegradable polymer, non-biodegradable polymers can also be used to evaluate expected outcomes of drug delivery carriers. This allows for the investigation of the drug delivery system without the concern of an additional factor of degradation.

Numerous vehicles have been characterized for their ability to be used for drug delivery applications. A few of the recent developments include microparticles, nanoparticles, and liposomes (18-21). Microparticles particles were not successful due to their poor ability to maneuver in the body. Nanoparticles are well suited for the human body due to the fact that the size of the particles is smaller than 5 μm, which is the size of the smallest capillary in the body (15). Not only will the microparticles be cleared from circulation they may also cause an immune response, such as a fibrotic encapsulation (22-24). Lipids seemed to offer a promising avenue for drug delivery. Liposomes are important because their properties are similar to that

of the body. They form a bilayer, so ideally drugs can be loaded inside the inner layer (21). Liposomes have shown low encapsulation efficiency, poor storage stability, and there was leakage of water-soluble drugs into the blood (25). Liposomes can be modified to improve their effectiveness as a drug delivery system (26). However, they are easily removed from circulation by the reticular endothelial system. Biodegradable nanoparticles have an advantage over liposomes with their ability to have a sustained drug release and remain within circulation for an extended amount of time.

There are some concerns when using nanoparticles and its effects on biological systems. Some studies suggest that nanomaterials affect biological behaviors at the cellular, subcellular, and protein levels (36). There are also concerns about the long-term side effects associated with nanoparticles (37). Studies are being conducted *in vitro;* however, we do not know how efficient the cellular uptake will be *in vivo*. *In vitro* experiments are under static conditions while *in vivo* experiments are under dynamic conditions. Due to the shear stress of dynamic conditions it can not be concluded that the particles will behave as in static conditions.

Additional factors to consider when formulating nanoparticles for drug delivery are the optimal particle size and the method to incorporate therapeutic drugs into the particles. The particle size is also determined based on the desired purpose. Microparticles are unable to disperse throughout the body. Due to their size the particles undergo phagocytosis following intravenous administration (15). The cellular uptake efficiency increases 15-250 fold when using nanoparticles as opposed to microparticles (27). However, microparticles have a better loading efficiency as compared to nanoparticles (28, 29). There are several different methods to incorporate therapeutic agents into nanoparticles. Drugs can be encapsulated within or attached to the particles. The encapsulation of drugs can occur by entrapment into the polymer matrix or encapsulation in the nanoparticle core. Drug can also be conjugated to the polymer or bound to the surface by adsorption (15). It can be deduced that it would be advantageous to investigate synthetic, biodegradable nanoparticles for controlled drug delivery. Therefore, we

will investigate the use of the synthetic polymer PLGA to form biodegradable nanoparticles in our studies.

Several techniques are used to formulate nanoparticles; the most common method is an emulsification-solvent evaporation technique. This technique consists of dissolving the polymer in an organic solvent (oil phase, o) while the hydrophilic drug is dissolved in water (water phase, w). The primary emulsion is made by adding the aqueous phase to the organic phase (w-o). Nanoparticles are induced by sonication. A stabilizer is dissolved in a separate water phase. The primary emulsion is added drop wise to the stabilizer solution to create a double emulsion (w-o-w). Following evaporation of the organic solvent, the nanoparticles are collected by centrifugation and lyophilization. The described protocol is for a double emulsion, but a single emulsion can be made as well (15, 31). Another method is nanoprecipitation, where the organic solvent is used to dissolve the polymer and drug. This solution is then added to the aqueous solution. The solvent is evaporated under reduced pressure; water is then added to the solution. This results in the formation of droplets of nanoparticles within an aqueous layer because the polymers spontaneously diffuse into the aqueous phase. The nanoparticles are collected through filtration or centrifugation (30). Although both methods involve toxic solvents, there are some advantages to using the emulsification-solvent evaporation technique. With this technique there is flexibility in the solvent and surfactant used for formulation (32). The emulsification technique results in a better size distribution and it does not rely on diffusion to formulate the nanoparticles (13). Accordingly PLGA nanoparticles will be formulated using the emulsification-solvent evaporation technique for our studies. After formulating the particles, conjugation can be used to target the nanoparticles for localized drug delivery.

Targeting nanoparticles can be accomplished by active or passive targeting (33-35). Active targeting requires the particle to be conjugated to the cell-specific ligands or antibodies. Passive targeting is achieved by coupling the therapeutic agent to a macromolecule or carrier

(34). The most common method for active targeting is avidin-biotin affinity (35). The carboxylic acid groups of the polymer are employed in this technique. The investigated carbodiimide is 1-ethyl-3-(3-dimethlaminopropyl) carbodiimide (EDC). EDC binds to the carboxylic acid groups of the polymer. The tetramer Avidin, a protein isolated from egg whites, is bound to the EDC. Biotin, vitamin H, is binded to the ligand or peptide. The biotinylated ligand is then conjugated to the avidin nanoparticles. This is possible due to avidin's high affinity for biotin. Following conjugation the nanoparticle will be bound to avidin, which will have four biotin binding sites. The avidin-biotin affinity will be used to conjugate the formulated nanoparticles to the peptide. The peptide will specifically bind to receptors on the smooth muscle cells.

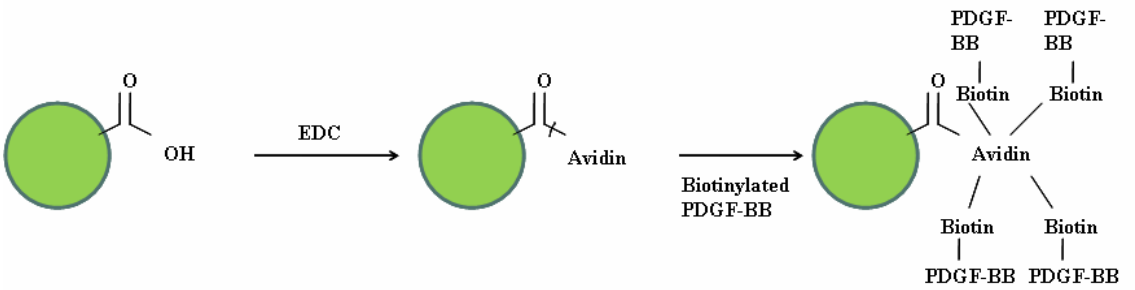

Figure 1.1. Schematic of avidin-biotin affinity used to conjugate nanoparticles to the peptide.

1.4 Targeting Smooth Muscle Cells

The artery is comprised of three layers: the outer adventitia, medial layer, and inner intima. Following vascular reconstruction and angioplasty the smooth muscle cells (SMC) migrate from the medial layer to the intima, resulting in neointimal hyperplasia (38). The SMC migration is stimulated to the intima by damage to the endothelium. As a result SMCs undergo a morphology change that causes uninhibited proliferation and eventually lesion. Typical SMCs found in the intima change from a 'contractile' to a 'synthetic' phenotype (41). In order to characterize the morphological changes in injured SMCs, animal studies have been conducted. It is found that in adult rats the phenotype of SMCs after balloon injury is similar to that of baby rats (38). Studies suggest that SMC migration begins three days after arterial injury (42). The SMC exit the quiescent state (G_0) and progress through the cell cycle and begin cellular

proliferation. The number of arterial smooth muscle cells tends to remain the same after two weeks (39). Therefore, it is pertinent to target a receptor that is expressed immediately following arterial injury. In normal blood vessels the SMCs expression of PDGF is low while after injury the PDGF expression increases (39).

1.5 Platelet Derived Growth Factor

Platelet derived growth factor (PDGF), hydrophilic cationic glycoprotein, is a potent chemoattractant for SMCs (48). It has three different isoforms: PDGF-AA, PDGF-AB, and PDGF-BB. The difference between the three forms is the binding receptor and binding components of the extracellular matrix (41). The two receptor subunits are PDGF-α and PDGF-β (41, 52). Figure 1.2 depicts the three isoforms of PDGF and their binding receptors. PDGF-BB has an advantage over the other isoforms due to its ability to bind to all forms of the receptor. Furthermore, PDGF-BB and PDGF-AB have similar mitogenic activity where as PDGF-AA has lower mitogenic activity (50, 51). Not only is PDGF-AA a poor mitogen it may act as an inhibitor to cellular migration (50, 51). There is a marked increase in the PDGF-α and PDGF-β receptors after balloon injury (51). PDGF-BB binds to both receptors with high affinity.

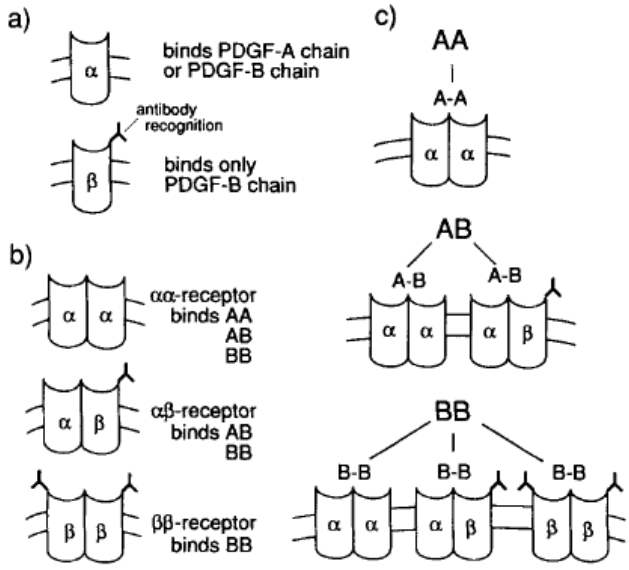

Figure 1.2. PDGF isoforms and receptors: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-α and PDGF-β, respectively (52).

The time and spatial expression of PDGF receptors suggests that PDGF-BB peptide may be a potential target ligand to prevent stimulates SMC proliferation in injured blood vessels. In normal vessels transcripts for PDGF-B, PDGF-A, and the PDGF receptors are expressed in low concentrations (39). Within the first four hours following arterial injury the PDGF-β decreases where as there is an increase in the PDGF-A mRNA (53). Between two to seven days there is an increase in the PDGF-β (38). When compared at seven to fourteen days there was an abundance of PDGF-β as compared to that of PDGF- α (38). This implies that larger amounts of PDGF-β are found within the SMCs after arterial injury. The principal effect of PDGF is the SMC migration into the intima (39). Therefore, it would be beneficial to investigate the effects of inhibiting the PDGF-β receptor. As shown in Figure 1.2, only PDGF-BB binds to both α and β PDGF receptors.

Several attempts have been made to target PDGF in treatment of arterial injury. These attempts include antibodies against PDGF and its receptors, receptor antisense oligonucleotides, low-molecular-weight PDGF receptor tyrosine kinase inhibitors, and local over expression of PDGF-β receptor in the extracellular domains (54). It has been demonstrated that PDGF antibody blocks SMC migration, this suggests that PDGF is a major component of SMC migration. Inhibition of PDGF with a blocking polyclonal antibody tremendously reduces the migration of SMC and intimal thickening (55). It can be deduced that PDGF would be a remarkable targeting receptor and PDGF-BB would be an ideal conjugating ligand due to their functions in cellular migration and proliferation. By binding PDGF-BB to the PDGF receptor mitogenesis was inhibited. So far there have been no attempts to target the PDGF receptor using the PDGF-BB ligand to target proliferative SMC and prevent SMC proliferation.

However, since we are conducting *in vitro* studies SMCs must be stimulated to express PDGF and receptor expression. Utilizing rat models, studies suggest that PDGF is expressed in SMCs without stimulation. The PDGF-B chain mRNA was detected in vitro with an increase in passage number (40). Furthermore, it is suggested that there is an abundance of PDGF-α

receptor mRNA and little to no amount of PDGF-B mRNA (40). Others have demonstrated that PDGF-BB have a potent chemotactic effect on SMCs *in vitro* (56). A limitation is that PDGF is expressed on several cell types. Cells that form PDGF include platelets, monocytes, vascular endothelial cells, arterial smooth muscle cells, and embryonic cells (57). However, it has also been noted that large vessel endothelial cells do not contain PDGF-β receptors (57).

1.6 Dexamethasone

In attempts to control intimal hyperplasia, pharmacological agents such as anti-platelet, anti-lipid, anti-hypertensive, and anti-inflammatory have been investigated (38). Glucocorticoids are anti-inflammatory agents that have an important role in maintaining and regulating the growth and differentiation of SMCs (43). The receptors for glucocorticoids are found within the cytoplasm. This is an important factor since the nanoparticles are internalized within the cytoplasm. The glucocorticoid receptor (GR) is localized in the cytoplasm until ligand binding, after which the ligand-receptor complex is translocated into the nucleus (43). Glucocorticoids appear to inhibit cellular proliferation through inhibition of key cell cycle regulatory genes (44). This is made possible when the ligand-receptor complex is inside the nucleus and it binds to specific DNA sequences. An example of a synthetic glucocorticoid is dexamethasone.

Dexamethasone (DEX) has been proven to be an immune suppressant that decreases the development of the intimal hyperplasia (45). DEX was shown to be effective in reducing SMC proliferation. Studies suggest that DEX induces a late G_1 phase arrest in the smooth muscle cell cycle (46, 47). Therefore, cells remain in the G_1 phase of the cell cycle and are not allowed to proceed into the S phase where cellular proliferation occurs. Unfortunately, a continuous drug concentration is needed because over a period of time the cells will progress into the S phase. With a sustained therapeutic concentration the cells will constantly be in a state of arrest. Figure 1 is a schematic of the SMC cell cycle and the site of action of DEX. In

the figure G_0 is growth phase$_0$, G_1 is growth phase$_1$, G_2 is growth phases$_2$, S is synthesis, and M is metaphase (2).

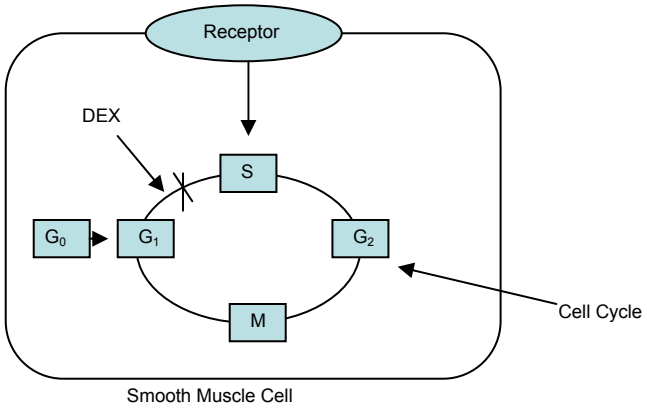

Figure 1.3. Schematic of DEX inhibition of the HASMC: DEX arrests the HASMC cell cycle at the late G_1 phase.

It can be concluded that the administration of DEX will inhibit cellular proliferation of human aortic smooth muscle cells (HASMC). However, it has been demonstrated that only prolonged exposures to DEX is effective for cellular inhibition. Short-term exposure to DEX results in proliferative effects (43). The mechanism behind this phenomenon is poorly understood. Since we are investigating sustained therapeutic release, DEX is the drug of choice. As of yet researchers have not investigated DEX encapsulated nanoparticles.

1.7 Overview of Research Project

The objective of this project is to use targeted nanoparticles encapsulated with a therapeutic agent to inhibit the proliferation of human aortic smooth muscles. The nanoparticles used in this research were formulated with poly ($_{D,L}$lactide-co-glycolide) (PLGA). PLGA was chosen as the polymer because of its ability to degrade via hydrolysis and well-characterized material properties. PLGA nanoparticles were produced using an evaporative emulsion technique and loaded with a fluorescent marker and therapeutic agent. The fluorescent marker used for the study was 6-coumarin and the selected therapeutic agent was dexamethasone (DEX). It has been demonstrated that DEX is an effective anti-proliferative and anti-

inflammatory agent. Knowing that the expression of PDGF receptors is increased after angioplasty it is the ideal targeting receptor. PDGF-BB has the capability to bind to all forms of the PDGF receptor, so PDGF-BB would be the perfect ligand. The nanoparticles may provide an avenue for sustained delivery of a therapeutic agent towards SMCs, thereby reducing cellular proliferation. Figure 1.3 is a schematic of the proposed project.

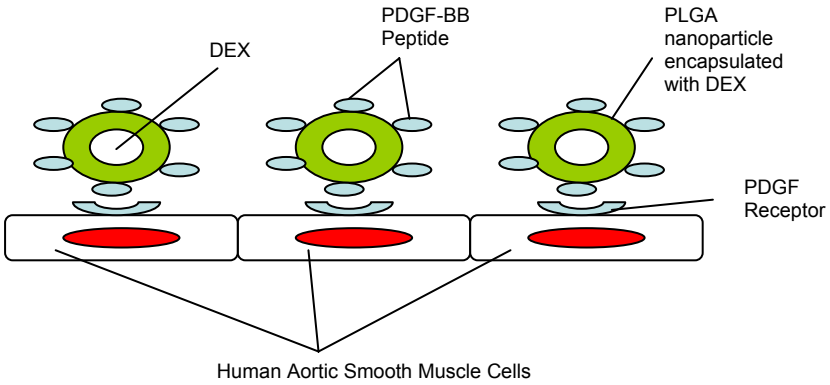

Figure 1.4. Schematic of project overview: PDGF-BB conjugated nanoparticles targeting the PDGF receptor of HASMC.

It is hypothesized that PDGF-BB conjugated nanoparticles (~100 nm) can target and bind to the human aortic smooth muscle cells, and provide a sustained release of DEX to inhibit cellular proliferation. This hypothesis is based on the following observations:

- Studies have shown that cellular uptake of nanoparticles is optimal with a particle diameter of 100 nm.
- Smooth muscle cells migration and proliferation are responsible for the cause of restenosis.
- It has been shown that DEX demonstrates SMC proliferation inhibition.
- PDGF receptor is shown to increase after angioplasty and is an important factor in SMC migration.

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

- Aim 1 – Develop PLGA nanoparticles and evaluate their efficiency in providing sustained drug release.
- Aim 2 – Evaluate the effectiveness of DEX encapsulated nanoparticles in inhibiting HASMC proliferation.
- Aim 3 – Conjugate PLGA nanoparticles with PDGF-BB and determine the effectiveness of cellular uptake when using PDGF-BB peptides as a targeting ligand.

The novelty of this project is the use of PDGF-BB conjugated nanoparticles as a potential treatment for restenosis. After conducting a literature review one can conclude that the PDGF receptor has a direct role in SMC proliferation following cardiovascular injuries. Therefore, the targeted nanoparticles would affect the injured area. Once the particles bind to the receptors the therapeutic agent, DEX will be released to inhibit HASMC proliferation. Moreover, the targeted drug delivery can service as an alternative treatment option for restenosis.

A successful outcome for this project would offer a method to transport therapeutic agents directly to human aortic smooth muscle cells that are proliferative. DEX encapsulated nanoparticles investigated in this project could either be developed as drug delivery devices or used as models for investigating other therapeutic agents. PDGF-BB can service as a model ligand to target receptors on HASMC.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Poly ($_{D,L}$lactide-co-glycolide) (MW ~40,000, copolymer ratio 50:50) was purchased from Lakeshore Biomaterials (Birmingham, AL). DEX and 6-coumarin were obtained from Sigma-Aldrich (St. Louis, MO). PDGF-BB peptide and antibody were purchased from Abcam Inc. (Cambridge, MA). Human aortic smooth muscle cells (HASMC) were purchased from Cascade Biologics (Portland, OR). Dulbecco's Modified Eagle's Medium (DMEM), serum, penicillin-streptomycin, and trypsin-EDTA were acquired from Invitrogen Corporation (Carlsbad, CA). Chloroform was purchased from Merck KGaA (Gibbstown, NJ). Other chemical such as Triton®-X 100, and polyvinyl alcohol (PVA) were purchased from Sigma-Aldrich.

2.2 Methods

2.2.1 Formulation of PLGA Nanoparticles

The PLGA nanoparticles were formulated using an evaporative emulsion technique consisting of a water-in-oil-in-water emulsion. To create PLGA nanoparticles, three solutions were prepared prior to the formulation. First, a 5% (w/v) polyvinyl alcohol (PVA) solution was produced by dissolving 0.6 g of PVA in 12 ml of deionized water (DI) overnight. The PVA solution was then filter using a 0.22 µm filter. Secondly, the 3% (w/v) PLGA solution was made by dissolving 0.09 g of PLGA into 3 ml of chloroform. To this solution 100 µl of 6-coumarin solution, (5 mg of 6-coumarin dissolved in 1 ml chloroform) was added. Third, a 10% (w/v) DEX solution was made by dissolving 0.03g of DEX into 300 µl of DI water. After the solutions were completely prepared, 100 µl of the DEX solution was added into the PLGA solution to form the primary water/oil (W/O) emulsion. The primary emulsion was produced using a sonicator

(Misonix Inc.,Farmingdale, NY), set at 30 W for 30 seconds. This emulsion was then added drop wise using a syringe to the aqueous PVA solution, and then further emulsified using a sonicator, set at 39 W for 1 minute cycles for a total of 3 minutes, to create a double W/O/W emulsion. The emulsion was then stirred overnight to allow for any excess solvent to be evaporated. The solution was then ultra centrifuged (Beckman Coulter, Inc., Fullerton, CA) at 30,000 rpm for 20 minutes at 15º C. The supernatant was collected and stored for later use, the indirect determination of the DEX loading efficiency. The pellet was resuspended in DI water, and then again sonicated for 30 seconds at 30 W. Following re-suspension, the solution was centrifuged (Thermo Fisher Scientific Inc., Waltham, MA) at 1000 rpm for 10 minutes to collect aggregates. The supernatant, containing the nanoparticles, was stored, and lyophilized for several days. Lyophilized nanoparticles were then used immediately or stored at -20 ºC until used for experiments. The same double emulsion technique was used to formulate several types of nanoparticles including: Nanoparticles with and without DEX and Nanoparticles with and without 6-coumarin.

2.2.2 Characterization of Nanoparticles

Nanoparticles were characterized for the morphology, particle size, and size distribution using transmission electron microscopy (TEM) and a dynamic light scattering technology. The size and shape of the synthesized DEX loaded PLGA nanoparticles were determined by TEM (JEOL 1200 EX Electron Microscope). For TEM, an aqueous dispersion of the nanoparticles was allowed to adsorb onto a carbon-coated formvar grid. The nanoparticles were then stained with 1% uranyl acetate (UA) and the grid was then dried at room temperature before viewing under the microscope. The range of particle size was further analyzed using a dynamic light scattering method (Nanotrac, Microtrac Inc.). Nanoparticles were suspending in DI water at a concentration of 1mg/mL, then sonicated at 30 W for 30 seconds. Using a pipette, small drops

were added to the loading well until the computer noted the sample amount to be sufficient. The solution was then scanned in order to determine the particle size distribution.

2.2.3 Culture of HASMC

HASMC were cultured in Dulbecco's Modified Eagle's Medium (DMEM) complete media unless otherwise noted. DMEM complete media was supplemented with 1% penicillin-streptomycin and 10% serum. Cells were grown until the flasks were 90% confluent. Upon confluence, cells were either passaged or used for experiments. All HASMC used in experiments were from passages three through eight. Procedures involving cell culturing were performed in a laminar flow hood using aseptic techniques unless stated otherwise. All incubations were performed at 37º C and 5% $C0_2$. For cell nanoparticle studies, low serum growth medium supplemented with 1% penicillin-streptomycin and 1% serum was used.

2.2.4 Nanoparticle Uptake Studies by Fluorescent Measurements

Cellular uptake was characterized using PLGA nanoparticles with 6-coumarin only. Uptake experiments included optimal time, dosage, and biocompatibility analysis. In order to evaluate cellular uptake of PLGA nanoparticles, HASMC were seeded on a 24 well plate (Falcon®, Becton Dickinson Labware) at a seeding density of 20,000 cells per well and grown for 2 days. A stock nanoparticle solution was prepared at a concentration of 2 mg/ml in low serum growth medium. The nanoparticle solution was sonicated at 30 W for 30 seconds after the nanoparticles were dissolved in the media. Uptake studies consisted of a time and dose study. For both studies, which were performed concurrently, the complete medium was replace with low serum medium at least 5 hours before conducting the experiments.

For the dose study, various dosages of the nanoparticle solution ranging from 0-1000 µg/ml were prepared from the stock solution. Low serum medium was removed from the 24-well plate and replaced with the various concentrations of nanoparticle solution. Cells were

incubated with these solutions for one hour (Forma Scientific water-jacketed incubator, Thermo Electron Corp.). After 1 hour of incubation, the media was collected, and cells were washed 3 times with phosphate buffered saline (PBS), and then lysed with 1% Triton®-X 100 for 30 minutes.

The time study had a constant concentration with a variance in the incubation time of the nanoparticle solution. The nanoparticle solution was added to the wells at a concentration of 200 µg/ml and incubated for 0-6 hours at 37 ºC. At the end of each time point, nanoparticle solutions were removed and the remaining cell monolayers were washed with PBS to remove any remaining nanoparticles. One percent Triton was then added to the 24 well plate and incubated for 30 minutes at 37 ºC in order to lyse the cells.

For both dose and time studies, the collected cell lysates were analyzed for total cell protein content using the Pierce BCA protein assay and for the nanoparticle uptake by measuring fluorescent intensities. For the Pierce BCA protein assay (Fisher Scientific), 25 µl of cell lysate from each sample was used. Working reagent was added to the lysate samples, and the absorbance of the sample was determined by a microplate reader (Infinite M200,Tecan USA, Inc.) at 562 nm. The total cell protein was determined against a BSA protein standard curve. To determine the amount of nanoparticles internalized by cells, 100 µl of cell lysate from each sample was added to a 96 well plate. The well plate was read on a microplate reader at EM 480 nm/EX 510 nm to determine the sample fluorescence intensity. Serial dilutions of stock nanoparticle solution in 1% Triton®-X 100 were also used to obtain a standard curve, and the sample particle concentration was determined based on the standards. The uptake of the nanoparticle by the HASMC was calculated by normalizing the particle concentration with the total cell protein of each sample. The determined optimal time and dosage were used for later studies.

2.2.5 Nanoparticle Uptake by Confocal Microscopy

Uptake of nanoparticles was also visualized using confocal microscopy. HASMC were seeded on glass cover slips (Fisherbrand®, Fisher Scientific) at a density of $2x10^4$ cells/cm^2 and grown similar to that of tissues culture well plates as described earlier. For confocal experiments, cells were incubated with 200 µg/ml of PLGA nanoparticle solution for 1 hour, and then washed with PBS. Following that the cells were then incubated with cell membrane dyes FM® 4-64 FX (Texas-Red® dye, Invitrogen) at 5 µg/ml in PBS for 10 minutes. Samples were observed using a Leica confocal laser scanning microscope equipped with a FITC filter (Ex(λ) 488 nm, Em(λ) 543 nm) and TRITC (Ex(λ) 465 nm, Em(λ) 744 nm) with the option of stacking sample sections.

2.2.6 Biocompatibility

To determine the biocompatibility of the PLGA nanoparticles against HASMC, a Lactate dehydrogenase cytotoxicity (LDH) assay (Promega Corp.) was performed. The media removed during dosage study (section 2.2.4) was utilized to measure the LDH released in the media from the damaged cells. 50 µl of media from each sample was place in a 96 well plate. To each well 50 µl of substrate was added. The positive control consisted of 100% dead cells treated with 1% Triton®-X 100; and the sample without nanoparticles served as a negative control. After incubating the well plate for 30 minutes, the well plate was read on a microplate reader at 490 nm. A background for each nanoparticle concentration was subtracted from the absorbance readings.

2.2.7 Drug Loading and Release Studies

The loading and release profiles of DEX from PLGA nanoparticles encapsulated with and without 6-coumarin were analyzed. DEX-loaded PLGA nanoparticles with and without coumarin were formulated using the protocol listed in section 2.2.1. The supernatant collected

from the nanoparticle formulation was used to determine the loading efficiency. This value was compared to the total amount of DEX used in the nanoparticle formulation protocol section 2.2.1 to determine the loading efficiency of the nanoparticles.

For the drug release studies, a stock solution containing 200 µg/ml of DEX-loaded nanoparticles was prepared in 0.1 M PBS for both samples. The solutions were sonicated at 30 W for 30 seconds. One milliliter of stock solution was placed inside dialysis bags (Spectrum Laboratories Inc.) with a molecular weight cutoff of 1,000 (the molecular weight of DEX is 392.47 AMU). Samples were dialyzed against 10 ml of PBS inside a shaker (Jeio TECH) with a temperature of 37 °C for a total of 21 days. At the various intervals, 1 ml of dialysate was removed from each sample and replaced with 1 ml of fresh PBS. The collected dialysate was stored at -20 °C for later analysis. To analyze the amount of drugs released, 100 µl of each sample was added to a 96 well plate, and the DEX absorbance was read at 242 nm. The amount of released drugs was determined against a standard DEX curve. That amount was then correlated to the loading efficiency to determine the cumulative percent drug release.

2.2.8 In Vitro HASMC Anti-proliferation Studies of DEX and DEX-loaded PLGA Nanoparticles

To determine the optimal dexamethasone dosage, HASMC were seeded on a 24 well plate at a density of 10,000 cells per well. The following day the medium was removed and medium with DEX solutions were added to the well plates. The DEX solutions consisted of DEX and complete DMEM with concentrations varying from 0-2000 nM. Cells were incubated with these DEX solutions for a total of four days. During that time the plate was observed daily using an inverted microscope, to evaluate cellular proliferation. After 4 days, the cells were lysed with 1% Triton®-X 100 for 30 minutes. The lysate was analyzed using a PicoGreen DNA assays (Ex(λ) 480 nm, Em(λ) 520 nm) to determine the total DNA which is corresponding to the number of cells present.

To compare the effectiveness of DEX in media or free DEX, and DEX released from our PLGA nanoparticles, the *in vitro* anti-proliferative study was conducted. For this study HASMC were seed overnight on a 48 well plate at a seeding density of 10, 000 cells per well. A nanoparticle solution of 600 µg/ml was prepared in low serum growth medium. For the free DEX, a dexamethasone solution of 25 µg/ml was prepared in complete DMEM. Half of the 48 well plate was treated with the nanoparticle solution and the other half was subjected to the DEX solution. The medium was changed on day two and every other day following that until the conclusion of the experiment, no additional drug was added. The experiment was conducted over a period of eight days with time points varying from day 0 to day 8. At each time point the medium was removed, and the cells were washed with PBS before being lysed with 1% Triton®-X 100 for 30 minutes. Analysis included using the Pierce BCA protein assay to determine the total cell protein which is corresponding to the total cell number as described earlier (section 2.2.4). A protein assay was used as oppose to the DNA assay as nanoparticles consisting of 6-coumarin dye may interfere with the PIcoGreen DNA assay.

2.2.9 Conjugation of Ligands onto Nanoparticles

The nanoparticles were conjugated with PDGF- BB peptide using avidin-biotin affinity. First, PDGF-BB peptide was biotinylated using the Biotin-X-NHS Kit (EMD Biosciences, Inc.). Sodium bicarbonate (0.1 ml) was added to the peptide solution (120 µl) with the desired concentration of peptide at 10 µg/ml. A 0.1 M solution of biotin/NHS was prepared by dissolving 11 mg of biotin/NHS into 0.25 ml of dimethylformamide. The biotin/NHS solution was then added to the protein solution. The protein/biotin solution was then incubated with gentle agitation using a rocker (Gyrotory® Water Bath Shaker) at room temperature for one hour. After incubation, the solution was dialyzed against 0.1 M PBS for one hour. Following that the solution was removed from the dialysis bag and stored at 4 °C until use.

Second, nanoparticles were incorporated with avidin through the activation of carboxyl groups on PLGA nanoparticles. For this reaction, an EDC/MES solution was prepared by adding 15 mg of EDC to 1 ml of 0.1 M MES buffer, pH 4.75. Approximately, 2.5 mg PLGA nanoparticles were measured out and added to the EDC/MES solution. The solution was then sonicated at 30 W for 10 seconds. The solution was allowed to react for five hours. During this time the solution was placed on a rocker with gentle agitation. After five hours, 500 µg of avidin (EMD Biosciences, Inc.) were added to the nanoparticle solution and allowed to dialyze overnight against 0.1 M sodium bicarbonate, pH 8.5. Once avidin attachment was completed, the biotinylated protein was added to the nanoparticle/avidin solution. The combined solution was incubated at room temperature for two hours with gentle agitation. Following the incubation period, the nanoparticle solution was dialyzed (100,000 MWCO) against PBS for three hours. The solution was then stored at 4 °C until analysis was performed.

To determine whether or not the ligands were successfully conjugated onto our nanoparticles, antibodies against PDGF-BB peptides were used. 100 µl of 30 µg/ml primary rabbit antibody monoclonal to PDGF-BB, was added to 100 µl of the ligand-conjugated nanoparticles solution prepared earlier. This solution was then incubated at room temperature one hour, and dialyzed for two hours against PBS. Next, was the addition of a fluorescent secondary antibody. 100 µl of 30 µg/ml fluorescent secondary antibody was added to the nanoparticle solution and followed the same procedure as for the primary antibody. The nanoparticle solution was then stored in a vial covered with foil at 4 °C until used for analysis.

2.2.10 Effectiveness of Targeted Nanoparticles

To determine if the PDGF receptor was expressed on the HASMCs, the cells were tested for the PDGF receptor. As described in earlier (section 2.2.5) cells were seeded at 2 x 10^4 cells/cm^2 and allowed to grow until confluency. The cells were incubated overnight at 4° C

with the primary antibody (10 µg/ml) for PDGF receptor. Following that the cells were further incubated for 1 hour with the secondary antibody (Texas Red) at room temperature.

In order to evaluate cellular uptake of PDGF-BB PLGA nanoparticles, HASMC were seeded on a 24 well plate (Falcon®, Becton Dickinson Labware) at a seeding density of 20,000 cells per well and grown for 2 days. The complete medium was replace with low serum medium at least 5 hours before conducting the experiments. In preparation for the uptake comparative study two stock solutions were made. The first stock solution was created using PLGA nanoparticles in low serum growth medium. The nanoparticle solution was sonicated at 30 W for 30 seconds after the nanoparticles were dissolved in the media. Secondly, a PDGF-BB nanoparticle solution was prepared in low serum growth medium. The stock solution was formulated using the protocol listed in section 2.2.9.

The optimal concentration and incubation time determined from the dose and time study, section 2.2.4, were used in this experiment. The cells were incubated at 800 µg/ml of nanoparticle solution for 4 hours at 37 ºC, low serum medium was removed from the 24-well plate and replaced either with low serum media, PDGF-BB conjugated nanoparticle solution, or PLGA nanoparticle solution. The aforementioned solutions were used to compare the control, peptide conjugated nanoparticles, and controlled release nanoparticles, respectively. After 4 hour of incubation, the media was collected, and cells were washed 3 times with PBS, and then lysed with 1% Triton®-X100 for 30 minutes. The collected cell lysates were analyzed for the total cell protein content using the Pierce BCA protein assay and for the nanoparticle uptake by measuring fluorescent intensities as described earlier (section 2.2.4).

CHAPTER 3

RESULTS

3.1 Nanoparticle Characterization

PLGA nanoparticles loaded with the therapeutic agent DEX were formulated using a double emulsion process. Using TEM, the average size of these nanoparticles was found to be about 130 nm (Figure 3.1). The formulate nanoparticles were further characterized by Nanotrac, to determine the size distribution. The particle size distribution ranged from 88-190 nm (Figure 3.2).

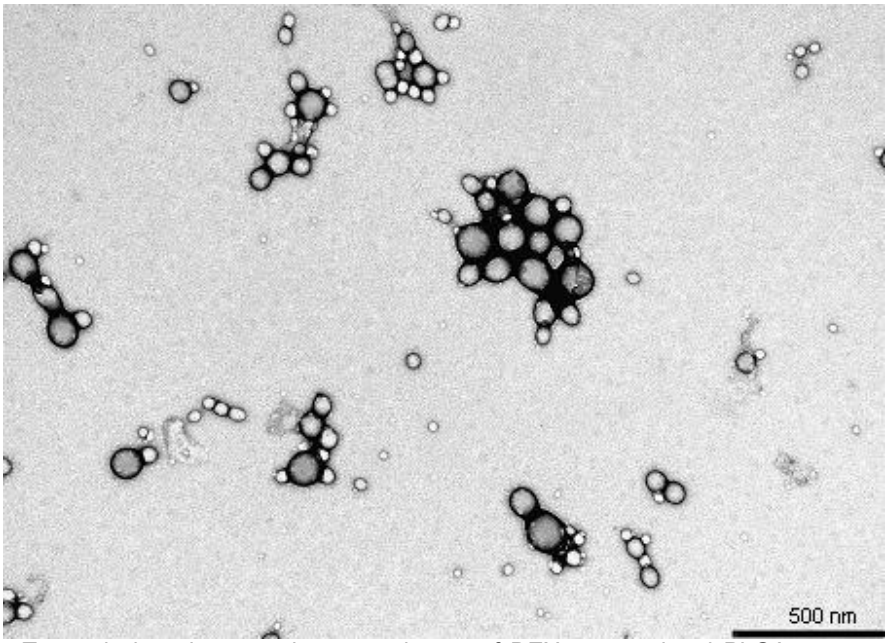

Figure 3.1. Transmission electron microscopy image of DEX encapsulated PLGA nanoparticles. The average size of the particles was about 130 nm.

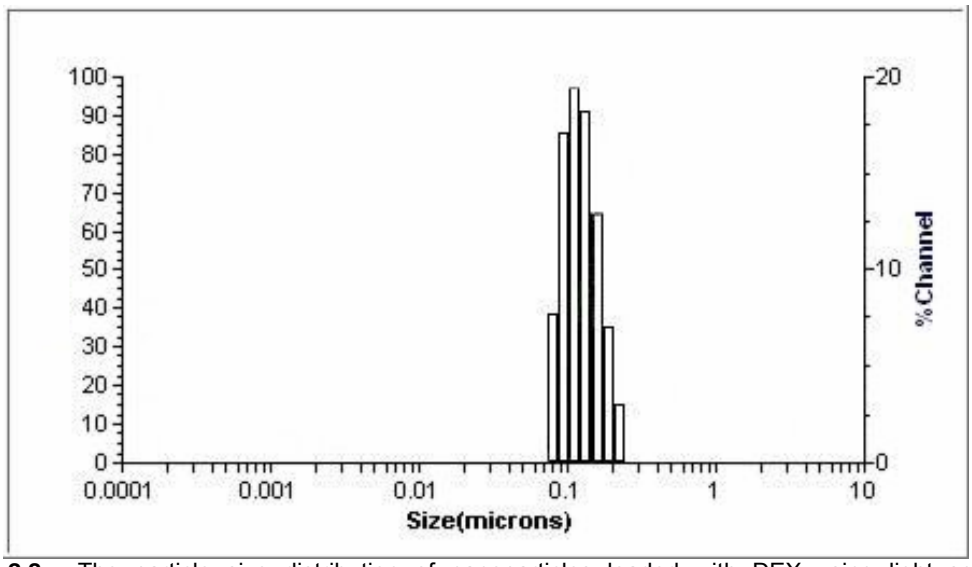

Figure 3.2. The particle size distribution of nanoparticles loaded with DEX using light scattering technology. The size rang of these nanoparticles was 88-190 nm.

3.2 Cellular Uptake

3.2.1 Uptake Studies by Fluorescent Measurements

To determine the optimal incubation time and concentration that the human aortic smooth muscle cells efficiently uptake the nanoparticles, cells were incubated with these particles over a range of time and concentration. The amounts of these particles within the cells were determined by fluorescent measurements and a protein assay. For the time study, it can be deduced that the optimal time was reached after four hours (Figure 3.3). As there is a linear trend in the increase of uptake until four hours is reached. As for the concentration study, there was also a linear trend shown until a point of saturation was reached at 800 µg/ml. Due to the similarities of the time and dose study only the graph for the time study is shown.

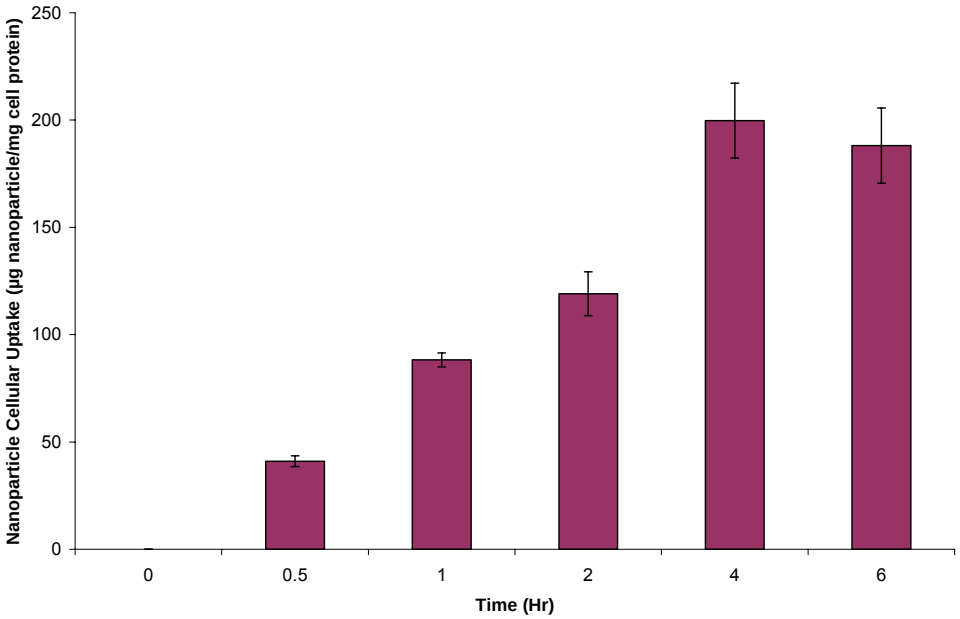

Figure 3.3. Nanoparticle Uptake Time Study: Uptake of nanoparticles in the HASMC is time-dependent. Values represent mean ± standard deviation (n=3).

3.2.2 Confocal Microscopy

Confocal microscopy was used to visually observe the cellular uptake of the PLGA nanoparticles. The FITC and TR filter were employed to visualize the PLGA nanoparticles loaded with 6-coumarin (green fluorescence) and the cellular membrane (red fluorescence), respectively. These two images at the center section of cells were overlapped, and results confirmed that the nanoparticles were localized within the cell cytoplasm (Figure 3.4).

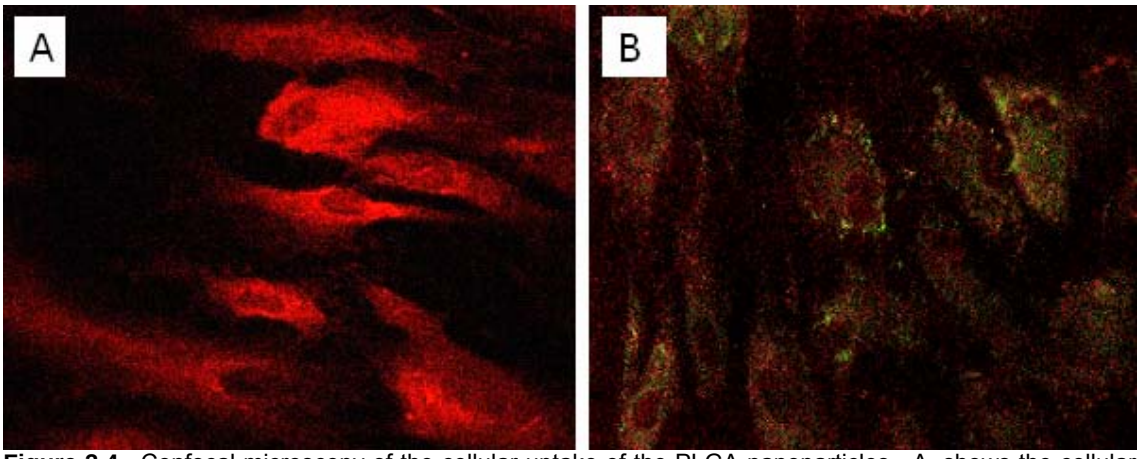

Figure 3.4. Confocal microscopy of the cellular uptake of the PLGA nanoparticles. A. shows the cellular membrane stained with Texas Red dye of the control samples (without nanoparticles). B. depicts the nanoparticles uptaken by the cellular membrane using the FITC and TR filter, respectively.

3.2.3 Biocompatibility

Following the uptake concentration study, the collected media was analyzed to determine the biocompatibility of PLGA nanoparticles. An LDH assay was used to find out if the various concentrations of nanoparticles are toxic to the cells. It was concluded that there was no more than 20% cell death for the different concentrations, and this percentage death is similar to the control samples that exposed to media only (Figure 3.5). All concentration values were found to be significantly less than the positive control; which was all dead cells. These results suggest that our PLGA nanoparticles are relatively biocompatible.

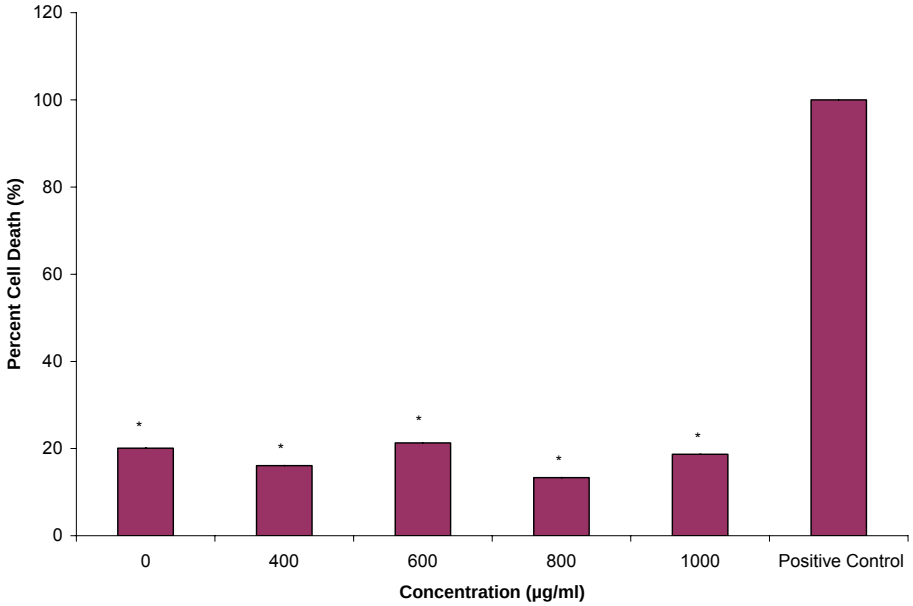

Figure 3.5. Biocompatibility of the PLGA nanoparticles with the HASMC. Values represent mean ± standard deviation (n=3). *Statistical difference versus positive controls.

3.3 Drug Characterization

3.3.1 Release Profile of Dexamethasone

In order to determine whether PLGA nanoparticle can effective deliver a sustained drug release or not, the release profile of DEX was analyzed. A comparative study was performed on DEX encapsulated nanoparticles versus DEX encapsulated particles with 6-coumarin to determine if the released 6-coumarin affected the evaluation of released DEX (Figure 3.6). The dialysate was used to determine the amount of DEX that was released from the particles. That value was then correlated to the particle loading efficiency to attain the cumulative drug release. Results indicate that there is relatively no difference between the release profiles of nanoparticles formulated with or without 6-coumarin. After 3 weeks DEX-NPs released about 12.54 % while DEX-NPs with coumarin released about 13.41 %. Similarly, loading efficiencies between these two nanoparticles is not significantly different. The loading efficiency DEX into the PLGA nanoparticles was about 65 and 64 % for DEX-NPs and DEX-NPs with 6-coumarin.

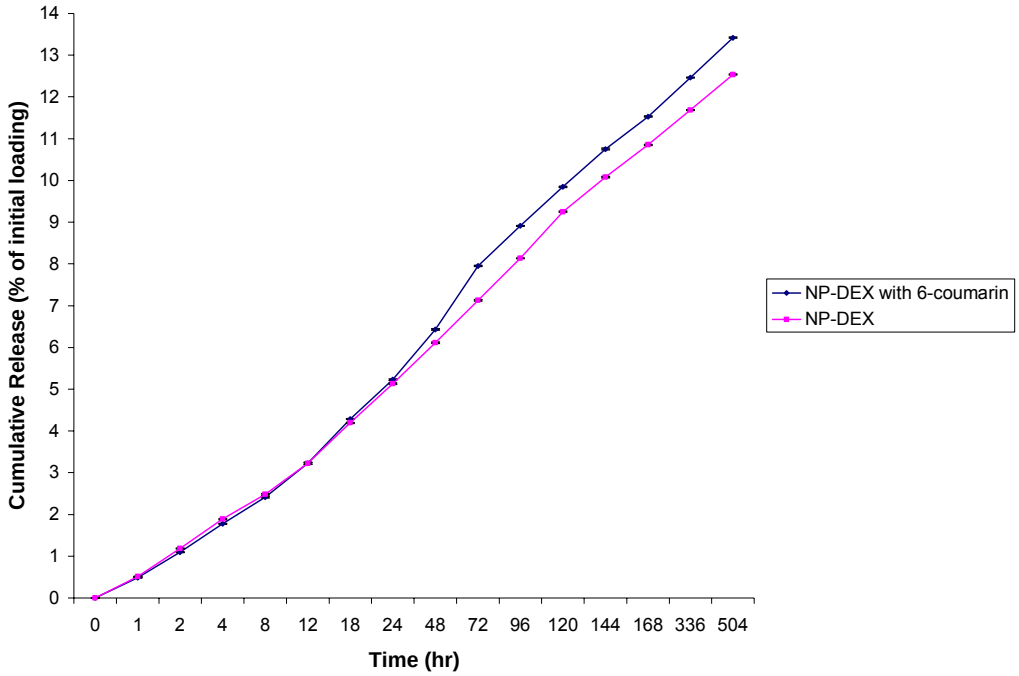

Figure 3.6. The release profile of nanoparticles formulated with DEX. After 3 weeks there was a cumulative release of about 12%. Values represent mean ± standard deviation (n=4).

3.3.2 Optimal Dexamethasone Dosage

As stated in the materials and methods section, the optimal dose of DEX to inhibit SMC proliferation was observed daily using a microscope (Figure 3.7). Utilizing a digital camera connected to an inverted microscope, pictures were taken to attain a visual observation of the cellular inhibition. While observing the cells it was clear to see that the control wells were steadily proliferating at a faster rate than the wells incubated with DEX solution. Over the course of the experiment some cell death was noted throughout the well plate; that could be attributed to the amount of time the cells were in the wells and the dosage of DEX. Images of the cells were only taken on Day 1; however the cells were monitored throughout the experiment. In addition, a DNA assay was used to determine the amount of DNA present in the cell lysate after 4 days of treatment. The DNA concentration relates to the amount of cells that were present. Figure 3.8, shows the corresponding amounts of DNA concentration to the

various sample concentrations. It is evident that the DEX inhibited HASMC proliferation and that the inhibitory effect is does-dependent.

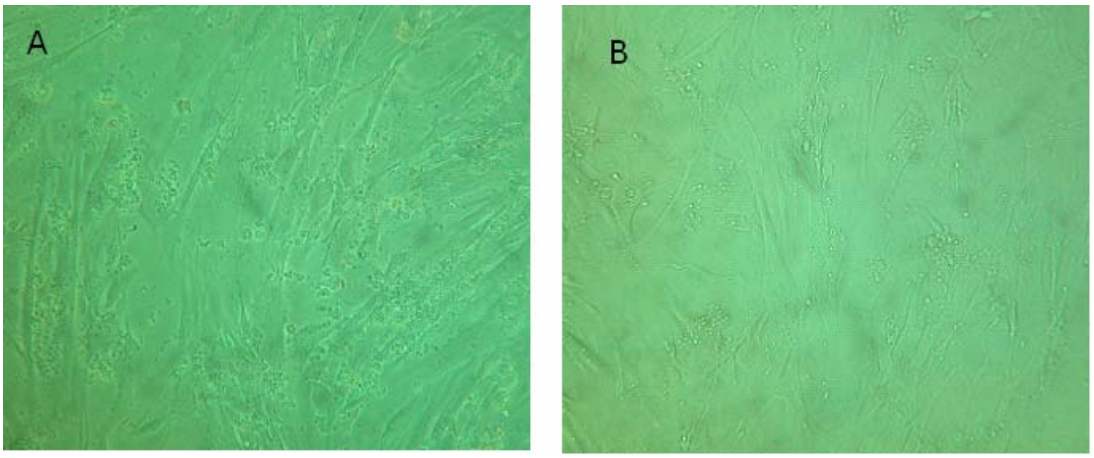

Figure 3.7. A visual representation of the DEX concentration study: The control, A, compared to the concentration of 1000 nm, B. Image was taken using an inverted microscope and digital camera.

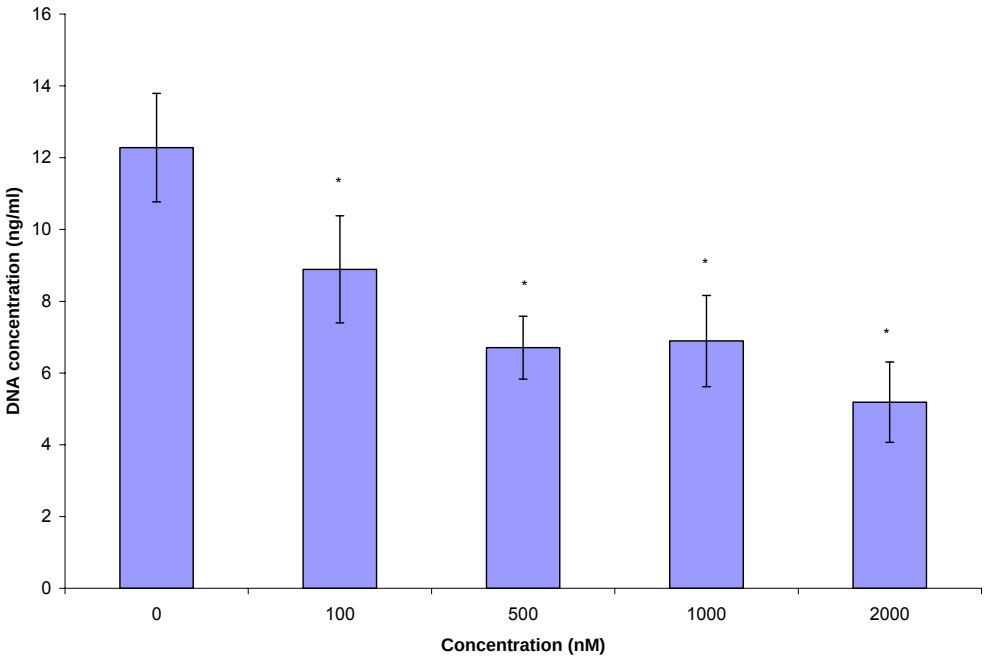

Figure 3.8. DEX Concentration Study: Determine the optimal concentration of DEX that will inhibit the HASMC proliferation. Values represent mean ± standard deviation (n=4). Statistical difference is represented by *.

3.4 Comparative Analysis between DEX and NP-DEX

HASMC were treated with dexamethasone in media and nanoparticles loaded with dexamethasone. It is clear to see that the NP-DEX showed more inhibition to cellular proliferation than the free DEX (Figure 3.9). From the figure it can be reasoned that after 5 days the effects of the therapeutic agent begins to decrease. The cellular proliferation showed a decreasing trend for both free DEX and DEX-NP until day 5. On day 5 the cellular proliferation increases and steadily increases until day 8. However, even though the cells were proliferating DEX-NP continued to have a better effect compared to that of free DEX.

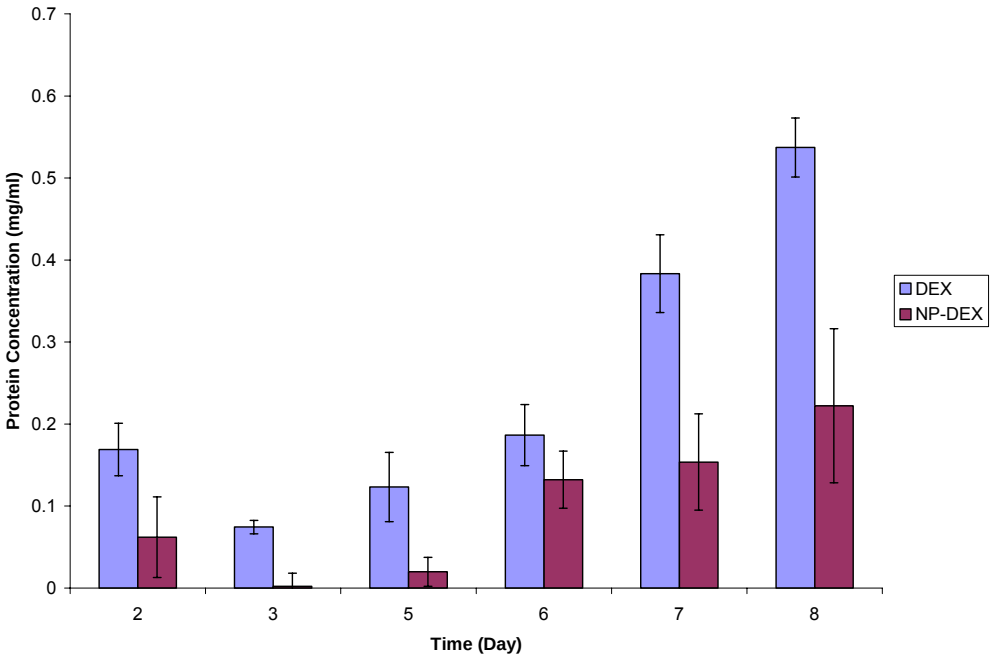

Figure 3.9. *In Vitro* Anti-proliferative Study: Comparative study amongst DEX and DEX encapsulated nanoparticles to determine which one more effectively inhibits HASMC proliferation. Values represent mean ± standard deviation (n=3).

3.5 Comparative Analysis between Targeted and Non-targeted Nanoparticles

In section 3.2.1 the optimal incubation and dosage that the cells would uptake PLGA nanoparticles was attained. Prior to conjugating the nanoparticles with PDGF-BB the HASMC were tested to see if they expressed the PDGF receptor. It was evident that the PDGF receptors are expressed throughout the cell membrane (Figure 3.10). Utilizing those results a comparative analysis was performed on PDGF-BB targeted nanoparticles and non-targeted nanoparticles (Figure 3.11). The amounts of particles uptaken by the cells were determined by fluorescent measurements and a protein assay. It was concluded that the PDGF-BB targeted nanoparticles significantly improved the cellular uptake. There was a fair amount of cellular uptake of the non-targeted nanoparticles (847 μg nanoparticle/mg cell protein) however that number was so small in comparison to the targeted nanoparticles (44045 μg nanoparticle/mg cell protein). Confocal microscopy was also used to image the uptake of the PDGF-BB targeted nanoparticles (Figure 3.12). Clearly, the uptake of the target nanoparticles is more effective than the non-targeted nanoparticles.

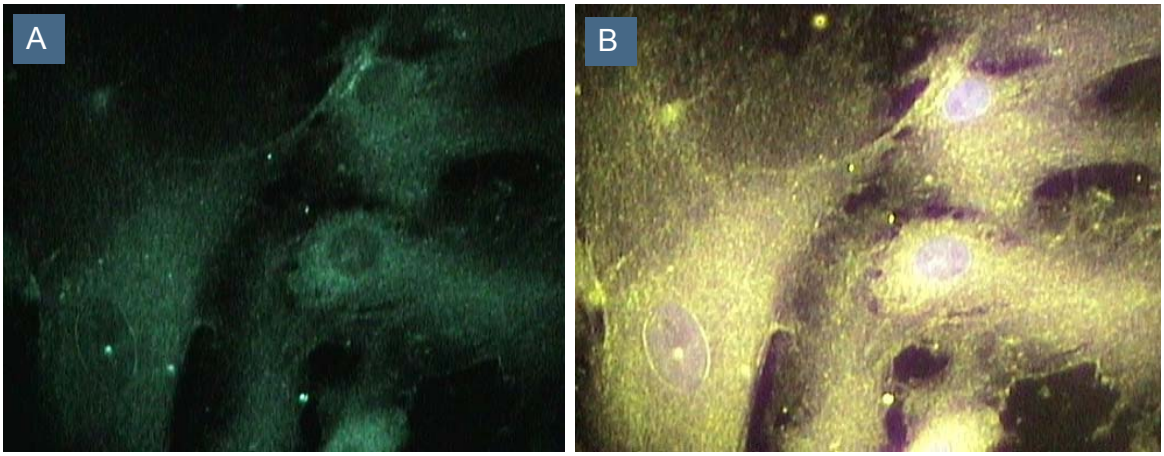

Figure 3.10. Testing for the PDGF receptors on the HASMC. The cells were incubated with the antibody and the cell membrane was stained with Texas Red and DAPI, respectively. A represents the green filter used to visualize the cell membrane , B is an overlap of the red and green filter.

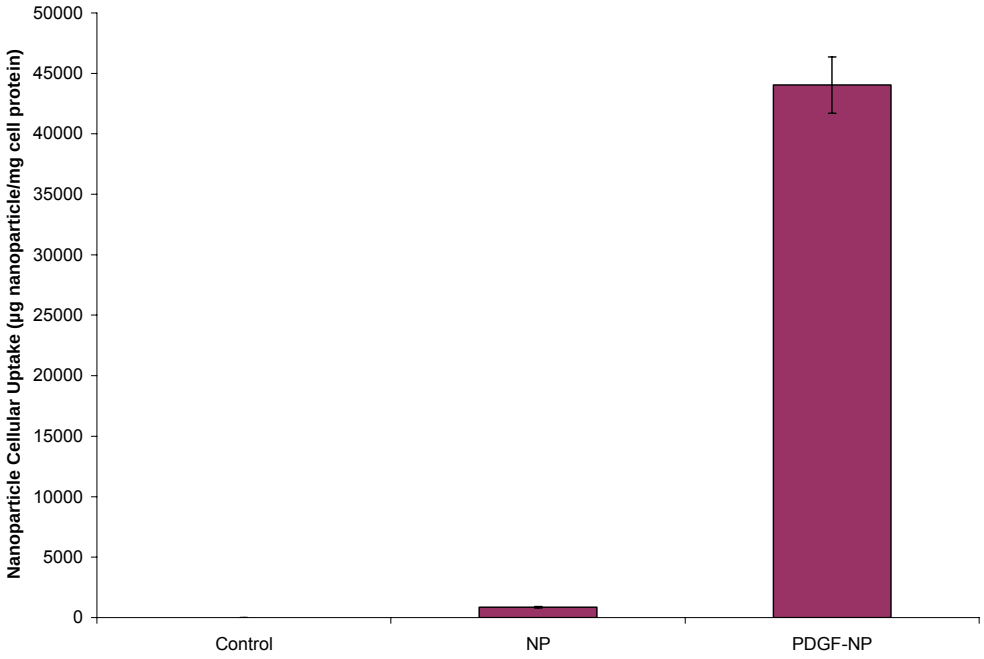

Figure 3.11. Comparative Uptake Study: The ligand conjugated PDGF-nanoparticles and PLGA nanoparticles were incubated at the determined optimal time and dosage. Values represent mean ± standard deviation (n=4).

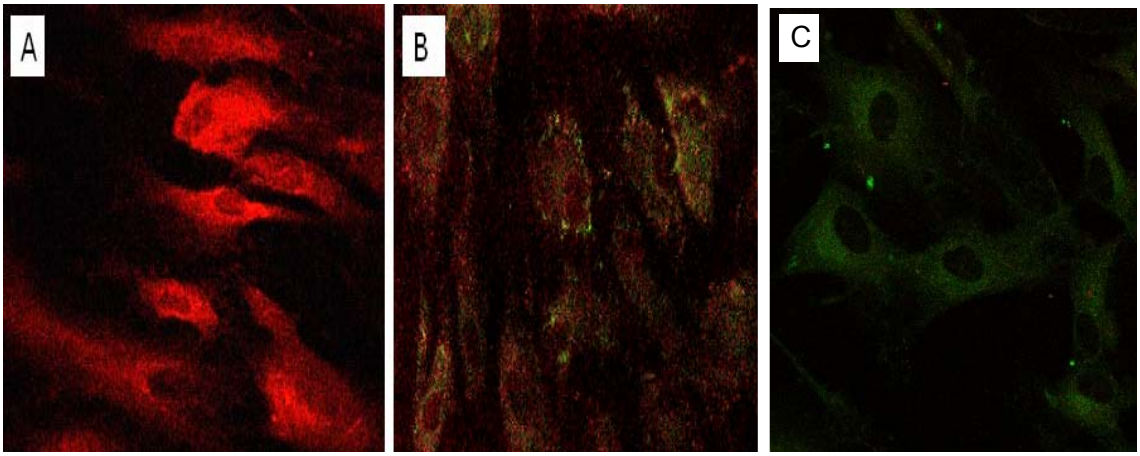

Figure 3.12. Confocal microscopy of PDGF-BB targeted nanoparticles. The nanoparticles uptaken by the cellular membrane was imaged using the FITC and TR filter for the nanoparticles and cellular membrane, respectively. A is the control cells, B is cells incubated with the nanoparticle solution (non-targeted), and C is cells incubated with the conjugated nanoparticle solution (targeted).

CHAPTER 4

DISCUSSION

4.1 Nanoparticle Characterization

In this research PLGA nanoparticles were developed for drug delivery. Approved by the Food and Drug Administration (FDA), PLGA has ideal materials to form drug delivery vehicles due to its biocompatibility and biodegradability (58). In addition to material properties, particle size is another important factor. The optimal particle size for drug delivery should be smaller than 5 μm; the smallest capillaries in the human body are 5-6 μm so the goal is to have particles significantly smaller than that (15). Studies have shown that smaller particles, on the nano scale, have a higher uptake than larger particles (59). Large particles have problems maneuvering throughout the body. Microparticles formulated for drug delivery were unable to cross the intestinal lumen into the lymphatic system when tested for oral delivery (15). Previous studies conducted within our laboratory showed that the optimal nanoparticle diameter should be about 100-200 nm. Thus, the aim of this project was to create nanoparticles that were 100-200 nm. Using TEM we were able to characterize the mean diameter of the nanoparticles encapsulated with DEX to be about 130 nm. These particles were further characterized using a Nanotrac that determined the size distribution to be in the range of 88 to 190 nm. The PLGA with and without DEX and 6-coumarin nanoparticles had a similar size distribution to the nanoparticles encapsulated with only DEX. The mean diameter and range of the nanoparticles are well within the optimal particle size range to circulate through the body.

Particle size not only effects the dispersion of the nanoparticles through the human body but it also affects the efficiency of therapeutic agent encapsulation. It has been found that smaller size particles have a higher cellular uptake than larger particles (60). The effectiveness of drug delivery using microparticles was low due to the fact that the particles

were being cleared by phagocytosis (15). The advantage of using nanoparticles is that the specific surface area increases as the size decreases and they are easily suspended in liquid solutions which makes them better suited for drug delivery. As the particle size decrease there is an increase in the molecules present on the particle surface. Furthermore, when large microparticles are suspended in liquid, they can precipitate out easier than nanoparticles due to the gravitational force (58).

It has been previously shown in our lab that PLGA nanoparticles have an anionic zeta potential. The zeta potential of PLGA nanoparticles changes in acidic medium, a low pH environment. The PLGA nanoparticles acquire a positive charge in an acidic medium such as that in endo-lysosomes; however in an alkaline medium, PLGA nanoparticles have a negative charge (61, 62). NPs are delivered to the lysosomes for degradation, following the intracellular uptake. NPs are able to escape from the endo-lysosomes into the cytosol because of the pH change (61, 62). Although majority of the particles escape into the cell cytoplasm some nanoparticles are ejected from the cell back into the medium while few particles are degraded by the lysosomes (Figure 4.1). The therapeutic agent is protected from the degradative cellular enzymes and released into the cytoplasm. Therefore, it can be concluded from studies in the literature that PLGA NPs would provide effective drug delivery.

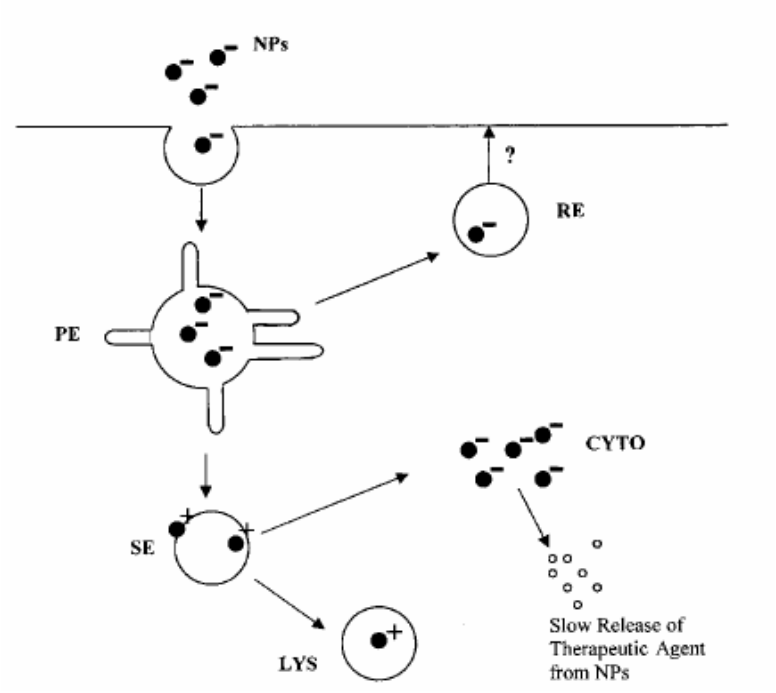

Figure 4.1. A depiction of the endocytosis/exocytosis cycle of PLGA's intracellular uptake (62). Nanoparticles (NP) are internalized and transported to primary endosomes (PE), from which they are sorted to recycling endosomes (RE) or secondary endosomes (SE). With a change in pH the majority of the NP escapes into the cytoplasm (CYTO) however a fraction is transported to lysosomes (LYS).

Another important factor in the cellular uptake of nanoparticles is the residual PVA within the particles. It has also been determined that the lower the amount of residual PVA the greater the cellular uptake (32). Our nanoparticles were formulated with a 5 % (w/v) PVA solution with chloroform as the solvent. It has been shown that the use of chloroform compared to other solvents such as acetone or dichloromethane results in a lower amount of residual PVA. The residual PVA tends to increase as the organic solvent miscibility with water increases (32). Five percent weight per volume PVA solutions proved to have a higher percentage of residual PVA ranging from 0.5 to 2 % (32). Although the 5% w/v PVA solution has more residual PVA than the lower weight per volume PVA solutions, it has many advantages: the mean particle diameter is smaller and the protein loading efficiency is greater (32). After analyzing the various factors of PLGA nanoparticles, it is concluded that PLGA nanoparticles formulated using 5% PVA evaporative emulsion method is an effective carrier for drug delivery.

4.2 Cellular Uptake

4.2.1 Uptake Studies by Fluorescent Measurements

It has been established that PLGA nanoparticles can be internalized by various cell types. These studies were completed to determine optimal incubation time and concentration of the PLGA nanoparticles for uptake of the particles by HASMC. The number of nanoparticles that the HASMC will uptake and the time it takes for the cells to uptake nanoparticles needs to be determined as other researchers have indicated that the nanoparticle uptake by cells is both time and dosage dependent (61). Our results indicated that after 4 hours of incubation time, there is no significant increase beyond this time point. At the various time points before the saturation there was a linear trend indicating that the amount of cellular uptake was increasing with time. The concentration of 800 µg/ml was found to be the optimal dosage of PLGA nanoparticles which the HASMC would internalize. There was no significant difference between 800 and 1000 µg/ml, therefore it was concluded that a saturation point was reached. As identified below, these findings have been verified by other researchers. It has been found that during the first few hours of incubation vascular SMC (VSMC) uptake of nanoparticles is achieved rapidly before saturation occurs (61). This group also determined that uptake efficiency was reduced and saturation was reached at concentrations ranging from 500-1000 µg/ml; lower concentrations tend to show a linear trend (61).

Cellular uptake can occur by phagocytosis, fluid phase pinocytosis, or receptor-mediated endocytosis (61). The effectiveness of internalized drug is modulated by the cellular uptake mechanism (65). Larger particles such as microparticles are taken into the cell by phagocytosis. Researchers have shown that when cellular uptakes are linear or saturable the process is fluid phase pinocytosis or receptor-mediated endocytosis, respectively (61). Our results indicate that the process of HASMC uptake of PLGA nanoparticles is both fluid phase

pinocytosis and receptor-mediated endocytosis. This conclusion is justified by the cellular uptake trends; the uptake of PLGA nanoparticles was both linear and saturable.

When performing the uptake studies, the complete DMEM was removed and replaced with low serum DMEM at least 5 hours before beginning the experiment. The use of low serum medium helped with the uptake of nanoparticles. The experiment was controlled by removing the complete medium at least 5 hours prior to the performing the experiment. It is believed that the use of complete DMEM plays a role in the exocytosis of the nanoparticles. Cellular uptake of nanoparticles follows an endocytosis/exocytosis cycle as described in section 4.1. The use low serum medium almost inhibits the exocytosis phase (61, 62). This suggests that the components of serum, albumin and growth factors, play a role in the exocytosis of nanoparticles.

4.2.2 Confocal Microscopy

Confocal microscopy was used to confirm the cellular uptake of the PLGA nanoparticles. The FITC and TRITC filter were utilized to image the nanoparticles and cellular membrane, respectively. The two images were overlapped to show that the nanoparticles were within the cellular membrane. The PLGA nanoparticles were loaded with 6-coumarin, therefore they would consist of a green fluorescence. 6-coumarin was proven by other researchers to be successful for fluorescent quantification. Coumarin dye was also found to leach less than 0.5% of the dye (66). This suggests that 6-coumarin is suitable as a fluorescent marker for nanoparticles as there are no given false measurements due to coumarin. Thus there would be no concern that the observed fluorescence was from the dye leaching. Texas red dye was used to stain the cellular membrane. The use of Texas red and 6-coumarin allowed for the study of nanoparticle intracellular distribution.

4.2.3 Biocompatibility

An ideal polymeric drug delivery system must be biocompatible. It is known that cells uptake nanoparticles through an endocytosis/exocytosis cycle. PLGA nanoparticles uptaken by the cells escape the endo-lysosomes through a change in surface charge, from anionic to cationic, which results in the particles escaping into the cytoplasm (61, 62). When escaping the endo-lysosomes, the nanoparticles may come in contact with other cellular components thus cell toxicity is possible. In our studies, nanoparticle concentrations of 0-1000 µg/ml showed 20% or less cell toxicity. A concentration of 0 µg/ml nanoparticle solution was used as a negative control to compare the biocompatibility of the cells that were exposed to the nanoparticle solution. The positive control resulted in 100 % dead cells; the cells were lysed using Triton®–X 100. The results indicated that the nanoparticle solutions were as biocompatible as the media itself. All concentrations of nanoparticles solution were found to be similar to the control group (media only) and significantly lower than that of the positive control. Similar to our study; other investigators also show that PLGA nanoparticles do not exhibit any cell toxicity with vascular smooth muscle cells (64, 66). It was also important to note that when testing PLGA nanoparticles biocompatibility, nanoparticles loaded with 6-coumarin were used. It has been found that 6-coumarin is not toxic to cells (66). For *in vivo* drug delivery applications, 6-coumarin is not included in the formulation process.

4.3 Drug Characterization

4.3.1 Release Profile of Dexamethasone

Thus far it has been shown that PLGA nanoparticles are biocompatible and capable of cellular uptake. In order to determine if PLGA is capable of controlled drug delivery, the release profile was determined to prove that DEX would be successful to be encapsulated and released fro PLGA nanoparticles for the long term drug delivery. Our results suggest that the PLGA loaded with the anti-proliferative agent DEX will be a successful drug delivery method. Over a

period of three weeks about 12 % of DEX was released from the nanoparticles. Since the cellular uptake studies were conducted using 6-coumarin loaded nanoparticles that release profile was also analyzed. For the 6-coumarin particles there was a release of about 13 % over three weeks. Both the DEX-NPs and DEX-NPs with coumarin had an initial burst release of about 4 % after 18 hours. These results indicate that DEX release profile from our nanoparticles is not affected by the incorporation of 6-coumarin. Our release profile is similar to that of other studies performed using anti-proliferative agents. One study using PLA nanoparticles was successful in drug delivery of anti-proliferative agents. The PLA nanoparticles were formulated with platelet-derived growth factor receptor β tyrophostin inhibitor (67). Other studies using poly (ethylene oxide)-PLGA nanoparticle had an initial burst release followed by a more sustained release over a period of one month (68). Another study using BSA as a model drug showed there was a cumulative release of about 20 % over a period of 90 days in 100 nm nanoparticles (64). Factors that may affect the drug release include the particle size. Larger particles tend to have a smaller initial burst release and are capable of sustaining the drug release over longer periods of time (15).

The loading efficiency determines the effectiveness of the drug release. It affects the rate of the drug release and the amount of the initial burst release (15). The loading efficiency was 65 and 64% for the DEX-NP and DEX-NP with 6-coumarin, respectively. Other researchers have shown a higher loading efficiency and release rate. For example, a 50 % release was shown over 30 days of tetanus toxoid from PLGA nanoparticles (13). It is possible that our results were lower due to the water solubility of the DEX used in this experiment. The loading efficiency may be affected when using hydrophilic drugs such as DEX. During emulsification there is rapid diffusion of the molecule into the outer aqueous layer when encapsulating hydrophilic proteins, thereby resulting in a poor drug loading efficiency (15). It is crucial to have an initial polymer membrane deposit during the first water-oil emulsion (15).

When formulating nanoparticles with hydrophilic drugs, a double emulsion is required. Contrarily when using a hydrophobic drug only a single emulsion is necessary.

The degradation rate of PLGA is important when characterizing the drug release properties. The PLGA used in this experiment had a 50:50 ratio of lactic to glycolic acid. The polymer ratio is a determining factor in the degradation rate. It has been demonstrated that degradation occurs faster when there is a larger amount of glycolic acid (69). This can lead to an increase in the rate of drug release. The amount of lactic acid is crucial because of its ability to make PLGA less susceptible to hydrolysis which leads to less water accessibility (69). An increase in the water accessibility will accelerate the drug release. However, since our PLGA had an equal amount of glycolic and lactic acid there was a sustained drug delivery. According to the manufactures our PLGA will degrade in 2 -4 weeks.

4.3.2 Optimal Dexamethasone Dosage

The smooth muscle migration and proliferation is a major focus in targeting restenosis. In this project, we examined the glucocorticoid dexamethasone as a therapeutic agent to inhibit smooth muscle cells. Glucocorticoids are known to play a fundamental role in the proliferation of cells through the regulation of cellular processes that are imperative for cellular growth (70). Our results indicated that DEX significantly reduces the smooth muscle cell proliferation at 100-2000 nM. This coincides with other researchers that have shown that DEX reduces cellular proliferation and DNA synthesis (46, 47). It was demonstrated that DEX inhibits SMC proliferation by preventing the cell cycle from continuing into the S phase (46, 47). Therefore, it is concluded that DEX can be used as a model drug to inhibit smooth muscle cell proliferation.

4.4 Comparative Analysis between DEX and NP-DEX

In order to further examine DEX, intracellular studies were conducted to determine if PLGA nanoparticles could effectively deliver DEX to the HASMC. As proven in the drug release

studies, PLGA nanoparticles provide a sustained drug release. We further investigated the effects of DEX released from DEX encapsulated PLGA nanoparticles versus non-encapsulated DEX (or free DEX) on HASMC proliferation. Our previous results indicate that DEX is more effective than the control. Results indicate that PLGA nanoparticles loaded with DEX was an efficient delivery method of DEX and that DEX encapsulated nanoparticles have better anti-proliferative effects than non-encapsulated DEX. These concepts were further confirmed by other scientists, who revealed that nanoparticles formulate with DEX reduces vascular SMC proliferation better than that of DEX in solution (63). In another study using PLGA scaffolds loaded with DEX, it was shown that the cell number was significantly reduced when the cells were seeded onto the scaffolds (20). PLGA has also been proven to effectively deliver therapeutic agent with a low molecular weight (63). Therefore, it can be deduced that PLGA nanoparticles is an effective carrier to deliver drugs that inhibit cellular proliferation of HASMC.

Initial studies with nanoparticles and cellular uptake were imperative in determining if the particles would be a successful drug delivery method. As stated early, nanoparticles are uptaken by the cells in the cytoplasm. It is also known that DEX acts pharmacologically by binding to the cellular cytoplasmic compartment through glucocorticoid receptors (71). Once the glucocorticoid receptor is binded to the ligand it is translocated into the nucleus (43). Therefore, once the nanoparticles are inside the cytoplasm, the drug is released. Then the drug will bind to the glucocorticoid receptors and be taken into the nucleus to inhibit the SMC proliferation.

The medium has plays an important role in cellular uptake. After the medium containing the nanoparticle solution and the DEX solution was removed, there was a potential reduction of DEX and DEX released from the nanoparticles in contact with the cells. When the medium containing the drug was replaced with fresh media, cellular proliferation increased as a result of no additional drug given to the cells in our studies. DEX has been known to increase cellular proliferation after short-term exposure (43). However, when DEX is given to cells over

long term exposure, cellular proliferation is inhibited (43). The removal of nanoparticles from the medium may be a factor in the endocytosis/exocytosis cycle of particle uptake in cells. This theory was confirmed by researchers analyzing the removed growth medium for nanoparticle concentration. It was found that the decrease in intracellular nanoparticle level correlated to that of the nanoparticles in the growth medium (61). The replacement of fresh medium lowers the concentration of intracellular nanoparticle, due to the halt in the endocytosisi/exocytosis cycle. It is also suggested that there is a biphasic drop in the intracellular nanoparticles levels: first, there is a rapid over turn of the nanoparticles and secondly, there is a slow over turn (61, 62).

4.5 Comparative Analysis between Targeted and Non-targeted Nanoparticles

Thus far we have demonstrated that PLGA is a successful method of drug delivery, DEX inhibits SMC proliferation, and DEX encapsulated nanoparticle are more efficient than free DEX. To improve this drug delivery system we conjugated PDGF-BB peptide to the nanoparticle to use for targeted drug delivery. It has been demonstrated that tissue factor ligand targeted nanoparticles can penetrate and bind to vascular SMCs (72). Interestingly, the expression levels of PDGF receptors in arteries undergoing cardiovascular interventions are increased significantly compared to those of normal arteries. Therefore, we hypothesis that PDGF-BB targeted nanoparticles will be able to penetrate and bind to HASMC. In this experiment PDGF-BB conjugated particles were compared against non-targeted particles. It was shown that the PDGF-BB nanoparticles were more effective than the non-targeted nanoparticles in cellular uptake studies. Our results are in accordance to other researchers, which suggest that targeted nanoparticles were better than non-targeted particles (24, 73, 74). It has been shown that tissue factor conjugated particles were more efficient when compared against non-targeted nanoparticles in smooth muscle cells (24). The non-targeted nanoparticles were uptaken by the cells but significantly less than the targeted particles (24).

Other methods have been used for targeting SMCs. Another study successfully used adenovirus to target heparan containing receptors on various cell types including smooth muscle cells (74). Based on our findings, we can conclude that PDGF-BB conjugated nanoparticles will be effective for targeting HASMC.

CHAPTER 5

CONCLUSION

The ideals presented throughout this research have proven to meet the requirements of an ideal drug delivery system. First, the PLGA nanoparticle appears to be an effective delivery method. PLGA nanoparticles were formulated to be a size of about 130 nm with a range of 88-190 nm. The determined particles diameter size is sufficient to successfully navigate through the body. It was also shown that the HASMC uptaken the nanoparticles. Optimal incubation time and dosage of PLGA nanoparticles were found to be 4 hours and 800 μg/ml, respectively for HASMC uptake studies. Relatively no cytotoxicity was found when cells were exposed to PLGA nanoparticles. Therefore, it can be concluded that PLGA nanoparticles are biocompatible with HASMC. Using dexamethasone as a model drug, the release profile of drugs from PLGA nanoparticles demonstrated a sustained drug release for three weeks. Hence, the PLGA nanoparticles could be used for intracellular drug delivery and controlled drug delivery of DEX to HASMC.

The therapeutic agent, DEX, inhibits the HASMC proliferation. DEX significantly inhibits HASMC proliferation at concentrations greater than 100 nM. However, when DEX was encapsulated with PLGA nanoparticles, the therapeutic effect was more efficient. Comparative studies concluded that nanoparticles formulated with DEX inhibited cellular proliferation more effectively than that of free DEX. Targeted drug delivery is proven to be a more valuable delivery method than non-targeted nanoparticles. The uptake of nanoparticles conjugated with PDGF-BB peptide was greater than the uptake of non-targeted nanoparticles. As PDGF-BB receptor is an excellent target receptor for HASMC, it can be concluded that targeted drug delivery using ligands that are specifically bound to proliferated HASMC is a successful avenue for drug delivery. In conclusion, results from this research project demonstrated that PLGA

nanoparticles could be used as a targeted and controlled drug delivery carrier to deliver drugs that inhibit the HASMC proliferation after cardiovascular interventions such as angioplasty and stenting to treat various vascular diseases.

CHAPTER 6

LIMITATIONS

Although the PDGF-BB nanoparticles used to target proliferative HASMC and encapsulate DEX are novel, there are still some limitations.

- PLGA nanoparticles are biocompatible as supported by our studies; however, there may be some complications with other cellular components such as blood, platelets, or other cell types. In order to test this potential, animal studies need to be conducted.
- The PLGA uptake within the body might differ from that of *in vitro* static conditions. Under static conditions the cellular uptakes studies were conducted in low serum media. Therefore, further studies need to be conducted in complete DMEM and in animals.
- There may be a low number of conjugation sites on the PLGA nanoparticles for peptide conjugation. The PLGA nanoparticles can be polymerized with other monomers to add more functional groups such as carboxyl and amine groups for bioconjugation.
- Due to the fact that the PDGF receptor is potentially expressed on cell lines other than that of smooth muscles, PDGF-BB peptide conjugated nanoparticle may be uptaken by other cell types. Uptake studies need to be performed on other cell lines such as endothelial cells, to ensure the uptake in HASMC is more effective.
- HASMC were tested for the uptake of PLGA nanoparticles only. The cells should be tested with other biocompatible polymers to ensure that they effective uptake all types of polymers. Different polymers will exhibit different properties which may interfere with the cellular uptake.

CHAPTER 7

FUTURE WORK

Future work includes improving the PDGF-BB conjugated and DEX formulated nanoparticles.

- Study the degradation rate of the PLGA nanoparticles. The degradation rate may interfere with the drug release and targeting strategy. As the PLGA nanoparticles degrade there may be functional and structural changes that interfere with the drug release and ligand binding.
- Formulate DEX encapsulated nanoparticles at the determined optimal therapeutic concentration range.
- The DEX nanoparticles have been conjugated with one peptide, PDGF-BB. However, the system may be more effective if multiple ligands were conjugated.
- Treatment of restenosis with the particle system may be improved using cocultures. Instead of targeting only HASMC, it may be better to target other cell types such as endothelial cells.
- Animal studies need to be performed to characterize how effective the DEX nanoparticles will be before human use.

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BIOGRAPHICAL INFORMATION

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