

**A BIOCOMPATIBLE, HEPARIN-BINDING POLYCATION FOR
THE CONTROLLED DELIVERY OF GROWTH FACTORS**

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**A BIOCOMPATIBLE, HEPARIN-BINDING POLYCATION FOR
THE CONTROLLED DELIVERY OF GROWTH FACTORS**

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NOMENCLATURE

FGF-2	Basic Fibroblast Growth Factor
DMAEMA	Dimethyl Aminoethyl Methacrylate
DMF	Dimethylformamide
DSC	Differential Scanning Calorimetry
ECM	Extracellular Matrix
FGFR	Fibroblast Growth Factor Receptor
GPC	Gel Permeation Chromatography
^{125}I	Iodine 125
^{125}I -FGF-2	Iodine 125 labeled FGF-2
IR	Infrared
NMR	Nuclear Magnetic Resonance
PAGS	Poly(Arginate Glycerol Succinate)
PAMAM	Polyamidoamine
PEG	Polyethylene Glycol
PEI	Polyethylenimine
PGA	Polyglycolic Acid
PLA	Polylactic Acid
PLGA	Copolymer of PGA and PLA
PLL	Poly-L-lysine
SEM	Scanning Electron Microscopy

SUMMARY

The delivery of growth factors has been attempted for a number of different therapies. The approach of delivering therapeutic growth factors in a safe and efficient manner is difficult and certain criteria should be met. These criteria include: bind the appropriate growth factors, maintain their bioactivity, and deliver these proteins with controllable release rates for an extended period of time. These criteria encompass a set of guidelines that hope to mimic the *in vivo* biological events (example: neovascularization). The central goal of this thesis is to meet these criteria by introducing a novel delivery strategy for growth factors using a biocompatible polycation and heparin complex.

At the onset, it was concluded that heparin should be used to bind and stabilize growth factors. Heparin was chosen because it allows the binding of numerous growth factors, will maintain the bioactivity of these growth factors for an extended period of time, and in some situations will provide a more bioactive response than the growth factor alone. Heparin accomplishes this because it has a native affinity for growth factors. In order to localize growth factor delivery, native heparin has to be precipitated out of solution. A synthetic polycation was chosen to complex heparin to form a water-insoluble matrix.

It was decided that this polycation had to be biocompatible for this delivery strategy to be successful. A delivery strategy needs to be biocompatible for use in *in vivo* applications. Heparin has already been used extensively for clinical applications, but the same cannot be said about synthetic polycations. To date, there has not been a reported synthetic polycation that is biocompatible enough to be used in this type of application.

To address this problem we set out to develop a synthesis strategy that mimicked the essential elements that make cationic peptides biocompatible. This resulted in a synthetic polycation that exhibited unprecedented biocompatibility *in vitro* and *in vivo*.

It was hypothesized that a polycation could interact with heparin to form a complex with the potential to deliver bioactive growth factors with a controllable release. I set out to test this hypothesis by examining the release kinetics of FGF-2 from the complex and investigating whether the released FGF-2 maintained its bioactivity. The [polycation:heparin:FGF-2] complex was formed by mixing the components in PBS, which resulted in a precipitate. This precipitate delivered FGF-2 with controllable release kinetics and the bioactivity of the released FGF-2 was comparable with bolus FGF-2 and heparin stabilized FGF-2. The system is expected to bind and deliver numerous heparin-binding growth factors.

In conclusion, the growth factor delivery system developed in this research provides a novel mechanism for controlled release of growth factors. This delivery strategy has met the criteria listed earlier and this research has laid the foundation for a successful delivery vehicle. Further, I designed a biocompatible polycation, a critical component of the delivery system. This polycation exhibited *in vitro* and *in vivo* biocompatibility that was order of magnitude higher than existing polycations and would likely be very useful for a variety of biomedical applications. This design principle is also expected to serve as a springboard to more biocompatible polycations.

CHAPTER 1

SPECIFIC AIMS

Introduction

Ischemic heart disease is the leading cause of mortality in the Western world and afflicts more than 10 million patients in the United States and hundreds of millions globally. The primary cause of ischemic heart disease is the accumulation atherosclerotic plaques within the coronary arteries. This can eventually lead to myocardial infarction which will result in damaged myocardial tissue. Since the mammalian heart has little or no capacity to regenerate, there is a great need for a cardiac regeneration therapy (1, 2).

Current therapies are aimed at reducing the extent of damage to cardiac tissue. Alternatively, it has been proposed cardiomyocyte repopulation and revascularization may lead to a restoration of cardiac function and represent an advancement over present therapies (3). Cell-based strategies have generated great enthusiasm but still face the challenge of poor host-tissue incorporation (4). Another approach to cardiac regeneration is the delivery of therapeutic proteins to stimulate neovascularization. These therapeutic factors have to be delivered in a local and controlled manner to be safe and efficient. One approach to achieve this type of delivery is an affinity-based delivery. This type of system uses non-covalent interactions between the therapeutic factor and delivery vehicle to regulate the rate of release. One of the best characterized examples of an affinity-based system is the heparin-binding delivery system.

In living tissue, a multitude of growth factors bind heparin in the extracellular matrix (ECM). Heparin serves as a storage for growth factors, allowing them to diffuse

out in a sustained manner while maintaining their bioactivity (5). There have been many growth factor delivery systems that have taken advantage of these attributes by incorporating heparin or heparin moieties (6, 7). However, most of these delivery systems do not use heparin in its native state and thus are not completely biomimetic. Also, the majority of these systems are made from components that do not allow a controllable release rate. This indicates a need for a growth factor delivery system that utilizes native heparin and allows for controllable release kinetics.

Our long-term goal is to establish a minimally invasive, controlled delivery system that will exploit heparin's ability to bind angiogenic growth factors. The objective of this application is to deliver FGF-2 in a local, controlled manner to promote the proliferation of endothelial cells. The central hypothesis is that an appropriately designed polycation will interact with heparin to form a network that will sustain a delivery of bioactive FGF-2 over an extended period of time. We also postulate that this resultant complex can be administered minimally invasively by injection. We have developed this hypothesis based on strong preliminary data indicating the arginine-based polymer interacts electrostatically with heparin to form non-covalent complexes. Also, these networks will use heparin's capacity to bind FGF-2 and serve as a growth factor reservoir. The rationale for this work is to develop a biocompatible delivery system that can deliver therapeutic growth factor over a specified period of time (days, weeks, etc.) while being minimally invasive. The overall objective will be accomplished by evaluating our central hypothesis in the following *specific aims*:

Specific Aim I

Synthesize and characterize a biodegradable, arginine-based polycation.

The *working hypothesis* is that a polycation that is both biodegradable and based on arginine will be biocompatible. The polycation will be synthesized by incorporating arginine into the polymer backbone via the synthetic linker's polycondensation reaction with the biomolecule. The polycation will be characterized by NMR and FTIR spectroscopy, gel permeation chromatography, and differential scanning calorimetry. Also, the polycation's biocompatibility will be investigated *in vitro* and *in vivo*.

Specific Aim II

Examine the potential of the polycation to complex heparin and deliver heparin-binding growth factors.

The *working hypothesis* is that the [polycation:heparin] complex will bind FGF-2, preserve its bioactivity, and release it in its active form in a controlled manner. We further hypothesized that the release of growth factor can be thermodynamically controlled through altering the composition of the network. The polycation's potential to form non-covalent networks with heparin will be analyzed via scanning electron microscopy and dynamic light scattering experiments. To investigate the network's ability to release growth factor, radio-labeled FGF-2 will be incorporated into the [polycation:heparin] complex and its release kinetics will be monitored. The [polycation:heparin] network's potential to maintain the bioactivity of the growth factors will be investigated through potency and functional assays using endothelial cells. FGF-2 release profiles will be used to examine changes in release rates by adjusting the components of the complex.

Innovation and Significance

This proposed research is innovative because it is the first time that a biocompatible polycation has been used for the controlled delivery of growth factors. This delivery vehicle has the advantages of being highly biocompatible and minimally invasive. This system also has an adjustable cation composition that allows for an adaptable release of growth factor. This work is expected to yield the following outcomes: First, we will synthesize and characterize the arginine-based polycation and establish its biocompatibility. We will optimize the polycation by modifying the synthetic linker and molecular weight to enhance complex formation. Second, we will examine the ability of this polycation to form electrostatic networks with heparin and release bioactive FGF-2 in a controlled manner. We will also evaluate how modifying the composition of the matrix will result in different release kinetics, creating a tunable delivery vehicle. Taken together, these outcomes will shed light on the potential of using this strategy to control the release of growth factors to treat a variety of human diseases including ischemic heart disease.

The thesis work detailed here entails synthesizing a biodegradable, arginine-based polycation. The polycation will then be used in conjunction with heparin as a growth factor delivery strategy. This [polycation:heparin] delivery strategy will have the potential to bind numerous growth factors and maintain their bioactivity through non-covalent interaction with heparin. The non-covalent interaction should also allow for more efficient loading and delivery of therapeutic factors. The overall goal of this research will be evaluated by the polycation's biocompatibility and the delivery vehicles ability to deliver bioactive FGF-2.

CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

A Biocompatible, Synthetic Polycation

Biomaterials

Biomaterials have played an important role in the treatment of diseases and healthcare in general. A biomaterial is a material (natural or synthetic) that comprises a therapeutic or diagnostic system intended to interact with biological systems. The earliest biomaterials can be dated back to the use of gold in dentistry over 2000 years ago, and other examples include the use of wooden teeth and glass eyes (8). Then in the late 1800's, the introduction of synthetic polymers introduced a new set of tools that would help advance the field of biomaterials. Example of these include: polymethylmethacrylate (hip replacement), polyethylene terephthalate (vascular grafts), and polyurethanes (artificial hearts) (9). While these biomaterials have greatly improved quality of life, they were not designed for their initial biomedical applications. This lack of design has resulted in complications with properties such as biocompatibility and responsiveness to its environment (8).

The challenge for a number of biomaterials is they are used to replace living tissue. Living tissue has spent centuries going through evolution to bring us to our present biological situation, and it would be difficult for a biomaterial to mimic all properties of living tissue. This problem is compounded when the material is not designed for its specific application. Presently, biomaterials science has moved in the direction of rationally designing materials for their application (10-12). This movement

has been aided with greater understanding of cellular signaling, cell interactions, and developmental biology (13, 14). A specific example of using knowledge of the biological environment to design novel biomaterials is the introduction of cell adhesion motifs, enzyme degradation sites, and growth factors into biomaterials for tissue engineering applications (15). With the paradigm shift in biomaterial methodologies, it is now possible to more efficiently direct tissue regeneration and deliver therapeutics safely and efficiently. With the progressive understanding of the biological environment, biomaterials will continue to evolve and lead to further breakthroughs in healthcare.

An example of one class of biomaterials that exemplifies the need for rationally designed materials is polycations. Synthetic polycations have been around for decades and are used in variety of different areas of biomedical engineering (16, 17). Synthetic polycations are well-known for being toxic in biological environments, and great lengths have been taken to modify them in the hope of increased biocompatibility (18). Presently, there has not been reported a synthetic polycation that has exhibited biocompatibility in both *in vitro* and *in vivo* environments. In order for a polycation to realize its full potential in therapeutic applications, it will have to encompass this type of biocompatibility. As seen with other biomaterials, a rational design can lead to improvements that help to revolutionize the field.

Synthetic Polycations in Biomedical Engineering

The use of synthetic polycations in biomedical engineering can be dated back approximately 50 years ago (16, 17). Two of the most extensively used and best characterized polycations in biomedical engineering are polylysine (PLL) and

polyethylenimine (PEI). PLL has been used in a variety of different applications but has been used most extensively in gene delivery and polyelectrolyte film applications (19-22). PEI is widely regarded as one of the most successful non-viral gene delivery vectors (23). PEI has also been used in applications including cell patterning (24), medicinal chemistry (25), and delivery of other biomolecules (26, 27). Both of these synthetic polycations have achieved levels of success in the aforementioned applications, but they have also been faced with the challenge of biocompatibility (28). It has been well documented that these polycations are not biocompatible because they are toxic to cells. This property of PLL and PEI has led to numerous other polycations being introduced over the years, as well as PLL and PEI derivatives.

In order to circumvent the issue of toxicity, numerous modifications to PLL and PEI have been made as well as creating many novel synthetic polycations in the process. Simple modifications of PLL and PEI include altering the molecular weight and structure (linear, branched, etc.) of the polymer (29). Adaptations have taken a step further in the form of using PLL and PEI motifs as part of a larger polycation. Examples of more extensive modifications include: PEG modified PEI and PLL (30-34), low molecular weight PEI cross-linked with biodegradable linkages (35, 36), polyhistidine graft-PLL (37), PEI conjugated to a biodegradable polyglutamic acid derivative (38), cholesterol modified PEI (39), lipid substituted PLL (40, 41), etc (42-45). This is by no means an exhaustive list of all the different modifications, but just a sample of the modifications that have been introduced over time. Some of these adaptations have met the goal of increased biocompatibility, but the issue of toxicity has never been overcome completely.

While efforts were focused on improving PLL and PEI, other research introduced other many polycations. Some of the polycations that have resulted from this research include DEAE(diethylaminoethyl)-dextran, polyamidoamine (PAMAM) (46), and dimethyl aminoethyl methacrylate (DMAEMA) (47). In the evolution of synthetic polycations, these polymers have also been modified to interact with their environment. These polymers are often referred to as “smart” biomaterials. These attributes can be seen in present polycations that are thermo-responsive (48, 49), pH responsive (50-52), and target different cellular functions (entry into the nucleus, escape endosome, etc.) (53-56). Nearly all of the synthetic polycations mentioned have been used for gene delivery applications, but there are also others that have been used in different areas of biomedical engineering such as tissue engineering and drug delivery.

One hypothesis of why these polycations remain toxic was because of their lack of biodegradability (57, 58). A class of synthetic polycations that have showed improved biocompatibility is the degradable polyester that carries a positive charge. This class of polycations has been used in gene delivery and as components of layer-by layer thin film applications. There have been a number of groups that have focused their efforts on the synthesis of biodegradable polycations, and this research has resulted in improvements in biocompatibility (59-62). Even given their biodegradable nature, these polycations have shown signs of toxicity at higher concentrations and have not been evaluated for biocompatibility in an in vivo setting. After these advancements, the search for a completely biocompatible polycations has yet to be achieved. In this pursuit, there has been a great amount of energy invested in elucidating the mechanism of polycation

toxicity. With a better understanding of how polycations elicit a toxic response, a more efficient synthesis strategy can be found.

Polycation Toxicity

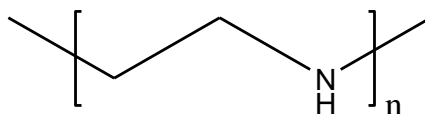
Synthetic polycations are well-known for being cytotoxic to biological environments. The exact mechanism that causes these toxic effects is not fully understood, but there have been many hypotheses to explain the occurrence. Biocompatibility for polycations has been hypothesized to be influenced by a number of different properties that include: molecular weight, charge density, order of amines present within polycation, structure (block, graft, branched, etc.), and conformational flexibility (18, 28, 63).

A standard observation with polycation toxicity is an increase in the molecular weight of the polycation, results in an increase in toxicity as well. This trend has been extensively studied and has been seen with numerous synthetic polycations. Examples of polycations that have exhibited this observation are PEI, PLL, Polyarginine, DEAD-dextran, and PAMAM dendrimers (28, 64-67). This trend in molecular weight is only seen within the same monomeric species of polycations and cannot be globalized across different polycations.

Because the molecular weight hypothesis cannot be used to compare different polycation species, the charge density of polymer's has been introduced as a means to predict cytotoxicity. Charge density is the number of positive charge carriers per monomer subunit. **Figure 2.1** illustrates an example of how charge density is calculated for PEI. The hypothesis of charge density predicting toxicity has been well documented

in PEI. PEI has a high charge density, as every third atom within the polymer is a charge carrying unit. This high charge density of PEI has demonstrated more deleterious effects than PLL, PAMAM, and other synthetic polycations with a lower charge density (18, 28). While using the use of charge density seems to be validated with this observation, there are issues with using it as an indicator of toxicity. The polymer comparisons listed earlier did not standardize molecular weight, polymer structure, among other properties. This makes it difficult to elucidate whether the toxicity primarily results from charge density or is their other factors that play a role.

PEI



Molecular Weight of Monomer Unit (MWM): 43

Charge Carrying Units per Monomer Unit (CCU): 1

Charge Density: $CCU/MWM = 0.0233$

Figure 2.1. Charge density calculation. The charge density of PEI is 0.0233. This is calculated by dividing the number of charge carrying units per monomer by the molecular weight of the monomer unit.

Other groups have hypothesized that the order of amines present in the polycation are critical in determining toxicity. PLL and its derivatives have been used to assess the role of amine order on toxicity (68). They demonstrated that primary amines are more

toxic than molecules that contain secondary and tertiary amines. This trend of primary amines being more toxic was also concluded by another group (69). This was accomplished by using a glutamic acid derivative based polycation in comparison to PEI and PLL. In this study, they found that polycations with primary amines were more toxic to red blood cells than the polycations with tertiary amines. Research on this topic is not as extensive as other hypotheses, and more is needed to prove its merit. None of these toxicity theories can be used as a governing law to predict biocompatibility, so a more thorough understanding of polycation-cell membrane interactions is needed.

There are many theories on why polycations elicit toxic effects, but investigating their interactions with cell surfaces can hopefully better explain their toxicity. The generally accepted mechanism for internalization of polycations is mediated by endocytosis (70). Endocytosis is a three-step process that includes binding with phospholipids and/or glycolipids of the cell membrane, internalization of the complex into the cell, and then either being released by the endosome, trafficked to a specific region of the cell, or targeted for degradation in the lysosome (71). Of these steps, there has been particular interest of the initial interactions between polycations with the cell membrane surface. A number of groups have attempted to model these interactions and have provided evidence for membrane disruption initiated by polycation contact. The knowledge of these mechanisms is not well understood, and this lack of understanding can be greatly attributed to the complexity and heterogeneity of the biomembrane matrix which makes it difficult to interpret results (70).

There are two main approaches to study the interactions between cationic macromolecules and membrane stability. One strategy is the use of tissue cultured or

fixed cells (72-74). This strategy has shown that the adsorption of polycations on cell membranes can result in critical physiological events such as: receptor clustering (75), changes in membrane permeability (76), and the functioning of ion channels (77). A theory on how this interaction is facilitated is through the conformation in which polycations adsorb onto the surface of membranes. It has been suggested that a three point attachment is necessary to achieve a biological response on cell membranes. It was then further hypothesized that the activity of the polymer to interact with the cell membrane will decrease as the space between the active amines is increased within the polymer structure (78). Under this same line of thinking, membrane interactions should also decrease as the polymer becomes more rigid in structure. This hypothesis is supported by DEAE-dextran being less toxic than PLL or protamine. DEAE-dextran is a rigid molecule, while PLL and protamine are linear, flexible molecules that can better neutralize the charge on the cell surface. This is then further supported by branched PEI being more toxic than all of the aforementioned polycations. The branched structure allows more accessibility of the cell membrane to interact with a positive charge within the PEI structure.

The second approach to investigate the interactions between a polycation and a cell membrane consists of using model systems to mimic some of the essential features of the cell membrane (76). The cell membrane is a selectively permeable bilayer that is composed primarily of lipids and proteins. The simplest cell mimetic model is a bilayer vesicle composed of phospholipids that carries a net negative charge. It has been demonstrated that polycation adsorption on liposomal membranes can result in migration of anionic lipids from the inner to the outer leaflet, lateral liposomal segregation, and

incorporation of polyelectrolytes into the membrane (79-81). Polycation adsorption at higher concentration can have more deleterious effects such as membrane leakage or complete disruption of liposomal formulations. This membrane disruption is not entirely a detergent-like effect (76). One model that has been proposed to explain how cationic dendrimers interact with cell membrane is the membrane bending model (**Figure 2.2**) (70). This model explains the interactions as being between a malleable anionic bilayer and a rigid, cationic dendrimer. These electrostatic forces induce a local region of inverse curvature, which in turns induces packing stresses to initiate lipid mixing. This lipid mixing eventually leads to membrane disruption. This model is biomimetic in the sense that protein-induced bending is thought to be an important factor in the fusion mechanism of the Influenza virus (82). This model is similar to the model from live cell experiments in that branched structures have a greater ability to interact with the cell membrane, thus creating a more toxic effect. A difference between these theories is that the membrane bending model assumes a polycation as a rigid object in contrast to a branched polycation being flexible. This flexibility then increases the probability of electrostatic interactions with the cell membrane.

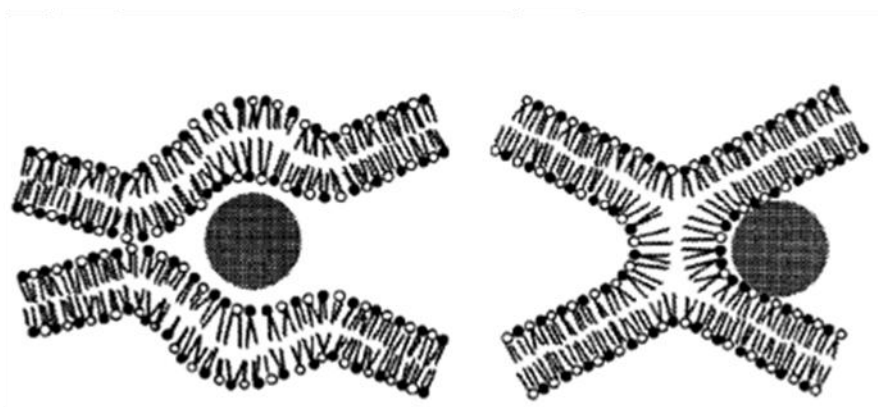


Figure 2.2. Membrane bending model. This is a schematic representation of the membrane bending model. Lipids represented with black head groups are anionic and lipids with white head groups are zwitterionic. **(A)** Cross-section of two lipid membranes cross-linked by a cationic dendrimer. Electrostatic forces between charged species bend the membrane and induce local regions of inverse curvature. This can lead to enhanced lipid mixing between vesicles. **(B)** Cross-section of cationic dendrimer bound two fused lipid membranes at the vesicle-vesicle contact point. This lipid mixing then leads to membrane rupture (70).

There has been great interest in elucidating the mechanism of polycation cytotoxicity. This interest has resulted in theories and hypotheses that have helped explain the toxicity of some polycations, but a theory that can encompass all polycation has not been found yet. There have also been efforts to model the interactions between a polycation and the cell membrane in an attempt to predict trends in toxicity. Both models listed here have their merits, but fail to accurately predict cytotoxicity for all polycations. Furthermore, it is still not completely known whether cell death from polycation interaction is mediated by necrosis or apoptosis. Given this situation, focusing on a positively charged polymer that is synthesized by the body might give rise to a synthesis template that would have increased biocompatibility.

Cationic Peptides

Cationic peptides are short polymers involved in numerous regulatory pathways. These regulatory pathways play an important role in maintaining biological homeostasis.

They generally consist of 12-50 amino acids and can be itself a biomolecule or be a cationic domain within a protein. These peptides consist of hydrophilic, positively charged domains and neutral, hydrophobic domains that are spatially separated (83, 84). They may also contain negatively charged domains as well. The positive charge of these peptides arises from the presence of arginine, lysine, and/or histidine residues. It is this positively charged domain that is used to biologically interact with its environment, most notably negatively charged biomolecules.

Cationic peptides are most often found within organisms as antimicrobial agents. These peptides were first discovered approximately 25 years ago. They were initially found in the skins of frogs, the lymph of insects, and human neutrophils. Presently, more than 600 cationic peptides have been discovered throughout all species (83). An example of an antimicrobial peptide found in humans is defensin (**Figure 2.3A**). Defensin is secreted by neutrophils and contributes to the killing of foreign microbes. It has even been proposed that defensins interfere with the ability of HIV to infect cells. These peptides have a broad range of antimicrobial activity including activity against bacteria, eukaryotic parasites, viruses, and fungi. The reason why cationic peptides are effective antimicrobial agents is because of their ability to interact with the membranes of these different threats (84, 85). As mentioned earlier, the hydrophilic and hydrophobic properties result in a three dimensional structure that is ideal to interact with membranes. This is because membranes are typically comprised of hydrophilic head groups with a hydrophobic core, a similar structure relative to cationic peptides.

Even though the majority of cationic peptides present in the body have antimicrobial roles, there are cationic proteins that facilitate other important biological functions. One example is the balancing of iron concentrations in the body. Iron is

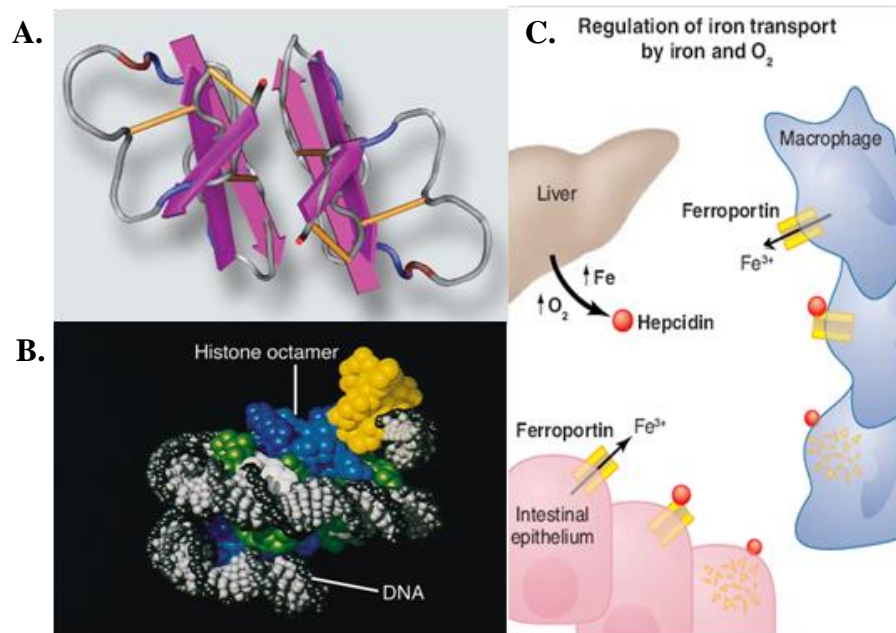


Figure 2.3. Examples of cationic peptides in nature. (A) Human neutrophils secrete the antimicrobial peptide, defensin, in response to bacterial infection. This peptide forms a dimeric structure that contains beta sheets (purple), disulphide bonds (orange), positively charged residues (blue), and negatively charged residues (red). (B) Histones are proteins that compact DNA and ultimately help to regulate gene regulation. Histones use cationic peptide sequences to interact with the negatively charged phosphates present within DNA. DNA (shown in black and grey) winds around an octamer of histones, shown in blue. (C) Hepcidin, a cationic peptide, is a key regulator of iron levels in the body. Iron levels control the secretion of hepcidin. Hepcidin then facilitates the

concentration of ferroportin on the surface of cells that regulate the storage of iron. Ferroportin is an iron receptor responsible for the transport of iron from inside to outside of these cells (85-87).

essential to life, but it is also highly reactive and can be toxic when in excess. Iron homeostasis is a complex biological process, as a number of different proteins respond not only to total iron levels in the body but also to stimuli such as hypoxia, anemia, and inflammation. One of the key regulators of this critical biological process is hepcidin (**Figure 2.3C**) (87, 88). Hepcidin is a peptide hormone secreted by the liver in response to iron loading and inflammation. Hepcidin is composed of 25 amino acids and has a structure similar to antimicrobial cationic peptides. It has been reported that the 5 amino-terminal amino acids of hepcidin are key to maintaining its bioactivity. This 5 N-terminal amino acid sequence is known as an amino terminal Cu(II) and Ni(II) binding motif (89, 90). Histidine is a key mediator of these interactions through the nitrogen on its imidazole group, which is present on the amino acid side chain. Decreased hepcidin levels leads to tissue iron overload, while excess hepcidin production results in hypoferremia and anemia of inflammation. Hepcidin regulates plasma iron levels by controlling the absorption of dietary iron from the intestine, the release of recycled hemoglobin iron from macrophages, and the movement of stored iron from hepatocytes. The process in which hepcidin operates is as follows (**Figure 2.3C**): iron regulates hepcidin secretion by the liver, which in turn controls the concentration of ferroportin on the cell surface. Ferroportin's main role is to transport iron from inside the cell to outside. When ferroportin's concentration decreases, less iron will be available.

As mentioned earlier, hepcidin is a mediator of innate immunity as well (87). The interplay between iron levels and immune defense can be seen with a pathogen's need of iron to survive, such as bacteria. Bacteria go to great lengths to obtain iron and a molecule like hepcidin can decrease iron levels within the body. It has been seen in patients with high iron levels that they are more susceptible to numerous intracellular and blood pathogens (91). Also, even a small increase in iron intake may diminish host resistance to infection. Hepcidin could also have an antimicrobial effect as seen with other cationic peptides given its structure. Hepcidin's role in immune response was evidenced in a reported study, where patients that developed a systematic infection had their hepcidin levels increase a 100 fold (91).

Examples of cationic peptide sequences contained within larger proteins are histones and heparin-binding motifs in growth factors. Chromatin is the complex combination of DNA, RNA, and proteins. Chromatin makes up the biological template of all eukaryotic genetic material (71, 92). The chief proteins present in chromatin are histones (**Figure 2.3B**). Histones serve as a tool for the compaction of the large amount of DNA present in the genome, but more importantly they serve as gatekeepers for gene regulation. Histones are a dynamic group of proteins that undergo post-translational modifications such as: acetylation, methylation, phosphorylation, among others (86, 93). These post-translational modifications are a complex language that plays a central role in gene regulation (94). The amino acid sequences found to interact with nucleosomal DNA consist of positive clusters of arginine, lysine, and histidine (95). Outside of histones, DNA binding sequences of other proteins contain similar amino acid sequences. For example, the zinc finger and leucine zipper motifs contain positive amino acid clusters

spatially separated from hydrophobic residues (96). Heparin-binding motifs are critical for growth factor-heparin interactions (5). This in turn aids in the regulation of events such as neovascularization and bone growth and maturation. Consensus sequence motifs that proteins use to interact with heparin and heparan sulfate are XBBXB and XBBBXXBX, where B is a basic amino acid and X is either a neutral or hydrophobic amino acid (97). These sequences interact with the negatively charged sulfates present on heparin. Heparin then allows for the sequestering of growth factors to be used during specific events while also maintaining their bioactivity.

From the specific examples given here, it can be seen that cationic peptides have an integral role in the body. Whether these cationic peptides are biomolecules themselves or present as domains within other proteins, their presence is felt from gene regulation to neovascularization. Not only do cationic peptides serve important roles in the body, but they make attractive targets to synthetically mimic. The reason they can serve as a template for synthetic targets is because they are positively charged and function within the body without eliciting a toxic response. Biocompatible polycations are important because there are numerous applications in biomedical engineering that use polycations, but existing polycations have limited biocompatibility.

Growth Factor Delivery for Therapeutic Neovascularization

Cardiovascular Disease

Cardiovascular disease is not only the leading cause of death, disability, and healthcare expenditure in the United States, but is also the leading cause of mortality in the world, with the exception of sub-Saharan Africa (1, 98). Coronary artery disease

(CAD) falls under the umbrella of cardiovascular disease and is the end result of accumulation of atherosclerotic plaques (**Figure 2.4**). These plaques then lead to the narrowing of the coronary arteries. CAD has a broad spectrum of manifestations that range from effort-induced angina without myocardial injury to irreversible myocardial damage resulting in congestive heart failure. The rate of progression of CAD is predominantly determined by the growth and/or rupture of these atherosclerotic plaques (99). In situations where myocardial tissue has been compromised, treatment outside of what the body can offer is needed. This need for treatment arises because the mammalian heart has little ability to regenerate cardiac tissue (2).

Presently, there are a number of invasive and noninvasive treatments for patients with CAD. Treatment options encompass mechanical revascularization (invasive examples: angioplasty and coronary bypass surgery) to medical therapies (noninvasive examples: nitrates and β -blockers). Mechanical revascularization physically restores flow to myocardial tissue, while medical therapies restore the perfusion supply and demand balance by reducing oxygen requirements of cardiac tissue. Despite the advancements that have been made in treating ischemic heart disease there is still a subset of patients that do not improve with current therapies and the patients that receive treatment are more likely to have reoccurring myocardial episodes. Also, this subset of patients is expected to increase with an aging population and the rise of diseases such as obesity and diabetes mellitus (3). Present therapies do not have the capacity to regenerate cardiac tissue and this presents a need for a therapy that can regenerate myocardial tissue. One approach to this growing problem is therapeutic neovascularization.

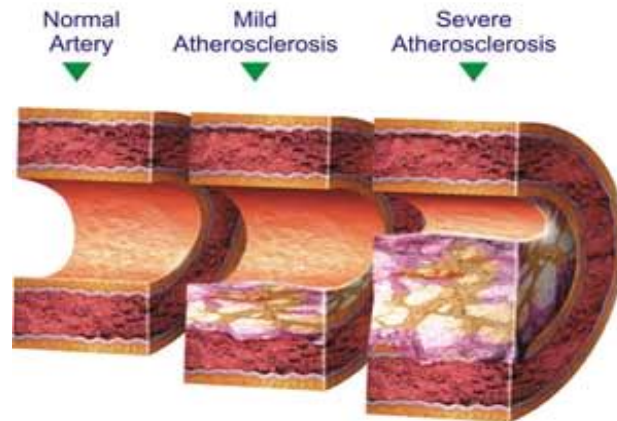


Figure 2.4. Cardiovascular disease. This is a schematic representation of the progression of atherosclerotic plaques. The accumulation of plaques is a chronic inflammatory response in arteries and eventually results in ischemic heart disease (100).

Therapeutic Neovascularization as a Promising Treatment for Ischemic Diseases

New blood vessel growth is referred to as neovascularization and is the result of several processes including angiogenesis, arteriogenesis, and, potentially vasculogenesis. Angiogenesis is defined as the sprouting of new capillaries from pre-existing vessels resulting in new capillary networks (101). In adults it is predominantly stimulated by tissue ischemia via the hypoxia-inducible factor (HIF-1 α) pathway. HIF-1 α in turn up-regulates the gene products of vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), angiopoietins, and other angiogenic factors (102, 103). These factors orchestrate the events that encompass angiogenesis, from degradation of the basement membrane to maturation of new vasculature. In contrast, arteriogenesis describes the growth of functional collateral arteries from pre-existing arterio-arteriolar anastomoses. Arteriogenesis typically occurs outside the area of ischemia in response to

physical forces such as altered shear forces. These forces generally appear within the collateral artery after an increase in blood flow (99, 101). Since arteriogenesis leads to the formation of arterial conduits, its potential to fully restore blood flow is much greater than angiogenesis. Finally, vasculogenesis is the process of formation of new blood vessels from circulating endothelial progenitor cells (104). The role of vasculogenesis in ischemic tissues has not been conclusively established and benefits have been reported from significant to none (105, 106). In the situations where native neovascularization is insufficient to restore arterial blood flow, such as CAD, myocardial tissue will become necrotic. In these situations, exogenous cues are needed to stimulate the formation of new blood vessels.

Neovascularization is a tightly regulated process and when uninhibited is implicated in a multitude of diseases. Because of neovascularization's central role in many disorders, there have been intensive efforts undertaken to develop therapeutic strategies to manipulate this process. Therapeutic neovascularization is the process of altering native vascular architecture under the direction of exogenous mediators (**Figure 2.5**). This type of therapy has had mixed results thus far in a clinical setting. The most successful therapeutics have come in the form of anti-cancer drugs that act as angiogenesis inhibitors. These angiogenesis inhibitors target predominantly endothelial cells with VEGF inhibitors, but have also been used to target mural and stromal cells (PDGF inhibitors) and haematopoietic cells (VEGFR-1 inhibitors) (107). In contrast, therapeutic strategies to promote revascularization of ischemic tissues have not achieved the desired results clinically. This sub-optimal outcome can be mostly attributed to the mode of delivery of biological agents, while other factors such as choice of therapeutic

agents and vasculature responsiveness also play important roles (98, 108). In response to the deficiency of an ideal delivery system, there has been a great deal of effort invested in achieving a strategy that has a sustained pro-neovascularization effect. Presently, there are two predominant thrusts being pursued in therapeutic neovascularization of myocardial tissue: cell-based and growth factor strategies.

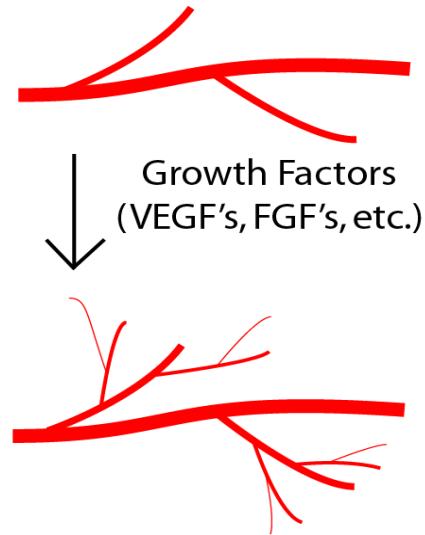


Figure 2.5. Therapeutic neovascularization. This is a schematic of therapeutic neovascularization using growth factors. The addition of exogenous growth factors results in the formation of new vasculature.

Myocardial Regeneration: Growth Factor vs. Cell-based Therapy

The lack of a successful delivery strategy for myocardial regeneration is a critical determinant in unsuccessful clinical trials. Currently, the two most investigated approaches to bridge the gap are cell-based and protein therapies. Over the past decade, there has been a variety of different cell types used to regenerate cardiac tissue. The cell types used include skeletal myoblasts, bone marrow cells, endothelial progenitor cells, mesenchymal stem cell, as well as others (4, 109). Even with the optimism this strategy

has generated, there are still many obstacles to overcome. The first challenge is to optimize the delivery of cells to the heart. It has been reported that approximately 90% of transplanted cells are lost to the circulation or leak out at the injection site (110). A second problem is the extensive death of injected populations regardless of cell type. Numerous studies have indicated that approximately 90% of cells delivered to the myocardium die within one week of injection (111). Another area of concern is nearly all studies of cell transplantation have found that scar tissue creates a barrier between damaged myocardium and delivered cells. This barrier then results in poor host-tissue integration of implanted cells (112). These challenges have left cell-based therapies without clinical success and are a reason why other alternatives are still being pursued for myocardial regeneration.

Growth factor-based therapy is another strategy for the regeneration of ischemic cardiac tissue and has been investigated more extensively in clinical trials compared to cell-based therapies. After being successful in a variety of animal models, therapeutic neovascularization via growth factor delivery has yet to attain the same level of achievement in a clinical setting. As stated earlier, there are a number of reasons why clinical trials have been unsuccessful. In these trials, growth factors were administered via bolus injection at the site of injury. This type of delivery is inefficient in that under 1% of growth factor injected is deposited in the myocardium at 1 hour and even less is present after 24 hours (108, 113). This time frame does not correspond with the time needed to alter native vasculature, which likely happens on a week to month timescale. As mentioned earlier, there are many factors involved with neovascularization and only two (VEGF, FGF) have been used in clinical studies, although many other growth factors

are being examined in animal studies. To achieve improved results, a cocktail of different therapeutic growth factors will most likely have to be used. Also, there may be a need for other factors (example: IGF) that could stimulate cardiomyocyte proliferation. Finally, endothelial dysfunction could also comprise a critical role in clinical trials failing (98, 99). Based on the criteria that has been listed, a strategy for growth factor delivery will have to encompass the following: a release of therapeutics over an extended period of time (weeks to months), delivery of a cocktail of different growth factors (VEGF, PDGF, etc.) (most likely at different concentrations and time scales), and find a way to circumvent endothelial dysfunction by choosing a more suitable patient subset for clinical trials or deliver factors (example: L-arginine) that could return function to damaged endothelium (114).

Polymeric Strategies for Growth Factor Delivery

Given the state of growth factor delivery for neovascularization, there are many researchers pursuing an improved solution. One approach is to deliver genetic material encoding growth factors, however *in vivo* gene delivery experiments have led to unpredictable results. Some of which have resulted in complications and even the death of patients (115). An alternative strategy to gene delivery is the delivery of the target growth factors. For a growth factor delivery strategy to be successful it should be able to mimic the native neovascularization process (116). The essential characteristics of the *in vivo* response include a delivery of different growth factors, the delivery should be under controllable release kinetics, and the target growth factors should maintain their bioactivity. A set of substrates that have made strides in duplicating neovascularization

in vivo events are polymeric delivery systems. Both synthetic and naturally derived polymeric systems have been used for the delivery of growth factors (**Figure 2.6A, B**) (117). Aliphatic polyesters (examples: poly(glycolic acid) (PGA), poly(lactic) acid (PLA), their copolymers (PLGA), and poly(ethylene glycol) (PEG)) (118) have been the most extensively used synthetic polymers for therapeutic delivery. These types of polymers allow for facile manipulation of their physiochemical properties, while also being readily available. In contrast, naturally-derived polymers (examples: collagen, hyaluronic acid, and alginate) are more limited in their scope of properties but often exhibit enhanced biocompatibility (119).

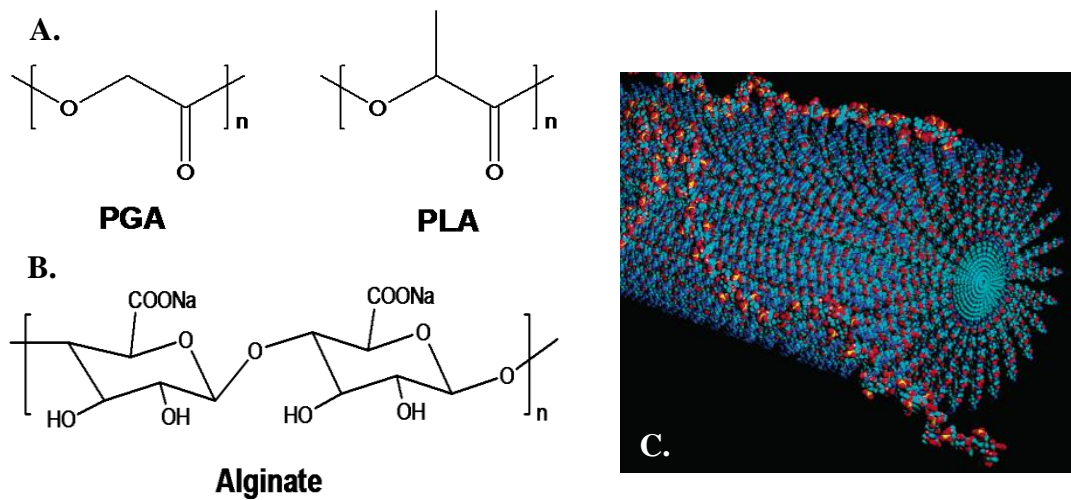


Figure 2.6. Examples of growth factor delivery strategies. Different strategies have been used to deliver growth factors safely and efficiently. Strategies can be classified as (A) synthetic polymers, (B) natural polymers, (C) and the combination of synthetic and natural strategies (7).

There have been numerous approaches used in polymeric delivery strategies. The simplest approach is the delivery of therapeutic growth factors alone. Examples of this type of delivery systems include micro/nano particles, micelles, microneedles, and hydrogels (120-123). These systems have take advantage of both synthetic and natural polymers to control release kinetics. Another approach is to delivery growth factors within a scaffold with the presence of cells (12, 124, 125). The delivered growth factors are then anticipated to help direct the fate of delivered cells. Scaffold materials, like other listed methods, use both synthetic and natural polymers.

Polymeric systems have made drastic improvements in growth factor delivery through the original goal of mimicking the *in vivo* neovascularization events. One way they have accomplished this is through physiochemical control of the delivery system. The release rate of therapeutics can be modulated by altering characteristics of the polymer such as molecular weight and cross-linking density (126). A sustained release over a period of weeks has been accomplished by adapting these polymer properties. Another improvement polymeric systems have provided is a localized delivery with spatial gradients (113). This type of delivery can be achieved by modifying polymer properties (example: cross-linking density) and substrate formation. These strategies also allow for the use of multiple growth factors at variable rates and gradients, similar to those in native tissue (127). Some problems with using these delivery strategies are that a number use harsh organic solvents that can limit the bioactivity of its growth factor cargo. It should also be noted that these systems have little to no affinity for their target growth factors. This can create limitations with loading capacity and efficiency of the delivery vehicles.

Finally, polymer strategies have used the combination of synthetic and natural polymers along with other biological motifs to create an environment similar to native ECM. These systems use non-covalent interactions to simulate ECM interactions with proteins (7, 128-130). Some examples include the use of growth factor binding motifs and native biomolecule incorporation (examples: alginate, heparin). One well characterized affinity-based delivery system is the heparin-based delivery system.

Heparin-based Delivery Systems

Heparan sulfate (HS) and heparin are glycosaminoglycans (GAGs) that are usually present as proteoglycans. In native tissues, heparan sulfate is widely distributed on cellular surfaces as well as in the ECM. Heparan sulfate and heparin are involved in a variety of biological functions. These functions include cell adhesion, migration, and mediating growth factor interactions. Heparin is a highly sulfated variant of heparan sulfate and is more commercially available. For these reasons, it has been used as a model agent in experimental studies which in a physiological setting would most likely involve heparan sulfate. Heparin has the highest negative charge density of any known biological macromolecule, and this property gives heparin the capacity to interact electrostatically with other biological molecules such as enzymes, ECM proteins, and various cytokines (5, 131). Also, heparin sequesters growth factors in the ECM, serving to localize growth factor activity, and in some instances increases the bioactivity through its interactions with the growth factor and its receptor (**Figure 2.7**). This function prevents growth factor degradation and keeps them readily available for biological events. Thus, heparin allows an environment for growth factors to remain bioactive over

an extended period of time. This is a valuable trait to have because it has been found that recombinant proteins have a very short half-life when introduced *in vivo* (132). These characteristics of heparin have made it an attractive candidate for use in growth factor delivery strategies. For example, heparin's capability for the controlled release of FGF-2 has been employed on heparin-sepharose beads. Other examples include functionalizing hydrogels and polymeric scaffolds with heparin or heparin moieties (7, 133-137) and incorporating heparin into polymeric microspheres/micelles (138-140). These delivery strategies have taken advantage of heparin to develop a system that is capable of a localized, sustained release for a multitude of therapeutic growth factors.

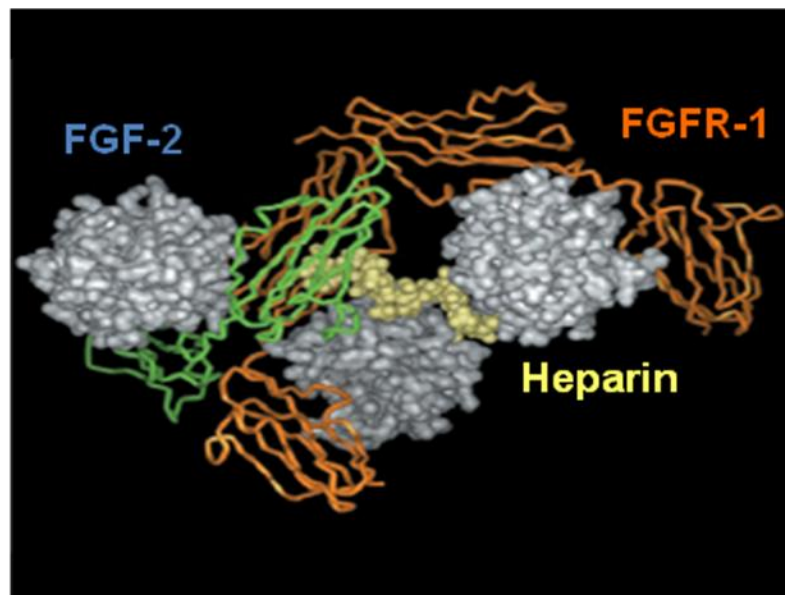


Figure 2.7. FGF-2 interactions. Heparin plays a key role in FGF-2 signaling by direct association with FGF-2 and its FGF receptor in a ternary complex on the surface of cells. Heparin is represented as the yellow molecule, FGF-2 as the bluish-grey molecule, and FGFR as the orange and green molecule (141).

Heparin-based delivery vehicles have generated much success in research, but there are still flaws that need improvement for clinical success. The majority of these systems do not incorporate heparin in its native form. This can lead to limitations with bioactivity. Also, these strategies do not have compositions that allow for easily tunable release kinetics of growth factor. A delivery strategy that uses native heparin and allows for a controllable release of growth factors is needed and would provide an improvement over these current systems. With continued improvement of delivery vehicles for therapeutic proteins, the present optimism should eventually lead to clinical realization.

Chapter 3

Synthesis of Poly(Arginate Glycerol Succinate) (PAGS)

Introduction

In the field of biomedical engineering there is still a need for the introduction of new biomaterials (8). A specific example of an area that would benefit from this is the synthesis of polycations. Presently, there are numerous synthetic polycations that are used in biomedical engineering ranging from tissue engineering to drug delivery (23, 142). These synthetic polycations have contributed greatly to the field over the years, but current polycations still have not met the challenge of biocompatibility (57). Present synthetic polycations have been modified extensively and a biocompatible polycation still has not been achieved. It is at this point where the introduction of a novel, rationally designed polycation could succeed where other polycations have failed. In the goal of designing a biocompatible polycation, it was decided to mimic cationic peptides because they are positively charged polymers that the body uses in a variety of different processes (91, 143). The rationale design of a polycation that had the essential traits of cationic peptides was hypothesized to yield a biocompatible polycation.

The essential traits of a cationic peptide include positively charged domains, biodegradability, and a naturally derived composition (83). It was hypothesized that combining these features within a polymer would yield a synthetic polycation that was biocompatible. We chose to instill biodegradability through the introduction hydrolysable ester linkages. The positive charge of the polycation originated through the incorporation of a positively charged amino acid. We chose a polycondensation reaction

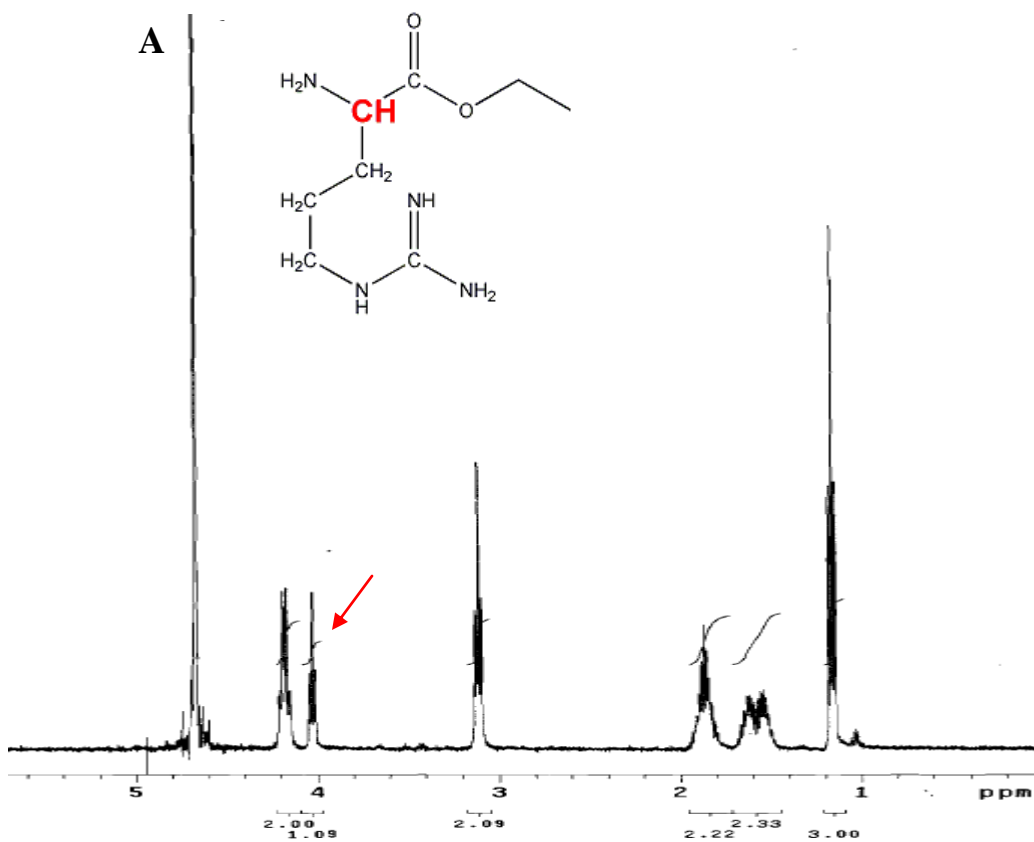
to carry out the polycation synthesis. The polymerization was mediated between the primary amine on the cationic amino acid and the epoxy of the synthetic linker through a ring opening mechanism. The path of synthesizing this polycation was one that went through a series of adaptations and modifications before settling on the final synthesis strategy.

Results and Discussion

Cationic Species

The first component decided upon was arginine and this was the positive charge carrying unit. Arginine is the most positively charged amino acid found in nature and there has been evidence that arginine heavy motifs are advantageous for cellular transport (entry into the cell and nucleus). In this synthesis, there are two monomers that needed to be brought into solution (arginine and the synthetic linker). An aqueous solvent was not an option because this type of polycondensation would not occur in this solvent. This reaction needed an organic solvent that would dissolve both monomers to be successful. DMF was chosen because it is a polar, aprotic solvent and had the best chance to dissolve arginine as well the organic synthetic linker. DMF was first used to dissolve arginine, but had little to no solubility in this solvent. It was then decided to use an arginine variant that would be more soluble in an organic solvent. We chose arginine ethyl ester and this compound proved to be soluble in DMF. The addition of the ethyl group through esterification of the acid on arginine is what led to this increase in solubility in DMF. Arginine ethyl ester comes as a salt (dihydrochloride) for stability issues and the hydrochloride present on the primary amine had to be liberated to ensure the reaction

would take place between this primary amine and the epoxide of the synthetic linker through a ring opening mechanism. The liberation of arginine ethyl ester is exhibited in **Figure 3.1**. Arginine ethyl ester was ensured to be liberated by the shift of the proton on the alpha carbon. After liberation this proton shifted up field from 4.08 ppm to 3.76 ppm. Once arginine ethyl ester has been liberated it can then be used for polymerization. Following polymerization, the incorporation of arginine ethyl ester was verified by presence of the guanidinium side chain via spectral analysis (**Figure 3.1B, C**).



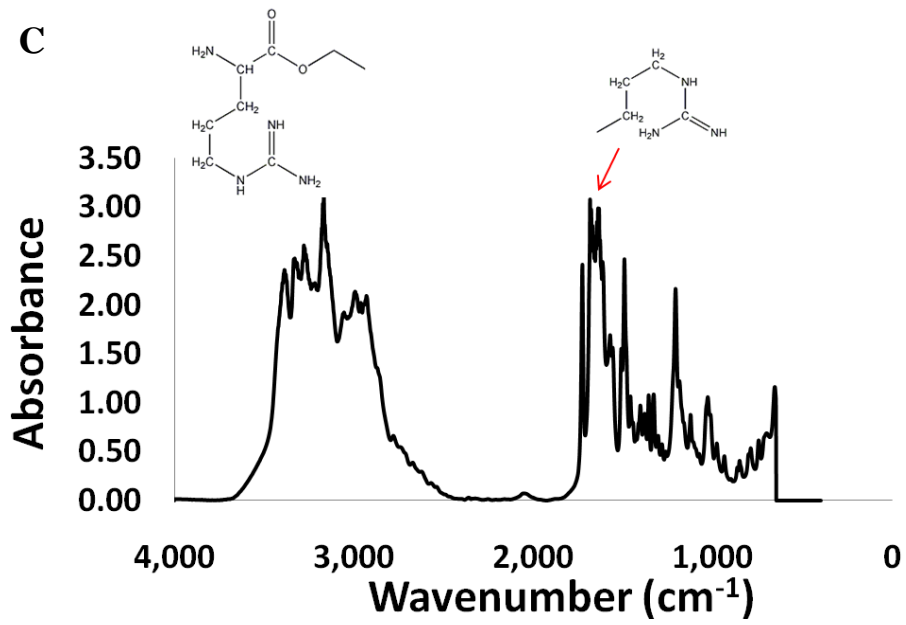
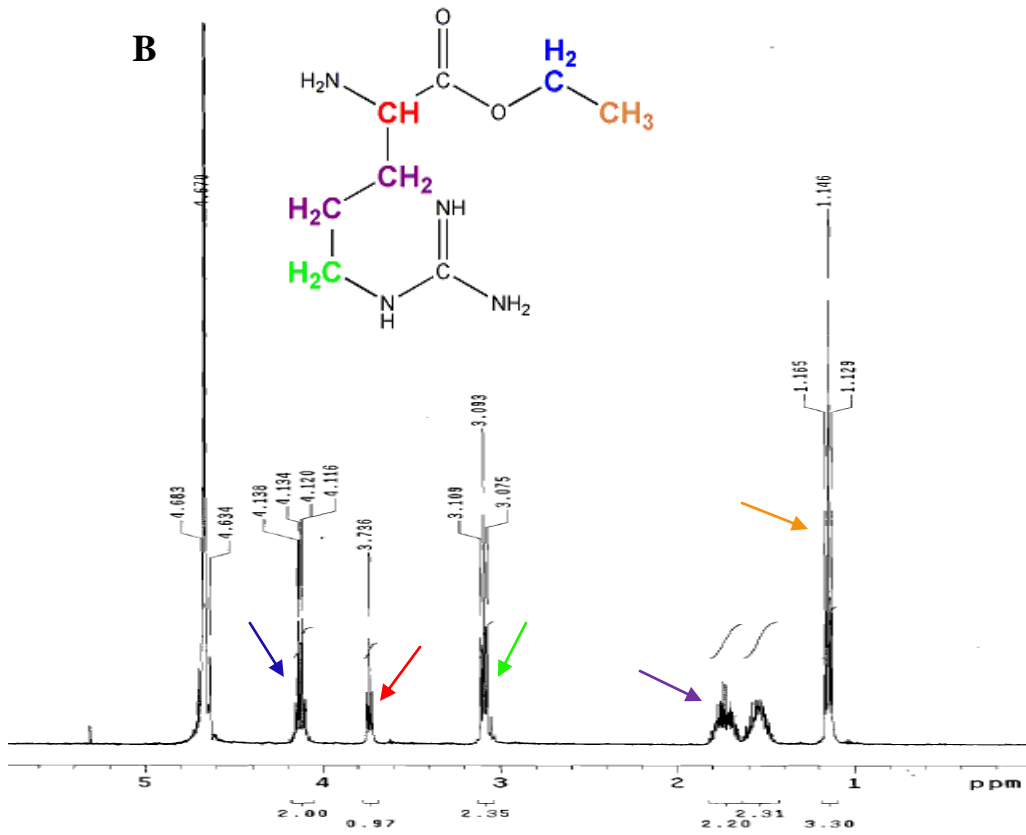


Figure 3.1. Spectral analysis of arginine ethyl ester. Before arginine ethyl ester could be used in the polymerization, it had to be ensured that the primary amine was liberated. (A) This is the NMR spectrum of unliberated arginine ethyl ester. The proton and alpha carbon are colored in red. (B) This is the NMR spectrum when the primary amine on arginine ethyl ester has been liberated. The key difference between the two spectra is the proton on the alpha amine has shifted upfield after it has been liberated. It shifts from 4.08 ppm in the unliberated form to 3.76 ppm in the liberated form. The spectrum in B also details the proton arrangement by the protons being colored as well as its attached carbon. (C) The IR spectrum of arginine details the guanidinium side chain (approximately 1673 cm^{-1} with a shoulder at 1635 cm^{-1}). Both NMR and IR spectra were used to ensure the incorporation of arginine into resultant polycations.

Synthetic Linker

The second component of the biodegradable polycation was an organic synthetic linker. The reactive groups on the synthetic linkers were glycidyl functional groups that facilitated the polycondensation reaction. There were many avenues pursued before settling on a monomer that resulted in a polycation that could be used for our desired applications. The first attempt used for the synthetic linker was 1,2 diglycidyl cyclohexane (**Figure 3.2**). This was chosen first because it was available commercially. Attempts to use this monomer never yielded a polymer with a molecular weight suitable for the delivery of anionic molecules. One reason this monomer did not yield a high molecular weight polymer was because the diglycidyl functional groups were in the 1, 2 conformation on the cyclohexane. This conformation will likely result in a cyclic

polymer where the polymerization would end before a suitable molecular weight was achieved.

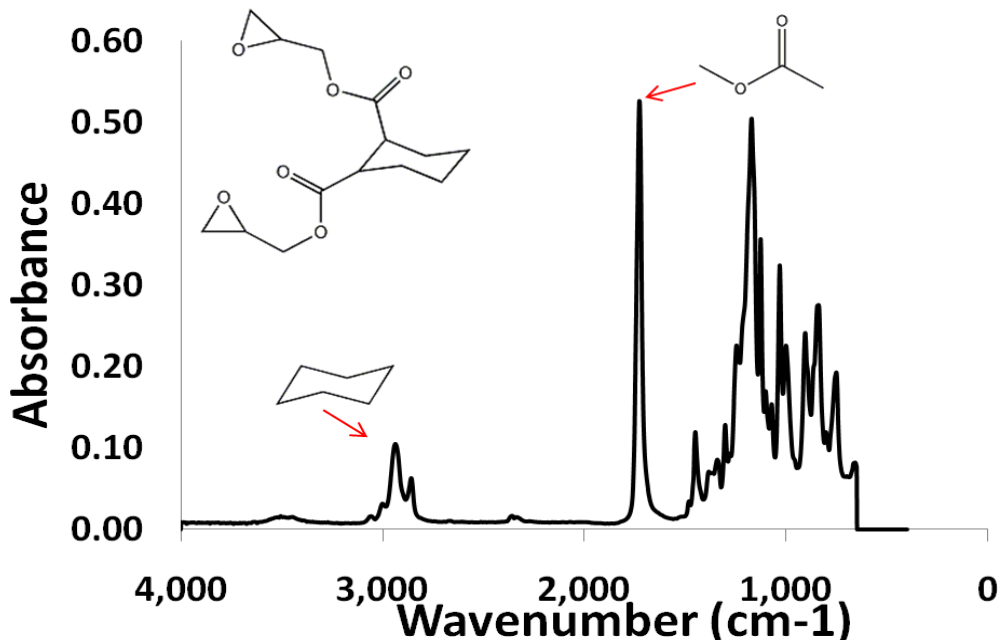


Figure 3.2. Infrared spectrum of 1, 2 diglycidyl cyclohexane. The IR spectrum of 1,2 diglycidyl cyclohexane was used to confirm the presence of this monomer in resultant polymers. Distinguishable absorptions of this monomer are noted with red arrows (cyclohexane at approximately 3000 cm⁻¹ and ester functional group at approximately 1700 cm⁻¹).

The next organic synthetic linker that was used was diglycidyl sebacate. This organic compound is a linear aliphatic ester that contains eight carbons between the ester functional groups. This monomer resulted in a polycation that was used for gene delivery. The sebacate-based polycation did not lead to high transfection efficiency in

gene delivery. It was hypothesized that a monomer that could yield a polycation with a higher charge density would improve the gene delivery results. A higher charged density could compact plasmid DNA with less polycation and these complexes could potentially be compacted into smaller nanoparticles. This in turn could potentially lead to more [polycation:DNA] complexes entering the cell.

The final synthetic organic linker attempted was diglycidyl succinate. Diglycidyl succinate is another linear aliphatic ester but it is smaller than sebacate, only containing two carbons between the ester functional groups. Diglycidyl succinate was elected as a synthetic linker because it ensured the highest charge density for the polymer while being a derivative of succinic acid. As mentioned prior, we hypothesized that a polycation synthesized from naturally derived compounds or derivatives of this compounds would result in better biocompatibility. Succinic acid is substance readily found in the body and plays a key role in cellular respiration by being a central substrate in the citric acid cycle. Succinate also allowed for a high charge density by having the shortest aliphatic chain that allowed for a benign degradation product. For example, diglycidyl oxalate was also considered but abandoned because of its capacity to chelate calcium and other divalent cations when used for *in vivo* applications. The final synthesis strategy used for our biodegradable polycation is exhibited in **Figure 3.3**.

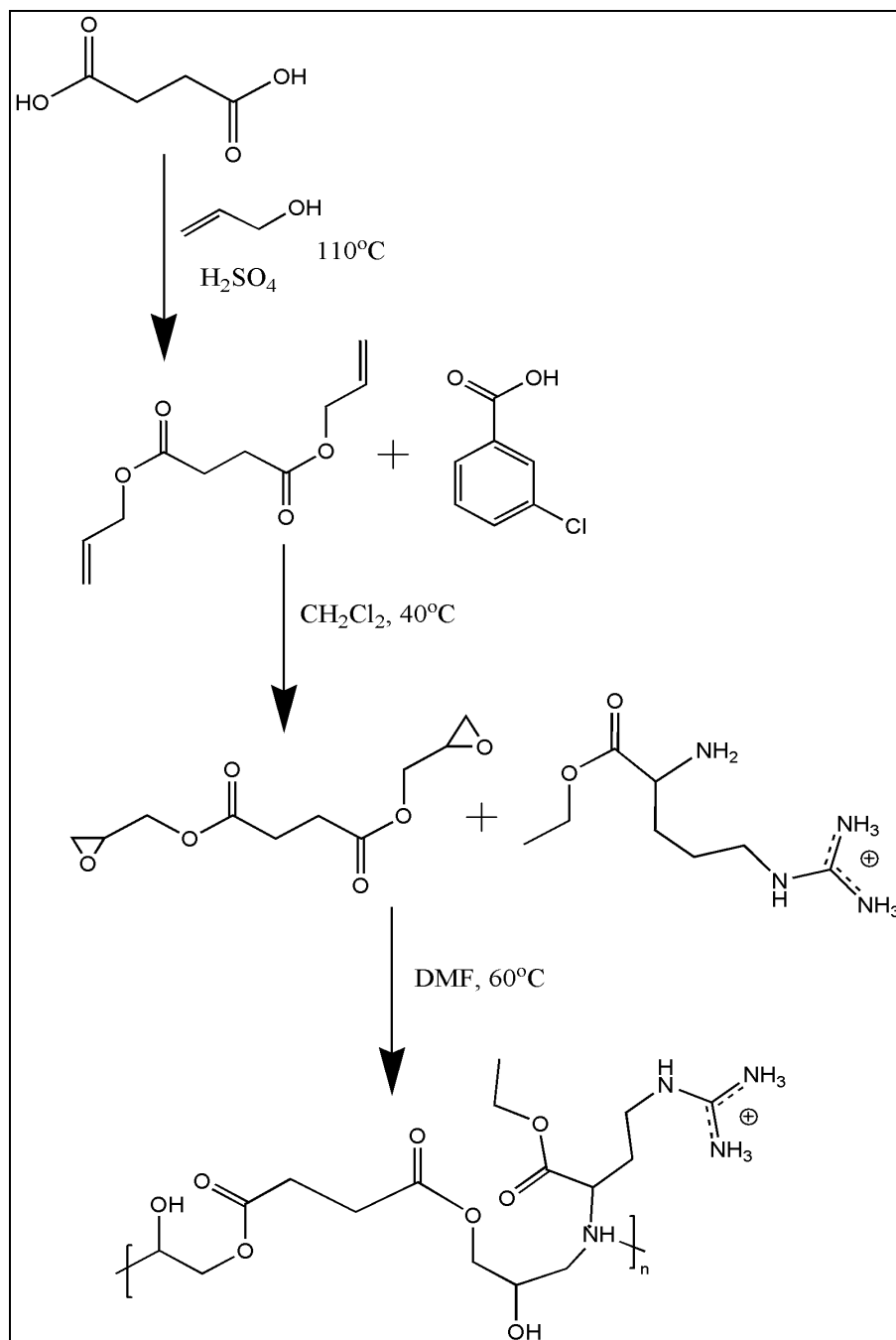


Figure 3.3. Synthesis of PAGES. The first step in PAGES synthesis is to synthesize the diallyl succinate from succinic acid. Then the diallyl succinate is oxidized by 1,3 mCPBA to yield the diglycidyl succinate. Finally, the diglycidyl succinate is combined with arginine ethyl ester to form PAGES.

Conclusion

The synthesis strategy for a synthetic, biodegradable polycation was accomplished through a great amount of effort. There were a number of different monomer and synthesis techniques attempted before a successful strategy was found. The period of time taken to accomplish may seem small compared to other parts of this thesis, but in reality took the longest. The synthesis template present here represents a strategy to improve the biocompatibility of a polycation through a rationale design. This rationale design attempted to mimic the essential traits of cationic peptides. The biocompatibility of this polycation was investigated in the next chapter.

Materials and Methods

Polymer Synthesis and Characterization

Diallyl succinate (**Figure 3.4**) was synthesized by esterification of succinic acid (5 g) in allyl alcohol (20 g) in the presence of catalytic amount of sulfuric acid (98%, 12 μ l). The reaction mixture was stirred and refluxed at 105°C overnight. Sodium bicarbonate was added to the reaction to neutralize the sulfuric acid and the reaction mixture was evaporated under vacuum. The organic phase was extracted using ethyl acetate, and dried by brine. The product was then exposed to anhydrous sodium sulfate overnight, and stored at room temperature. ^1H NMR ($\text{CDCl}_3\text{-d}_1$): 5.87 (m, 2H), 5.23 (m, 4H), 4.57 (d, 4H), 2.65 (s, 4H).

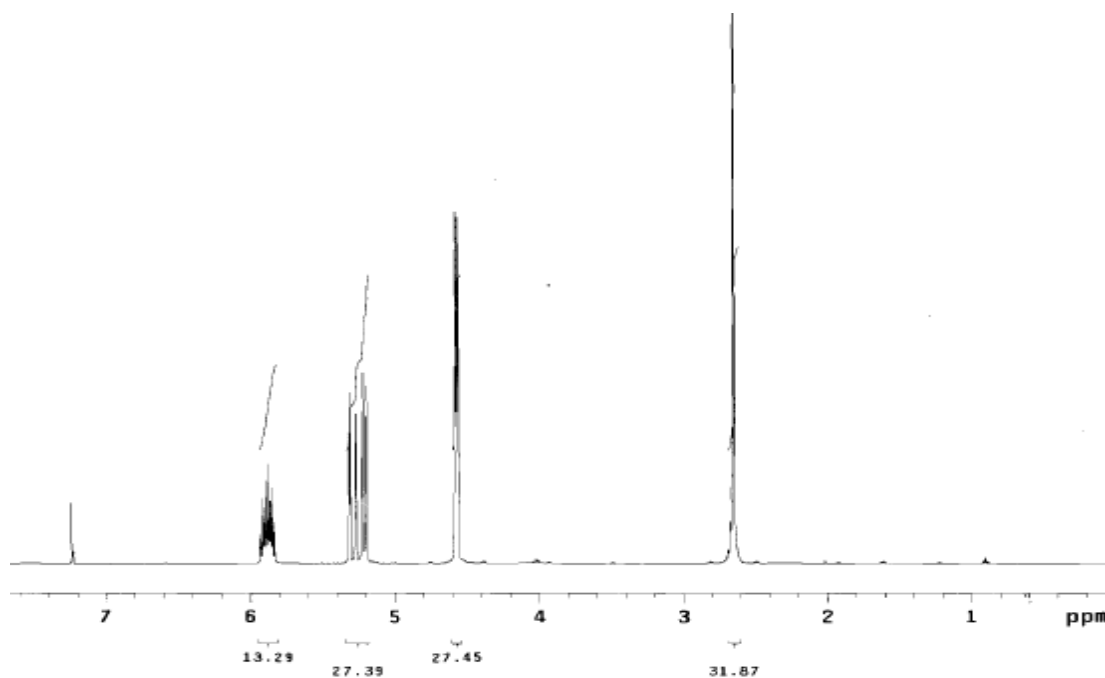


Figure 3.4. NMR spectrum of diallyl succinate.

Diglycidyl succinate (**Figure 3.5**) was synthesized by the epoxidation of diallyl succinate (1.3 g) with meta chloro peroxy benzoic acid (mCPBA, 5 g) in dichloromethane (100 ml). The reaction was stirred and refluxed at 40°C overnight. Reaction mixture was then run through an ionic resin column containing tertiary amine beads. Diglycidyl succinate was further purified using flash chromatography and the final product was stored under N₂ at -20°C. ¹H NMR (CDCl₃-d₁): 4.40 (dd, 2H), 3.95 (q, 2H), 3.20 (m, 2H), 2.84 (t, 2H), 2.68 (s, 4H), 2.63 (q, 2H).

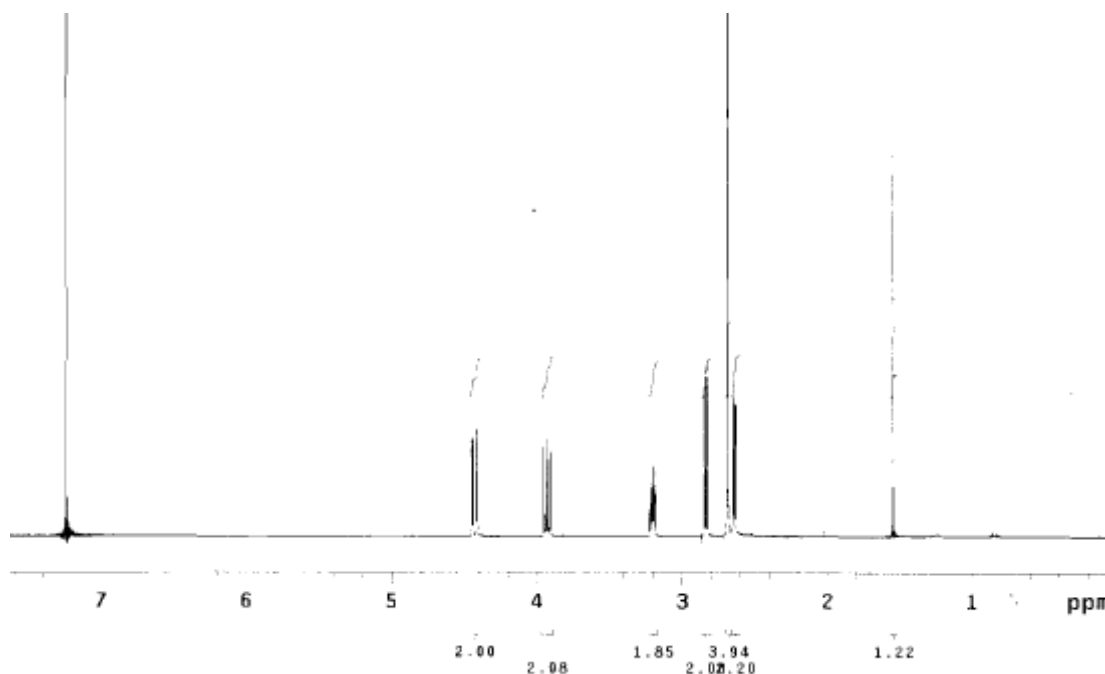


Figure 3.5. NMR spectrum of diglycidyl succinate.

The arginine-based polymer (PAGS) was synthesized via polycondensation reaction of a 1:1 molar ratio of diglycidyl succinate and arginine ethyl ester in anhydrous N,N-dimethylformamide under N₂. The reaction mixture was stirred and kept at 60°C for 7 days. The resultant polymer was placed under vacuum and heated to 60°C for 24 hours to remove solvent. The residual dimethylformamide was removed by dissolving the polymer in methanol, precipitating polymer out with ethyl acetate, and dried under vacuum at 60°C overnight. Ethyl acetate wash is used to remove unreacted product and oligomers. The polymer was characterized by FTNMR, FTIR, differential scanning calorimetry, and gel permeation chromatography.

CHAPTER 4

Characterization of PAGS

Abstract

Cationic peptides participate in many critical events in the body. Their importance in gene regulation, host defense, and many other processes has made them ideal targets to synthetically mimic. Over the years, a multitude of polycations have been synthesized, but a synthetic polycation that is biocompatible has yet to be realized. We created a synthesis platform that has resulted in a synthetic polycation that is orders of magnitude more biocompatible than existing polymers. This design template was inspired by the fundamental characteristics (biodegradability, natural composition) that make native cationic proteins biocompatible. This synthetic, biocompatible polycation holds the potential to improve a wide array of applications in areas from drug delivery to tissue engineering.

Introduction

Cationic peptides are key mediators in a variety of homeostatic processes critical for survival, including histone that regulates DNA replication and gene expression, hepcidin that balances iron concentrations, heparin-binding domains that control growth-factor stability and activity, and defensins that act as anti-microbial agents in host defense. The important functions of cationic peptides in numerous biological pathways have generated great interests in synthetic polycations.

Cationic peptides are a series of amino acids linked through peptide bonds yielding a hydrolyzable polymer. These resultant polypeptides are composed of hydrophobic and positively charged domains that control their physiological functions (84). Cationic peptides make ideal targets to mimic because polycations are used widely in biomedical applications from drug delivery to tissue engineering. There have been numerous attempts to synthetically duplicate the characteristics of cationic peptides to take advantage of their potential (23, 144), however these existing polycations are limited by their biocompatibility (57, 58). Here we report a design platform that gives rise to a polycation that is orders of magnitude more compatible than existing polycations.

We hypothesized that mimicry of the essential chemical structure of cationic peptides would result in a biocompatible polycation. Firstly, natural cationic peptides can be digested by appropriate proteases into molecules that can be recycled by the body or at least elicit an acceptable host response. Secondly, the positive charge of cationic peptides arises from endogenous amino acids such as arginine and lysine. A minimalistic mimicry of a cationic peptide is to design a synthetic polycation with hydrolytic degradability and

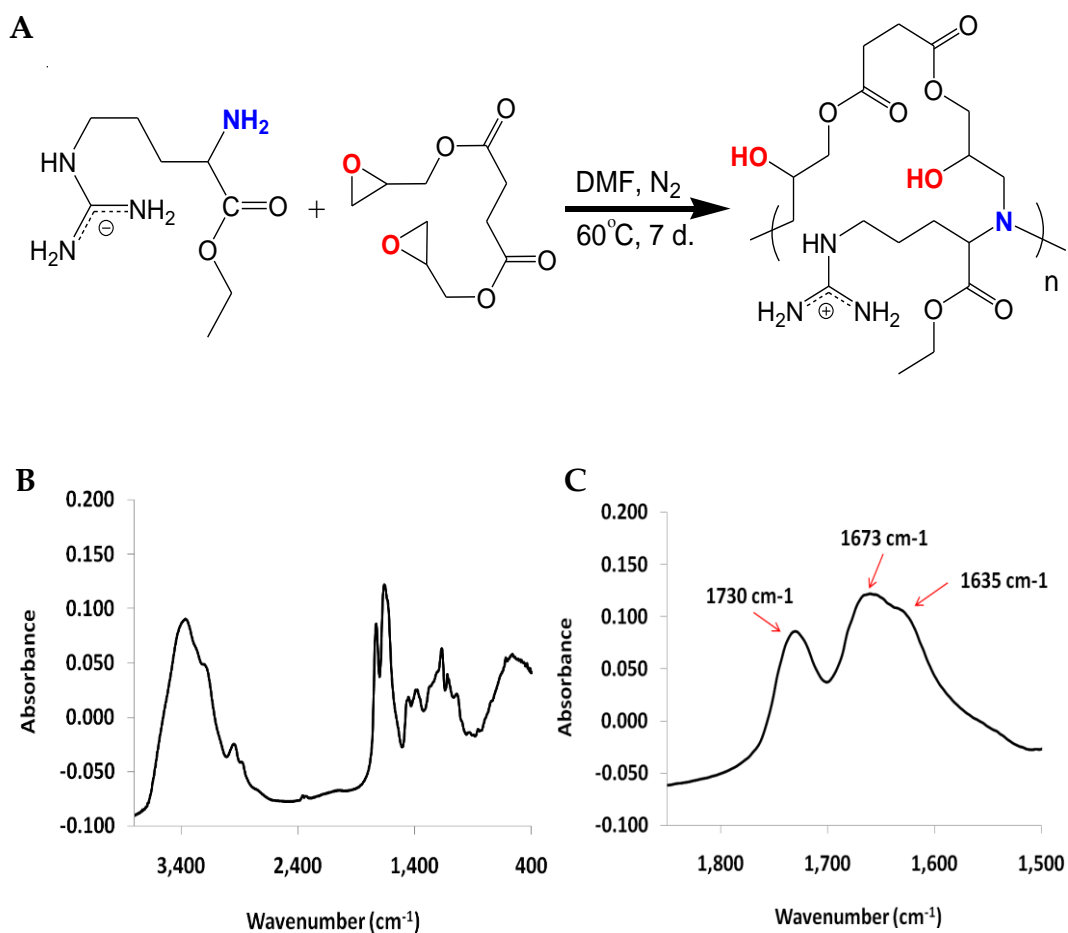
to incorporate a positive charge through an amino acid. We chose to use ester functional groups in the polymer to introduce susceptibility to hydrolysis because of its well defined synthetic routes and familiarity to the biomaterials community (145). We chose to use arginine because of its prevalence in endogenous polyanion-binding domains. Furthermore, arginine carries the most positive charge among the 20 naturally occurring amino acids. The incorporation of arginine facilitates ionization of the resultant polycations at a neutral pH. We chose to integrate arginine into a polyester using a ring opening reaction between the α -amino group of arginine and the epoxy ring of diglycidyl succinate (**Figure 4.1A**). The resultant polycation is referred to as poly(argininate glyceryl succinate), or PAGS. Outside of arginine, the other building blocks of PAGS are derived from succinic acid and glycerol. Succinic acid is readily found in the body, and plays a key role in cellular respiration as a central substrate in the citric acid cycle (71). Glycerol derivatives are prevalent throughout the body in lipids and many signaling molecules. We anticipate that using derivatives of endogenous building blocks are necessary to design a polycation with good biocompatibility.

Results and Discussion

Polycation Synthesis

The polycation reported here is synthesized through a polycondensation reaction between arginine ethyl ester and diglycidyl succinate in N,N-dimethylformamide. The resultant polymer, PAGS, is a pale yellow powder soluble in water. The polymer was purified by repetitive evaporation under vacuum and solvent washes using methanol and ethyl acetate. Nuclear magnetic resonance spectroscopy (NMR) revealed a change in

chemical shift from approximately 3.2 ppm in the diglycidyl ester to approximately 4.0 ppm in PAGS (**Figure 4.1B**). This shift corresponds to the opening of the epoxy ring in the diglycidyl ester. The intense C=O stretch at 1735 cm^{-1} in the Fourier transform infrared spectroscopy (FTIR) confirmed the formation of ester bonds, and the intense band at 1673 cm^{-1} with a shoulder at 1635 cm^{-1} (**Figure 4.1C**) indicated the presence of the guanidinium side chain of arginine (146). PAGS has a glass transition temperature of 42.8 $^{\circ}\text{C}$ and a melting temperature of 88.1 $^{\circ}\text{C}$ as revealed by differential scanning calorimetry measurements.



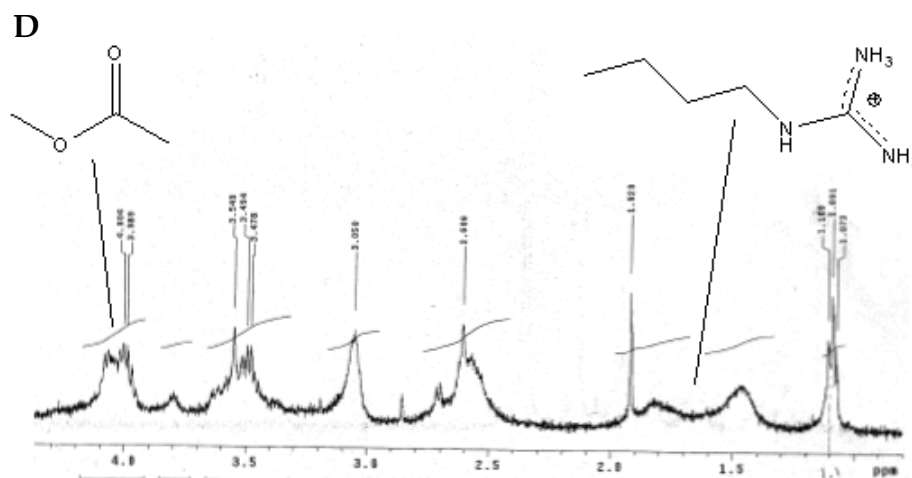
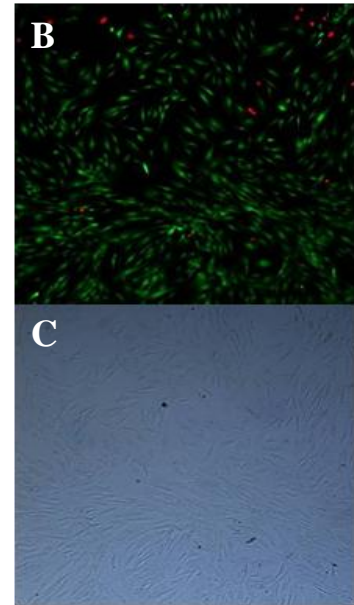
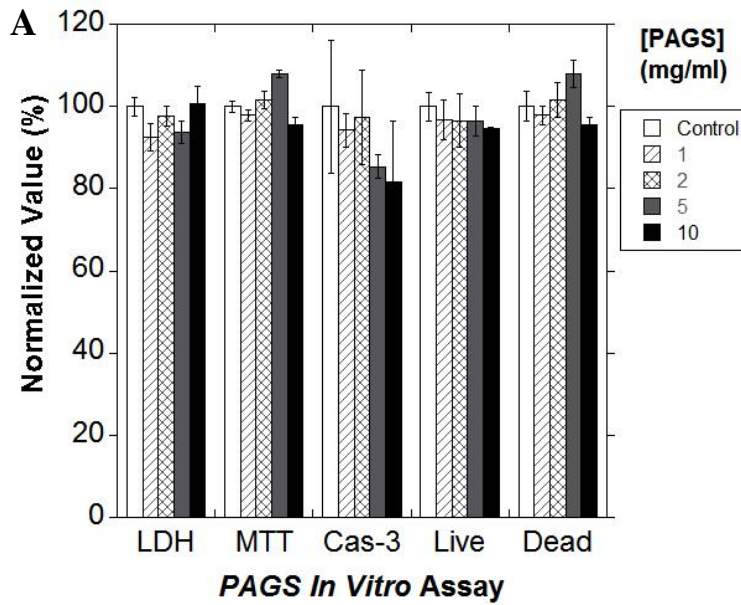


Figure 4.1. Synthesis and characterization of PAGES. **A.** PAGES was synthesized by a polycondensation between equimolar amounts of arginine ethyl ester and diglycidyl succinate. The starting materials are derivatives of arginine, glycerol, and succinic acid. The reactive groups (glycidyl and amine functional groups) involved in the polycondensation reaction are colored in red and blue respectively. **B and C.** The FTIR spectrum of PAGES showed an intense absorbance at 1730 cm^{-1} indicating the formation of esters, and a strong absorption at 1673 cm^{-1} with a shoulder at 1635 cm^{-1} suggesting the presence of guanidinium groups (147). **D.** The NMR spectrum of PAGES demonstrated the shift in the protons of the ester and the incorporation of the guanidinium side chain of arginine ethyl ester.

PAGES was synthesized from an amino acid and an aliphatic diglycidyl ester. This synthesis platform can be applied to virtually any primary amine and diglycidyl ester. The flexibility in polymer architecture allows the control of biomaterial properties, making it adaptable for various applications. For example, the backbone of the diglycidyl ester can be modified to a longer aliphatic chain, aromatic, or cyclic aliphatic.

Each modification will bring unique properties to the resultant polymer. Other biomolecules that have been introduced through amine groups include acetylcholine (148) and dopamine (149).



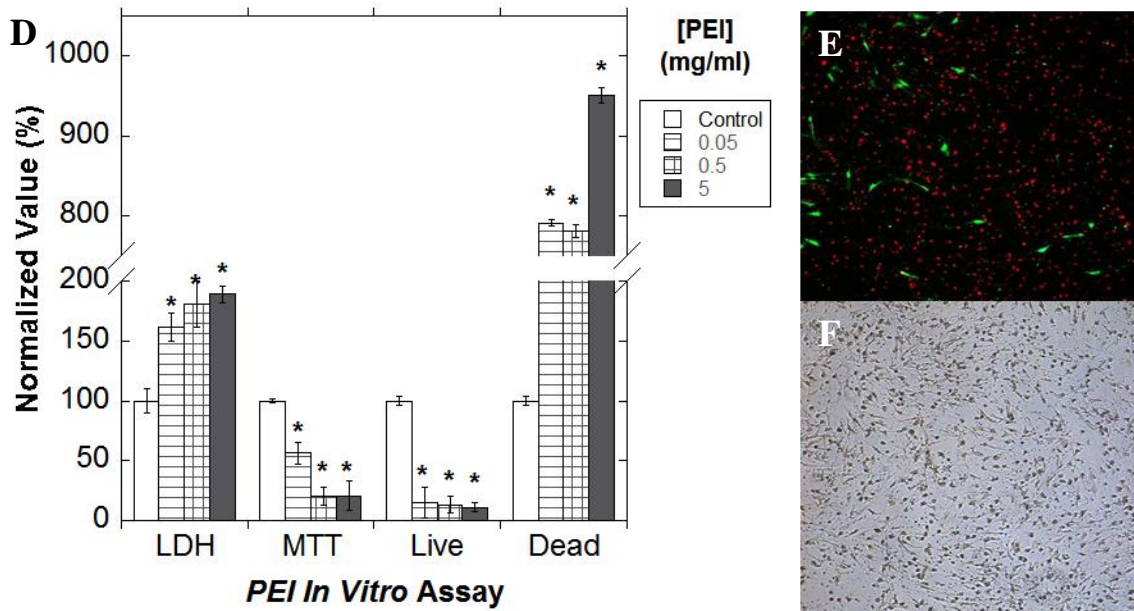


Figure 4.2. *In vitro* biocompatibility of PAGES. **A.** PAGES experimental groups are normalized to the control (SMCs exposed to normal cell culture medium) groups. The assays showed that the cells had intact membrane, exhibited normal metabolic activities and were viable when exposed to up to at least 10 mg/ml of PAGES supplemented media. Cells were incubated for 4 hours except in live/dead assay, which was incubated for 24 hours. **B** and **C.** Fluorescent (10x) and phase contrast (10x) images were captured during live/dead assay: live cells are stained green (calcein AM) and dead cells are stained red (ethidium homodimer-1). SMCs had high viability and expressed normal morphology even after a 24 hour incubation with PAGES. **D.** PEI exhibited toxicity at a concentration as low as 0.05 mg/ml under identical conditions using identical assays. **E** and **F.** A majority of cells exposed to 0.05 mg/ml of PEI exhibited cell death. Multicomparison ANOVA, Tukey method, $p < 0.05$ was considered statistically significant. Any statistically significant difference between the experimental and the control group is noted by an “*”.

***In vitro* Biocompatibility**

We investigated the biocompatibility of PAGES *in vitro* using primary baboon smooth muscle cells (SMCs). *In vitro* biocompatibility assays of polycations often use cell lines, which tend to be more robust than primary cells. We chose primary cells because they are likely to be more accurate indicators of the *in vivo* biocompatibility of PAGES. PEI of nearly identical molecular weight served as the control because it is a well studied polycation and has been used in a wide variety of biomedical applications. There is no standardized procedure to determine a polycation's biocompatibility *in vitro*, so we chose four assays that can evaluate toxicity from different perspectives and at progressively more severe levels. We examined the effects of PAGES on cell membrane integrity using lactate dehydrogenase (LDH) assay, metabolic activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, apoptosis using caspase-3 assay, and cell viability using calcein AM and ethidium homodimer-1 staining followed by fluorescence measurements (live/dead assay). Many existing polycations disrupt cell membranes within minutes of contact, and consequently the extracellular concentration of LDH, an intracellular enzyme, will increase (150). MTT, metabolic activity assay, provides a good indication to the level of stress cells experience (151). If the stress crosses a threshold, cell apoptosis will occur and caspase-3 levels correlate with the severity of apoptosis (152). Finally, differences in cell viability resulted from apoptosis and necrosis were evaluated by live/dead assay (153).

We examined the *in vitro* biocompatibility of PAGES by subjecting the cells to media with increasing concentrations of either PAGES (MW = 10,500 Da) or PEI (MW = 10,000 Da). The PAGES concentrations used were 1, 2, 5, and 10 mg/ml, and the PEI

concentrations used were 0.05, 0.5, and 5 mg/ml. Cell incubation time with polycation supplemented media was either 4 hours or 24 hours. The 4 hour incubation time was used to analyze earlier stresses and the 24 hour incubation was used to assess cell death. A 4 hour incubation time demonstrated that PAGES was non-toxic up to at least 10 mg/ml (**Figure 4.2A**) as determined by LDH, MTT, and caspase-3 assays. Live/dead assay indicated that even after a 24 hour exposure to a PAGES concentration of 10 mg/ml, cells were as viable as control populations subjected to normal culture medium (**Figure 4.2A**). At this concentration of PAGES, a majority of cells (**Figure 4.2B**) were observed to be alive and displayed normal morphology (**Figure 4.2C**). In contrast, PEI induced significant cell toxicity as indicated by LDH and MTT assays at a concentration as low as 0.05 mg/ml (**Figure 4.2D**). Furthermore, extensive cell death was observed by the expression of ethidium homodimer-1 at 0.05 mg/ml (**Figure 4.2E**). The morphology of cells exposed to PEI was consistent with the results of the viability assay indicating massive cell death (**Figure 4.2F**). These assays revealed that PAGES has excellent *in vitro* biocompatibility and is orders of magnitude improvement over PEI.

Many state-of-the-art polycations have been tested *in vitro* at concentrations hundreds of times lower than PAGES with the same type of assays using various cell lines. To the best of our knowledge, none displayed biocompatibility close to that of PAGES. Polycations are known to disrupt cell membranes, resulting in the leakage of LDH. Polycations such as polylysine have been shown to release significant amounts of LDH within 30 minutes of exposure (18, 28). For PAGES, no difference in LDH level was observed between the normal culture medium and PAGES media after a 4 hour exposure. Several propositions suggested that polycation toxicity increases with increasing

molecular weight and charge density [expressed as (number of charge carriers)/(molecular weight of the polymer repeating unit)] and decreasing orders of amines respectively (18, 69, 78). PAGES exhibited orders of magnitude higher biocompatibility than existing polycations regardless of which proposed criterion was used. Relative to PEI (10,000 Da), which was cytotoxic at a concentration as low as 0.05 mg/ml, PAGES (10,500 Da) exhibited no toxicity up to at least 10 mg/ml. Thus PAGES was at least 200 times more compatible than PEI of near identical MW. The charge density differs between PEI and PAGES. Each PEI repeating unit (43 g/mol) has one positive-charge carrier (amine). This leads to a charge density of 0.0233. Each PAGES repeating unit (418 g/mol) has two positive-charge carriers (guanidine and amine). This leads to a charge density of 0.00478. Thus the charge density of PEI is approximately 5 times that of the PAGES. A synthetic polycation with a similar charge density to PAGES is poly(vinyl pyridinium bromide) (PvPBr, charge density: 0.0054), which was shown to be cytotoxic to a fibroblast cell line at 0.1 mg/ml (21). Thus PAGES was at least 100 times more compatible than a polycation with approximately the same charge density. For order of amines, the third proposed criterion, PAGES contained tertiary amines and guanidiniums. We could not find a synthetic polycation that contained both. Thus we compared PAGES with polyarginine and polyamidoamine (PAMAM). These two polycations contain guanidiniums and tertiary amines respectively. Polyarginine was cytotoxic to HeLa cells at 0.03 mg/ml (64). Other arginine-rich polymers, such as an arginine-modified, proline-based biodendrimer, exhibited cytotoxicity to HeLa cells at 0.22 mg/ml (67). Poly(3-guanidinopropyl methacrylate), a guanidinium-containing polymer was toxic to COS-7 cells at 0.03 mg/ml (66). Finally, PAMAM exhibited toxicity to L929 murine fibroblasts

at 1 mg/ml (18). Again, this demonstrated that PAGES possessed unprecedented biocompatibility relative to existing polycations.

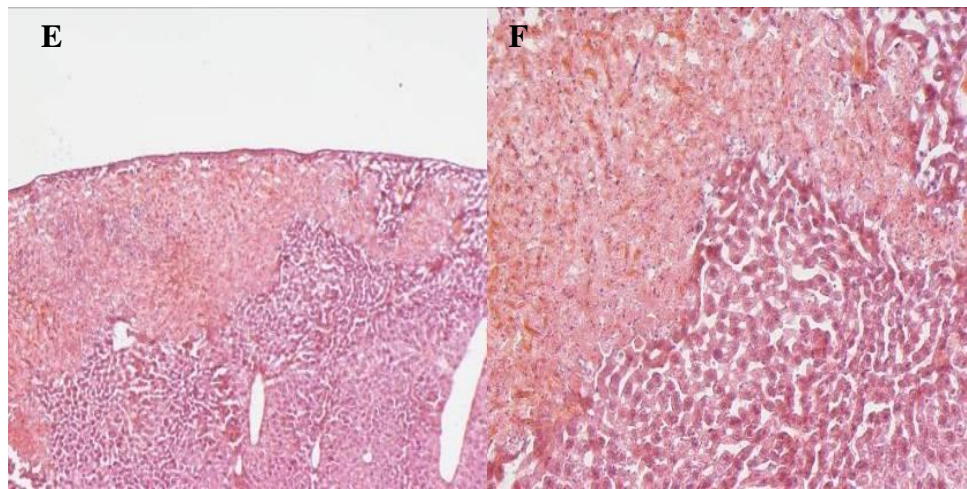
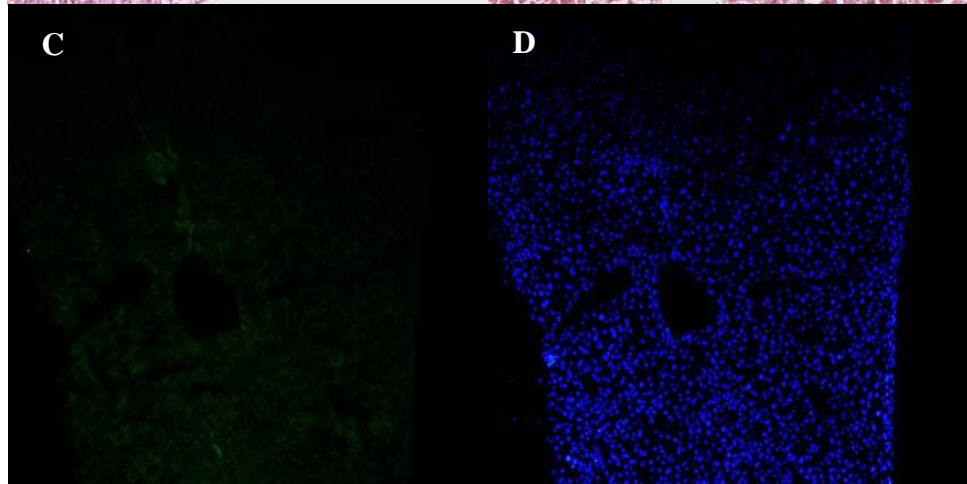
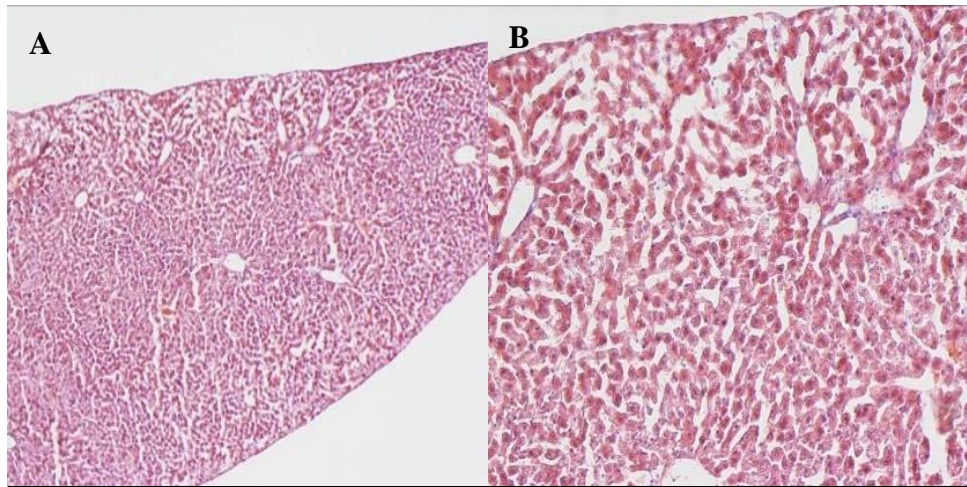
***In vivo* Biocompatibility**

In vitro biocompatibility can provide valuable indications of overall biocompatibility and help determine the mechanism of toxicity. Complex responses at the organ and whole body level can only be obtained through *in vivo* analyses. We investigated the *in vivo* biocompatibility of PAGES by intraperitoneal injection of 150 μ l of polycation solution in saline in six-week old BALB/c mice that weighed approximately 20 g. Each mouse was weighed and injected with either a PAGES solution (8 mg/20 g body weight), PEI solution (0.2 mg/20 g body weight), or saline. Organs including heart, liver, lungs, spleen, kidneys, and bladder were collected on day 1, 5 and 30 after injection. Tissues were fixed and stained by hematoxylin and eosin (H and E) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. All slides were analyzed blindly by Dr. Adeboye Osunkoya at Emory University. The organs were analyzed for tissue damage (H and E) and cell apoptosis (TUNEL).

The animals injected with PAGES showed normal tissue architecture relative to saline control throughout all time points (**Figure 4.3A, B**). Mice injected with PAGES showed no observable change in apoptosis compared to control mice (**Figure 4.3C**). Mice injected with PEI were the only animals that showed signs of toxicity. Of the organs harvested, only liver tissues harvested on day 1 suffered significant damage (**Figure 4.3E, F**). On average, these liver tissues exhibited 15% necrosis. Similarly, extensive apoptosis was observed in livers of the PEI, day 1 group only (**Figure 4.3G**).

All other PEI tissues at other time points did not exhibit apoptosis. Also, all tissues in the PAGES group at all time points showed no signs of apoptosis. The tissues from all the organs except livers in the PEI, day 1 post-injection were indistinguishable histopathologically from healthy controls in terms of inflammation and necrosis. The PEI, day 1 group liver exhibited 15% focal centrilobular necrosis on average. Hepatocytes surrounding the necrotic areas were characterized by grade 2 (out of 3 grades of severity) inflammation. The inflammation is predominantly acute in nature with infiltrate containing mostly neutrophils and expression of a small number of lymphocytes. Bile ducts were spared from the inflammatory responses. There was no significant bile stasis, bridging fibrosis or cirrhosis identified. No micro- or macro-vesicular steatosis was noted. Also, there was no associated atypical or malignant transformation of hepatocytes. Hepatocytes in the uninvolved areas were normal.

The amount of PAGES used for the *in vivo* experiments was 40 times higher than that of PEI. Even so, animals exposed to PAGES displayed no toxicity and tissues maintained normal histological architecture. The charge density of PAGES is approximately 1/5 that of PEI. Thus the total charges in the PAGES sample was approximately 8 times that of PEI. Therefore PAGES is orders magnitude more compatible than PEI either by matching MW or total charge. Because of arginine's size (MW = 174), it is impossible to create an arginine-based polycation that equates the charge density of PEI (monomer MW = 43). In summary, the *in vitro* and *in vivo* evaluations demonstrated that PAGES possesses excellent biocompatibility unfound in existing polycations.



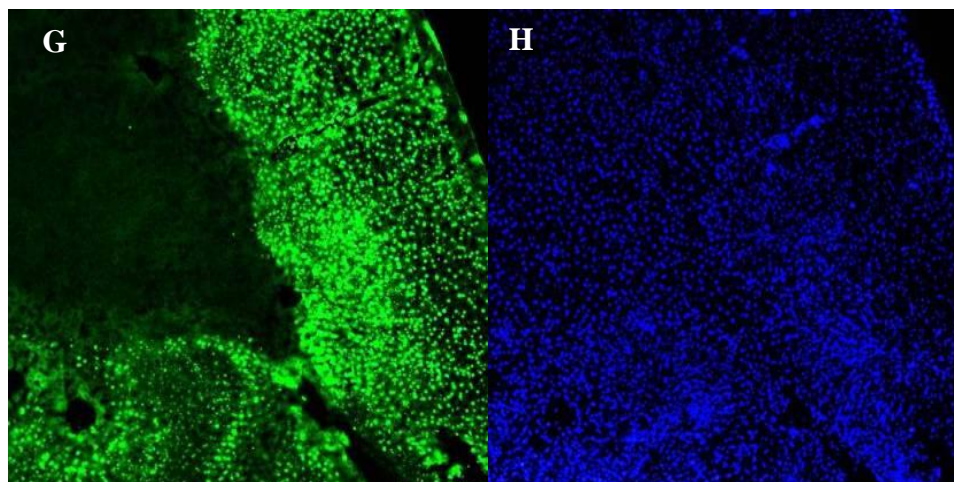


Figure 4.3. *In vivo* biocompatibility analysis of PAGS. Mice were either injected with 8 mg of PAGS or 0.2 mg of PEI intraperitoneally. Of all the organs isolated, liver was the only one that showed any significant histological change. A representative image of liver tissue in the PAGS group, H and E staining (**A**, 10x and **B**, 20x) revealed normal tissue architecture and TUNEL (green fluorescence, **C**) revealed no apoptotic response. Image **D** is the same tissue represented in **C** stained with DAPI to reveal nuclei. For PEI at 0.2 mg/animal, H and E staining (**E** and **F**) revealed that liver tissue suffered approximately 15% necrosis. TUNEL staining (**G**) exhibited extensive apoptosis in the livers of the PEI groups. Image **H** is the same tissue represented in **G** stained with DAPI. All images were from samples isolated day 1 post injection. All image acquisition parameters are identical for PAGS and PEI.

We set out to investigate the importance of biodegradability and use of endogenous building blocks on the biocompatibility of polycations. Many polycations satisfy one of the parameters. To the best of our knowledge, none meets both. Polylysine

(11.2 kDa) has been shown to be highly toxic (0.1 mg/ml) and induce apoptosis in a wide range of cells (28, 65). Polyarginine has shown toxicity to HeLa cells at a concentration of 0.03 mg/ml (64). These polycations are built from endogenous amino acids, but are orders of magnitude less biocompatible than PAGES. The ester bonds in PAGES are more susceptible to hydrolysis than the amide bonds in polyarginine and polylysine. This difference likely contributed to the significant improvement of PAGES's biocompatibility. To improve biocompatibility, many synthetic polycations have been designed to be hydrolyzable (57-62). These polycations have in general displayed higher biocompatibility than non-degradable ones. The highest reported concentration for their *in vitro* tests was 1 mg/ml. To the best of our knowledge, there has been no report on the *in vivo* biocompatibility of these existing hydrolyzable polycations. The essential difference between these hydrolyzable polycations and PAGES is the charge carrier in PAGES is derived from arginine, an endogenous molecule. This supports the hypothesis that biodegradability and the use of endogenous building blocks are necessary for a biocompatible polycation.

The important functions of cationic peptides in numerous biological events make them attractive molecules to synthetically mimic. Synthetic polycations are commonly used to complex polyanions through Coulomb interactions. One can take advantage of these interactions between oppositely charged macromolecules to precipitate negatively charged biomacromolecules such as nucleic acids and glycosaminoglycans (7). Synthetic polycations are widely used in non-viral gene delivery to increase transfection efficiency of genetic materials. The binding with a polycation protects the DNA from damaging enzymes and there is tentative evidence that the complexes might move along

microtubules to reach the nucleus thus helps to direct the path of the nucleic acid (154). PAGES interacts with plasmid DNA electrostatically to form complexes that are approximately 70 nm in diameter (**Figure 4.4A**), a size suitable to be endocytosed for gene delivery. As more PAGES was added to the DNA solution, the charge was neutralized as indicated by the titration curve of the zeta potential (**Figure 4.4B**). The nonlinearity of the charge neutralization curve likely resulted from the summation of changes in pH and the particle size.

The affinity associated with polycations can also allow negatively charged glycosaminoglycans such as heparin to form complexes with polycations. The resultant polycation/heparin complex can be used to deliver heparin-binding growth factors. The interaction between heparin and heparin-binding growth factors is expected to stabilize and potentially activate the growth factors upon binding with the corresponding growth factor receptors. Furthermore, it is possible to control the release of the growth factors by adjusting the polyvalency between the polycation and heparin. PAGES complexes heparin through Coulomb forces and precipitates it out of an aqueous solution as a fibrillar matrix (**Figure 4.4C**). The diameter of the fibrils ranged from approximately 1 μm to sub-micron in diameter and sheets range from 5-20 μm in diameter. Globular structures were also observed dispersed among the fibrils. As more PAGES was added to the heparin solution, the zeta potential increased following a nearly sigmoidal curve (**Figure 4.4D**). The nonlinearity of the charge neutralization is likely a result of the summation of pH changes and the progressive precipitation of heparin. The ability of complexation in an aqueous buffer is expected to increase the bioactivity of the growth factors compared to procedures involving organic solvents.

Another application for a biocompatible polycation is cell encapsulation. Presently, alginate hydrogels are the most frequently used biomaterial for cell immobilization. Alginate can interact with a polycation to form a hydrogel with increased bioactivity and improved mechanical properties such as faster gel kinetics and a higher compressive modulus (155). Polycations can also stabilize and control the molecular weight cut-off of the alginate microcapsule membrane (156). Additionally, polycations can mediate cell encapsulation by forming multilayers through Coulomb interactions with polyanions. Polyelectrolyte multilayers are advantageous for cell encapsulation because it allows control of the local biochemical environment. Polyelectrolyte multilayers have also been used to condense genetic material for gene delivery (157). Polycation toxicity has been a great concern in these applications as well, and a more biocompatible polycation is expected to significantly advance the clinical translation of cell encapsulation and polyelectrolyte multilayers (142, 158).

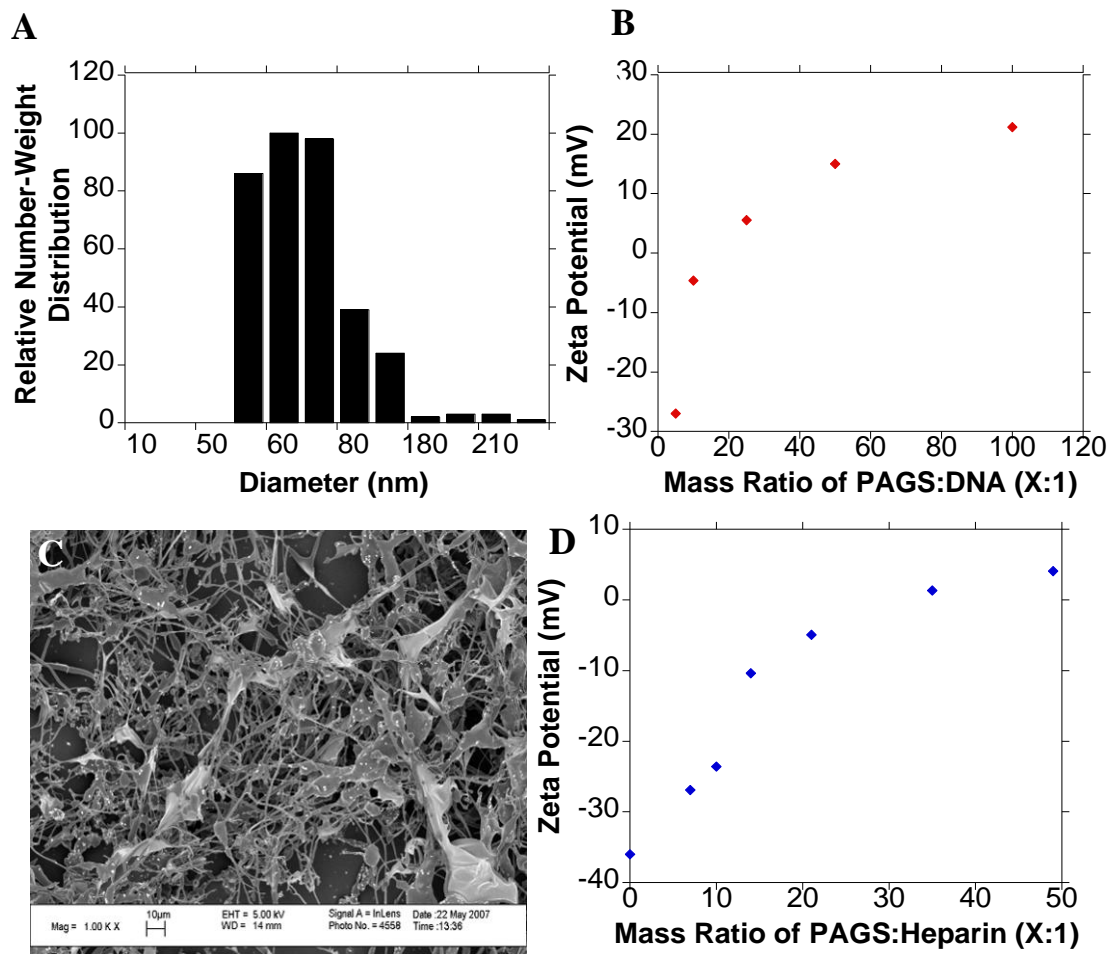


Figure 4.4. Biomedical application of PAGS. **A** and **B.** PAGS can be used to compact plasmid DNA to particles under 100 nm. PAGS:DNA complex size was measured by dynamic light scattering (68 nm +/- 15 nm, fit error: 1.28). **B** The positive charge of PAGS has shown that it can neutralize negatively charged plasmid DNA. **C** and **D.** PAGS can also precipitate heparin out of solution to form a fibrillar matrix with the potential to deliver therapeutic proteins. PAGS:heparin complexes were visualized by SEM at a magnification of 1000x. PAGS uses its positive charge to interact with heparin and this is exhibited by the heparin titration with PAGS. Titration of DNA and heparin was analyzed by zeta potential measurements.

Conclusion

Polycations are implemented in a variety of biomedical applications. They have been utilized in areas from drug delivery to tissue engineering. Even though they have experienced some success in these fields, synthetic polycations still face the issue of biocompatibility. Given this predicament, research has been focused on finding a completely biocompatible polycation. With the synthesis reported here, we have shown a biocompatible polycation is achievable by mimicking nature's own version of a cationic polymer. This biomimetic strategy resulted in PAGES exhibiting exceptional *in vitro* and *in vivo* biocompatibility, specifically when compared to other synthetic polycations. The design philosophy employed by PAGES will hopefully serve as template for a new generation of biocompatible polycations.

Materials and Methods

Chemicals and General Methods

Succinic acid (TCI, Tokyo, Japan), arginine ethyl ester dihydrochloride (Research Organics, Cleveland, OH), 1,3 meta chloro peroxy benzoic acid (Acros Organics, Morris Plains, NJ), and all other chemicals (Alfa Aesar, Medford, MA) were used without purification, except for 1,3 meta chloro peroxy benzoic acid. This compound was lyophilized overnight to remove water. Flash chromatography was performed on a Buchi Fraction Collector C-660 equipped with a UV photometer C-635 (Flawil, Switzerland). Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Varian Mercury-400BB NMR. Fourier-transform infrared (FTIR) spectra were recorded on a Thermo Nicolet IR-100 spectrometer (Madison, WI). Gel permeation chromatography

was performed on a Viscotek GPCmax VE2001 GPC Solvent/Sample Module with 270 Dual Detector (RALS and RI) using a Viscotek Viscogel I-MBMMW column (Houston, TX). The molecular weight and polydispersity of the polymer are reported relative to polystyrene standards. Lyophilization was performed on a Labconco FreeZone 2.5 (Kansas City, MO). *In vitro* biocompatibility assays include: Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR), EnzCheck Caspase-3 Assay Kit (Molecular Probes, Eugene, OR), and Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). TUNEL staining was done using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI). Fluorescence and absorbance measurements were performed on a Nikon Eclipse TE2000-U (Melville, NY) equipped with X-cite 120 Fluorescence Illumination System and a 4 MP Diagnostics Spot Flex digital camera (Sterling Heights, MI).

PAGS Synthesis

The arginine-based polymer (PAGS) was synthesized via polycondensation reaction of a 1:1 molar ratio of diglycidyl succinate and arginine ethyl ester in anhydrous N,N-dimethylformamide under N₂. The reaction mixture was stirred and kept at 60°C for 7 days. The resultant polymer was placed under vacuum and heated to 60°C for 24 hours to remove solvent. The residual dimethylformamide was removed by dissolving the polymer in methanol, precipitating polymer out with ethyl acetate, and dried under vacuum at 60°C overnight. Ethyl acetate wash is used to remove unreacted product and oligomers.

The polymer was characterized by FTNMR, FTIR, differential scanning calorimetry, and gel permeation chromatography.

***In Vitro* Biocompatibility**

Cell Membrane Interaction

Primary baboon smooth muscle cells (SMCs, passage 12-14) were cultured in 96-well tissue culture treated polystyrene plates at a seeding density of approximately 8,000 cells per well in 200 μ l of MCDB 131 growth medium with 10% FBS and 1% glutamine. Cells were grown overnight up to approximately 70% confluency and the growth medium was replaced with appropriate amounts of 0.2 μ m filtered PAGS dissolved in 200 μ l growth media. Cells were incubated at 37°C, 5% CO₂ for 4 hours, with polycation containing media. Samples were then analyzed for LDH present in media. The manufacturer's procedure was used for CytoTox96 Non-radioactive Cytotoxicity Assay (Promega, Madison, WI). All data was standardized to the control, which was baboon SMCs that were not exposed to polycation media.

Metabolic Activity

Baboon SMCs were prepared and exposed to PAGS media in the same manner as in measuring metabolic activity. Cells were incubated at 37°C, 5% CO₂ for 4 hours, with polycation containing media before the culture medium was replaced with 400 μ l growth medium and 100 μ l MTT solution (5 mg/ml), and incubated at 37°C for 4 hours. MTT media was removed, and replaced with 500 μ l of lysis buffer (10% w/v sodium dodecyl sulfate in 0.01M HCl) following a D-PBS wash. Cell digest was incubated at room temperature for 90 minutes and absorbance of each sample was measured at 560 nm. All

data was standardized to the control, which was baboon SMCs that were not exposed to polycation media.

Apoptotic Activity

Baboon SMCs were prepared and exposed to PAGES media in the same manner as in measuring metabolic activity. After the 4 hour incubation with PAGES media, caspase-3 levels were examined by fluorescence measurements at 496 nm. All data was standardized to the control, which was baboon SMCs that were not exposed to PAGES media.

Cell Viability

Baboon SMCs were prepared and exposed to PAGES media in the same manner as in measuring metabolic activity. After the 4 hour incubation with PAGES media, cell viability was examined by fluorescence measurements at 494 nm. All data was standardized to the control, which was baboon SMCs that were not exposed to PAGES media. Cell morphology and fluorescence images were then visualized for cell morphology and fluorescence.

In Vivo Biocompatibility

Male Balb/C (Harlan, Indianapolis, IN) mice weighing 19-21 g were injected intraperitoneally with PAGES/saline solution, PEI/saline solution, or saline. Twenty four animals received intraperitoneal injections of either PAGES (n=10), PEI (n=9), or control animals (n=3). Animals were cared for in compliance with protocols approved by the Committee on Animal Care of the Georgia Institute of Technology following NIH guidelines for the care and use of laboratory animals (NIH publication No. 85-23 rev.

1985). Organs were harvested at 1, 5, and 30 days post-injection. The organs harvested include heart, liver, lung, kidney, spleen, and bladder with prostate gland. These organs were rinsed with PBS and fixed in 10% neutral buffered formalin overnight then submersed in 30% sucrose overnight at 4°C prior to histological analysis. All samples were embedded in optimal cutting temperature compound (O.C.T, Tissue-Tex, Sakura Finetek U.S.A. Torrance, CA) for cryosection. Cross-sections (10 µm thick, the longitudinal axis cut) were stained with hematoxylin and eosin (H and E) staining method. Sections were stained using a standard protocol for haematoxylin and eosin (H and E) and analyzed for degree of inflammation and fibrosis. Sections were also stained for apoptosis using manufacturer's procedure for TUNEL staining. TUNEL stained sections were then visualized and assessed for apoptosis.

Statistical Analysis

For each variable group tested there were four replicates for the experimental and control samples. Multicomparisons ANOVA, Tukey Method, was used to statistically compare the different experimental values; $p < 0.05$ was considered statistically significant. The results are reported as mean values with standard deviations.

CHAPTER 5
A [Polycation:Heparin] Complex for the Controlled Release
of Growth Factors

Abstract

Therapeutic neovascularization facilitated by growth factors could serve as a new alternative to the treatment of cardiovascular disease. Neovascularization is a complicated biological response regulated by a series of different growth factors. In order for a growth factor delivery strategy to be successful it should meet certain criteria: bind relevant growth factor or cocktail of growth factors, have controllable release kinetics, and maintain the bioactivity of the therapeutic growth factors. We hypothesize that a delivery strategy consisting of a biocompatible polycation and heparin would meet these criteria. Heparin provides the ability to bind a multitude of growth factors and has been shown to maintain their bioactivity for an extended period of time. Our biocompatible polycation has exhibited the ability to complex heparin and form water-insoluble complexes. These resultant complexes have demonstrated controllable release kinetics of FGF-2 by altering the molecular weight of the polycation. The FGF-2 released from the delivery complex maintained its bioactivity and provided comparable cellular responses to bolus FGF-2 and heparin stabilized FGF-2. The delivery system described here represents an attractive new therapeutic delivery vehicle for the treatment of cardiovascular diseases because it has the potential to deliver heparin-binding growth factors in a bioactive form with controllable release rates.

Introduction

Cardiovascular disease is the leading cause for death of men and women in the United States, accounting for nearly 2,400 deaths daily (159). Worldwide, cardiovascular disease is the cause of more than seven million deaths annually (1). Cardiovascular disease encompasses many forms, ischemic heart disease being the deadliest. Ischemic heart disease can potentially lead to myocardial infarction and ultimately a damaged myocardium (98, 99). Given that the mammalian heart has little capacity to regenerate this damaged tissue, there has been great effort to develop treatments to alleviate this problem (2). Current therapies (invasive and noninvasive strategies) have made improvements in extending the life of these patients but do little to regenerate the damaged tissue (3). Patients who have undergone these therapies have a greater chance of experiencing additional myocardial episodes in the future, compared to people who have never experienced a heart attack (159). Furthermore, there is a subset of patients whose conditions do not improve despite these therapies. This same subset of patients is expected to increase with an aging population and the rise of diseases such as obesity and diabetes mellitus (4). It is clear that current therapies for ischemic heart disease do not help every patient and the patients that do benefit are more likely to have reoccurring symptoms in the future. To improve the effectiveness of myocardial therapies it is important to heal the heart on a tissue level. A potential treatment is therapeutic neovascularization (113).

Therapeutic neovascularization is the process of altering native vascular architecture under the direction of exogenous mediators. One approach to therapeutic neovascularization is the delivery of therapeutic proteins, and this has been pursued to the

level of clinical trials (108). These pursuits were determined inconclusive and led to reevaluating strategies to deliver therapeutic proteins. For therapeutic neovascularization to be successful, a delivery strategy should attempt to mimic the *in vivo* neovascularization response. In order to mimic the *in vivo* neovascularization response, certain criteria should be met. These criteria include the ability to bind appropriate growth factors, control the release of these growth factors, and maintain their bioactivity.

There have been numerous attempts to delivery growth factors in a safe, efficient manner. These delivery strategies can be classified as consisting of natural (chitosan, alginate, collagen, etc.) or synthetic (PLG, PLA, PEG, etc.) polymers, and the combination of the two (self-assembling amphiphile, PEG-heparin, etc.) (7, 118, 119). These strategies have had some success but have also faced challenges. A major challenge of some of these delivery strategies is the use of organic solvents. The use of harsh organic solvents can result in denatured proteins, ultimately limiting bioactivity. Another challenge in some of these strategies is the delivery vehicles have little to no affinity for the growth factors. This can lead to decreased loading capacity and efficiency. A delivery complex that bypasses these issues could be an attractive candidate for therapeutic neovascularization.

We hypothesized a system governed by polyvalent interactions between the delivery matrix, heparin, and growth factors will enable a controlled release and preserve the bioactivity of the growth factors. We chose to test this hypothesis by combining a polycation (poly(arginate glycerol succinate), PAGES), a glycosaminoglycan (heparin), and FGF-2 to form a delivery matrix. All three components are water soluble and their interactions result in a precipitate insoluble in water. Having water-soluble compounds

avoids the use of organic solvents. The resultant complex will be capable of binding multiple heparin-binding growth factors through non-covalent interactions. The use of heparin in its native form is also expected to provide a more potent growth factor response. For example, FGF-2 has been shown to have a more potent effect when it binds its receptor as a ternary complex with heparin (141, 160).

In this study we investigated whether the [polycation:heparin] complex (**Figure 5.1**) could bind FGF-2 and subsequently control its release in a bioactive form. We found that the loading efficiency and release kinetics can be controlled by the molecular weight of PAGES. Also, this system is controlled by the polyvalent interactions between the polycation, heparin, and growth factor and all of these components are soluble in water. These characteristics combined were anticipated to preserve growth factor bioactivity. This preservation of bioactivity was exhibited by two different methods: an endothelial cell proliferation assay and an endothelial tube formation assay. The results indicated that the [PAGES:heparin] complex holds great potential for therapeutic neovascularization.

Results and Discussion

[PAGES:heparin] Complex Characterization

The charge of [PAGES:heparin] complex was examined by zeta potential titration by adding a PAGES solution into a heparin solution (**Figure 5.2A**). A solution of heparin alone had a charge of approximately -30 mV. An increasing amount of PAGES was added to the heparin solution, and the resultant zeta potential increased following a nearly sigmoidal curve. A ratio of [35:1] of PAGES to heparin resulted in a complex closest to

neutral. This ratio was then used for subsequent experiments because it was hypothesized this ratio would result in the greatest amount of precipitate.

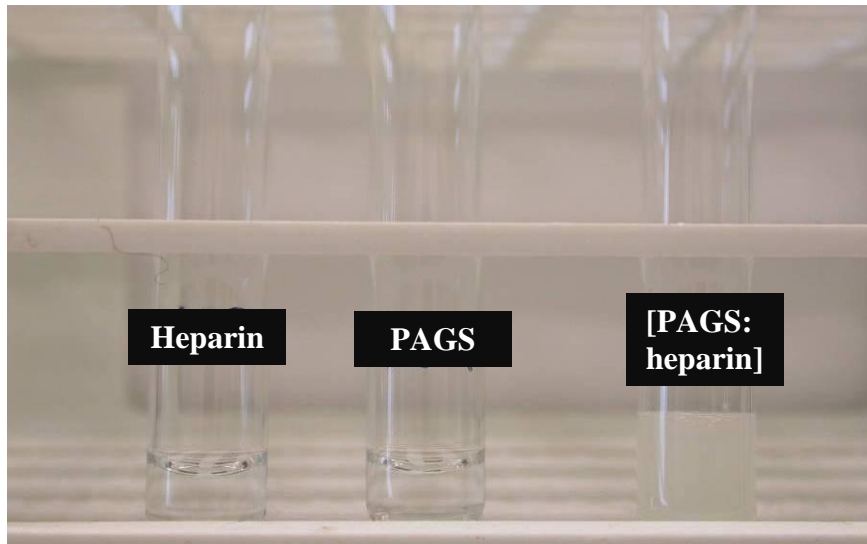


Figure 5.1. The [PAGS:heparin] complex. The interactions between PAGS and heparin resulted in a white precipitate when combined in an aqueous solution.

Both PAGS and heparin are soluble in water. When the two are combined in solution, a white precipitate forms. To further characterize complex formation, scanning electron microscopy was used. Scanning electron microscopy revealed [PAGS:heparin] complexes as a matrix composed of fibers, sheets, and beads (**Figure 5.2B**). Fiber diameters were approximately 1 μm to sub-micron and the sheets ranged from 5-20 μm in size. Examination of the matrix at 25000x magnification (**Figure 5.2C**) revealed the fibers were more like thin ribbons and the apparent beads were in fact rings.

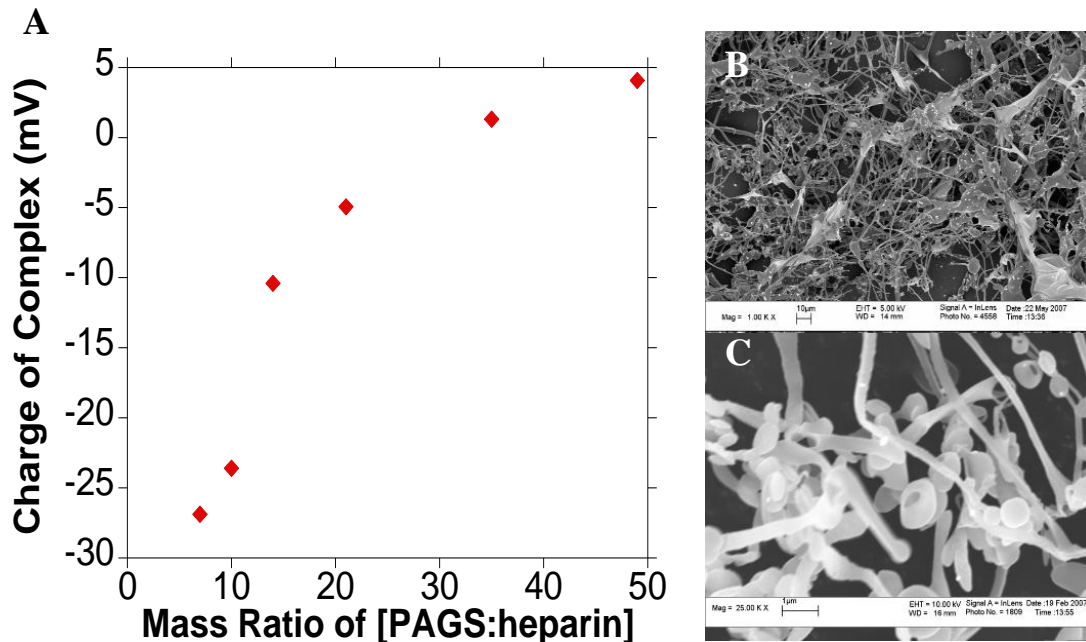


Figure 5.2. [PAGS:heparin] complex characterization. (A) The charge of the [PAGS:heparin] complex was analyzed by zeta potential measurements. The charge of the suspension increased as PAGS was incrementally added and exhibited a sigmoidal shape. A mass ratio of [35:1] resulted in a complex closest to an overall neutral charge. Scanning electron microscopy was used to characterize the morphology of the [PAGS:heparin] complexes and revealed them as a matrix composed of fibers and rounded sheets (**B, 1000x**). Fiber diameters measured approximately 1 μm to sub-micron, and the sheets range from 5-20 μm in size. (**C, 25000x**) Examination of the matrix at higher magnification revealed the fibers were more like thin ribbons and the apparent beads were in fact rings.

FGF-2 Incorporation

The loading capacity and efficiency of [PAGS:heparin] complexes was characterized through the use of radiolabeled FGF-2. When preparing complexes, 0.1% to 1% of the growth factor was ^{125}I -labeled FGF-2. Loading efficiency was investigated using two different molecular weights of PAGS and at different [PAGS:heparin] ratios. The high molecular weight (HMW, M_n : 73,947 Da, M_w : 238,802 Da) [35:1] ratio corresponded to the highest loading efficiency at 66%, corresponding to 132 ng out of the attempted 200 ng of FGF-2 (**Figure 5.3A**). It should be noted that FGF-2 incorporation was calculated by measuring the amount of radioactivity present within the samples. Ratios of [21:1] and [7:1] resulted in loading efficiencies of approximately 59% (118 ng) for the HMW species. Mass ratios lower than [7:1] did not form visible precipitates. A higher mass ratio of [49:1] was also attempted but did not improve upon the loading efficiency of the [35:1] ratio (data not shown). Low molecular weight (LMW, M_n : 63,944 Da, M_w : 182,023 Da) [35:1] ratio corresponded to a loading efficiency of 50% (100 ng) of the attempted 200 ng. LMW ratios of [21:1] and [7:1] resulted in efficiencies of 43% (86 ng) and 40% (80 ng) respectively. The higher molecular weight species of PAGS corresponded to a higher loading efficiency across all ratios relative to lower molecular weight species. HMW PAGS loaded approximately 15% more FGF-2 across the aforementioned ratios. The loading capacity of [PAGS:heparin] complexes were also examined. This was done using a [35:1] ratio of LMW PAGS and increasing amounts of attempted loaded FGF-2 (**Figure 5.3B**). The attempted amount of FGF-2 loaded was increased from 20 to 2000 ng. In all cases, FGF-2 was loaded with the same efficiency of 50%. This efficiency corresponds to loading range of 10 ng to 1000 ng. From these

experiments, it was demonstrated the [PAGS:heparin] complexes can incorporate at least 1000 ng FGF-2.

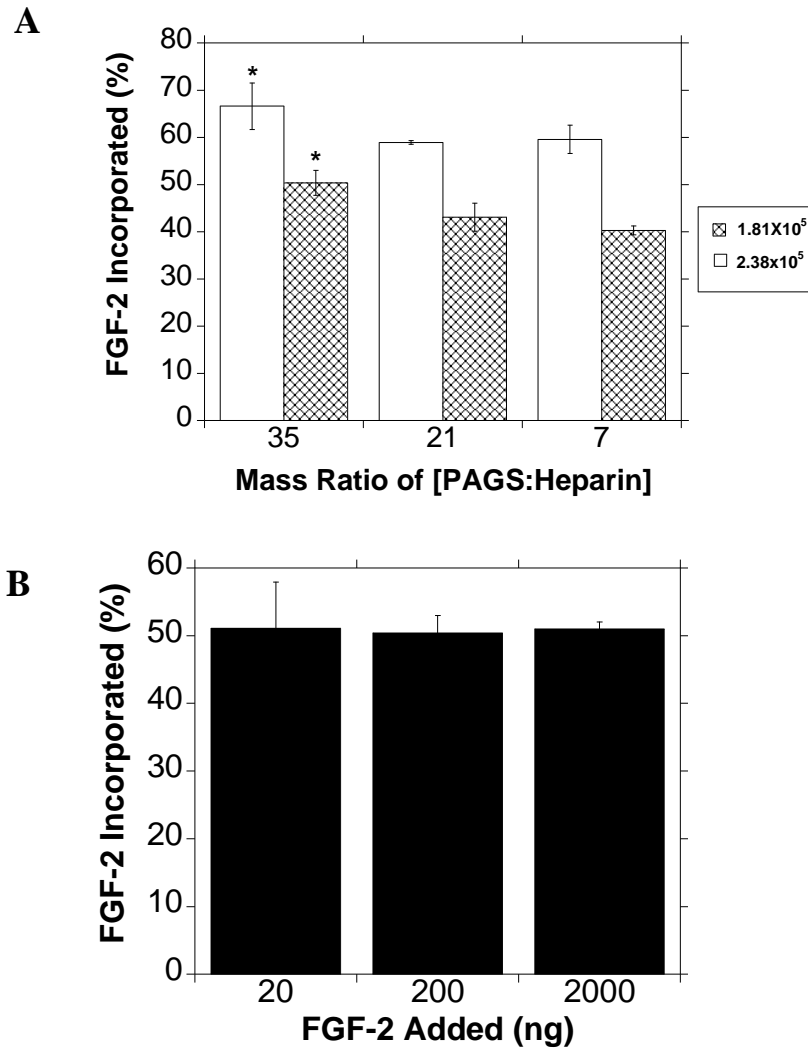


Figure 5.3. [PAGS:heparin:FGF-2] complex loading analysis. Complex loading efficiency and capacity was investigated using ¹²⁵I-FGF-2. Loading efficiencies (A) of different molecular weight PAGS was investigated for different [PAGS:heparin] ratios. The higher molecular weight PAGS was more efficient at incorporating FGF-2 at all [PAGS:heparin] ratios than the lower molecular weight species. A ratio of [35:1] was the most efficient at incorporating FGF-2 for both molecular weight species. The loading

capacity (**B**) of complexes was investigated for a [35:1] ratio of low molecular weight PAPS and demonstrated a loading efficiency of 50%. From this experiment, it was exhibited that FGF-2 could be incorporated up to at least 1000 ng. Statistical significance between control and other experimental groups was noted as “*”, $p < 0.05$.

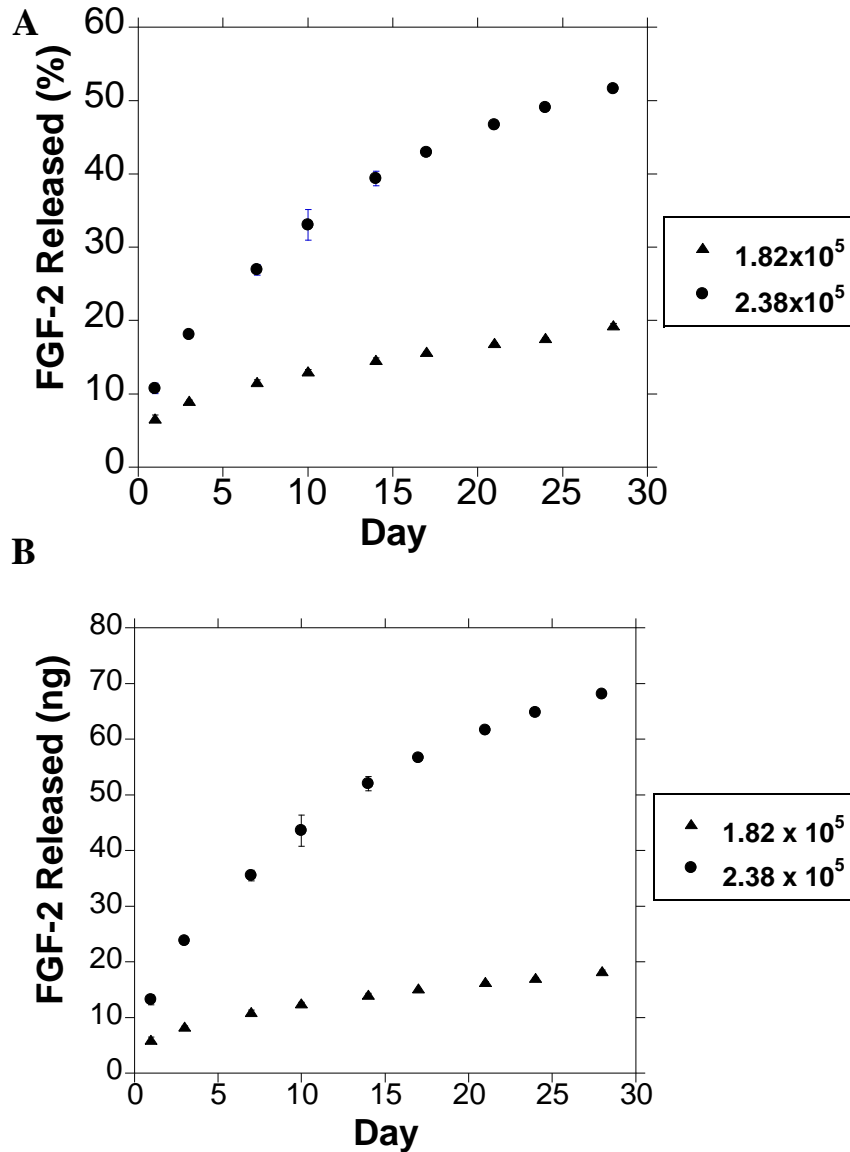


Figure 5.4. [PAGS:heparin:FGF-2] complex release kinetics. Release kinetics were examined by measuring the amount of ^{125}I -FGF-2 released from complexes. The percent of FGF-2 released from complexes (**A**) was monitored over a period of 28 days. Two different molecular weight species of PAGS were used to characterize whether release kinetics were controllable. The LMW species of PAGS released nearly 20% of its loaded growth factor, while the HMW species of PAGS released approximately 50% of incorporated growth factor over the same period of time. The amount of growth factor (ng) is also shown (**B**) to further illustrate the ability to control the amount of FGF-2 released by altering the molecular weight of PAGS. HMW PAGS released 69 ng of FGF-2 over 28 days, compared to 17 ng of FGF-2 released by the LMW PAGS.

FGF-2 Release Kinetics

The FGF-2 release profile from the [PAGS:heparin] complexes was examined using ^{125}I -labeled growth factor (1%). The subsequent release of radiolabeled FGF-2 from complexes was monitored for a period of 28 days. Two different molecular weight species of PAGS were used for these release experiments. These different molecular weights were used to investigate if the release kinetics could be controlled through molecular weight. The release kinetics (**Figure 5.4A**) of both PAGS species starts with a small initial burst and then has a sustained release over the remainder 28 days. The initial burst (7% of loaded FGF-2) was approximately the same for both molecular weight species. The release profile of the HMW PAGS follows that of a power law and 52% of the loaded FGF-2 was released over these 28 days. If this release profile continues along the pattern exhibited in the first 28 days then it should release growth factor up to 110

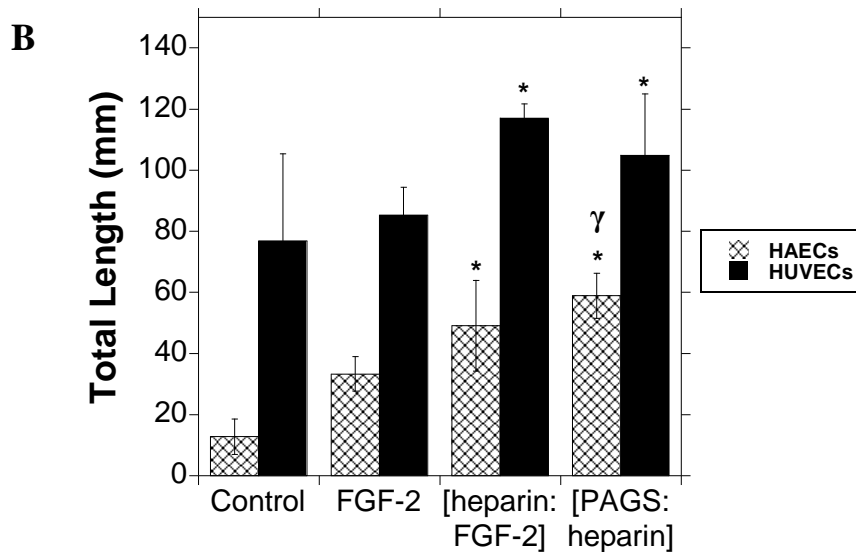
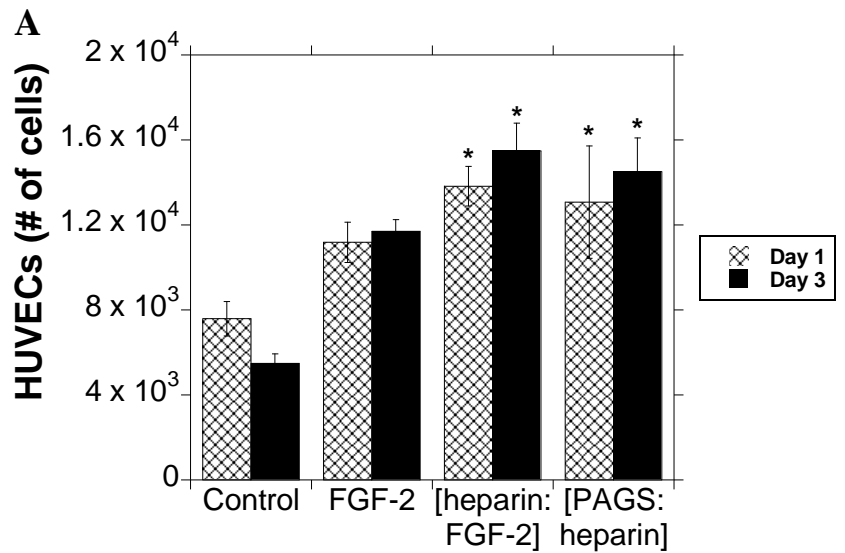
days. The lower molecular weight species resulted in releasing 19% of loaded FGF-2 over this same time period.

The release profile was further characterized to express the specific amount of growth factor released from the different complexes. Once again it should be noted the amount of FGF-2 released was calculated by measuring the amount of radioactivity present within the supernatant. The specific amount of FGF-2 released from both molecular weight species exhibited similar profiles relative to the percentage of loaded FGF-2 released (**Figure 5.4B**). In comparing the different PAGES molecular weights, the HMW species released 18 ng of FGF-2 within 3 days and the LMW species released the same amount of FGF-2 over a period of 28 days. This observation further demonstrated that modifying PAGES's molecular weight results in controllable release kinetics.

Bioactivity of Released FGF-2

The bioactivity of released FGF-2 was investigated by two different assays. The initial examination was the number of endothelial cells present after being exposed to different media conditions. Basic fibroblast growth factor's ability to stimulate proliferation of endothelial cells has been well documented. In this assay, if FGF-2 released from [PAGES:heparin] complexes maintains its bioactivity, then HUVECs exposed to this media will proliferate at a rate similar to that of bolus FGF-2. HUVECs were exposed to different media conditions and cell number was assessed using a Coulter Counter after 48 hours of incubation with this media (**Figure 5.5A**). Media conditions included: [PAGES:heparin] release media, media without FGF-2 (control), FGF-2 supplemented media, and [heparin:FGF-2] supplemented media. HUVECs incubated

with [PAGS:heparin] day 1 release media and [FGF-2:heparin] media resulted in approximately a 1.7 fold increase over control groups at the end of the 48 hour incubation. The FGF-2 released from the [PAGS:heparin] complexes proved to have a proliferative effect on the same level as FGF-2 added directly to culture media. When HUVECs were incubated with [PAGS:heparin] day 3 release media, it resulted in approximately a 3 fold increase over control groups. As seen with the day 1 release media, day 3 release media also a similar proliferative effect as bolus FGF-2.



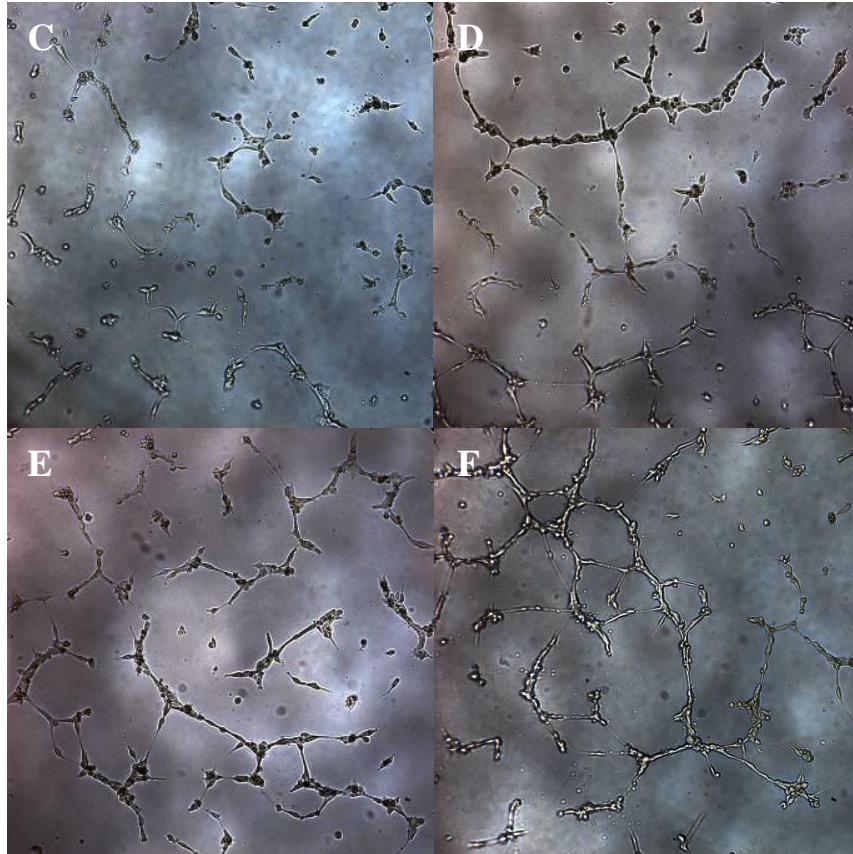


Figure 5.5. Bioactivity of released FGF-2. The bioactivity of released FGF-2 was examined by potency and functionality assays. (A) HUVECs exposed to different types of conditioned medium were analyzed by cell number to evaluate the potency of the FGF-2 released from complexes. [PAGS:heparin:FGF-2] release media from days 1 and 3 stimulated HUVEC proliferation that approached [heparin:FGF-2] supplemented media. (B) The bioactivity of released FGF-2 was also assessed by examining its ability to stimulate endothelial tube formation. The FGF-2 released from [PAGS:heparin:FGF-2] complexes stimulated approximately the same amount of tube formation as [heparin:FGF-2] supplemented media and bolus FGF-2 media. These trends were seen in

both HUVECs and HAECs. Visualization of HUVECs exposed to the different media conditions: **C** – control, **D** – bolus FGF-2, **E** – [heparin:FGF-2], and **F** – [PAGS:heparin:FGF-2] release media. Statistical significance between control and other experimental groups was noted as “*”, $p < 0.05$. γ denotes the statistical significance between an experimental groups and the bolus FGF-2 group, $p < 0.05$.

Bioactivity of released FGF-2 was further analyzed using a functional assay of endothelial cells. It has been shown that endothelial cells grown on Matrigel[®] form tube-like structures. An angiogenic growth factor such as FGF-2 should stimulate the formation of tubes if presented in a bioactive form. Both HUVECs and HAECs were exposed to the same media conditions as the proliferation assay but cultured on matrigel (**Figure 5.5B**). These endothelial cells were then investigated for the formation of tubes and the total tube length was measured using Image Pro. HAECs incubated with [PAGS:heparin:FGF-2] release media exhibited approximately a 4.5 fold increase in measured tube length compared to the control group. HUVECs exposed to this same release media also displayed an increase (1.2 fold) relative to control group but not as extensive. Also, as seen in the proliferative assay, the trend that FGF-2 released from [PAGS:heparin:FGF-2] complexes stimulated a response similar to heparin stabilized FGF-2 media was demonstrated (**Figure 5.5C, D**). It should be noted that [PAGS:heparin:FGF-2] media in HAEC's stimulated more tube formation than bolus FGF-2 supplemented media.

Preliminary *In Vivo* Biocompatibility of [PAGS:heparin] Complexes

The [PAGS:heparin] delivery system will eventually be evaluated for *in vivo* applications. As a preliminary *in vivo* biocompatibility examination, Sprague–Dawley rats were exposed to the [PAGS:heparin] networks. The animals were injected subcutaneously in their hindlimbs with [PAGS:heparin] solutions. These solutions consisted of [PAGS:heparin] ratios of [35:1]. Rats were sacrificed and examined for acute tissue toxicity on days 3, 7, and 12 via H and E staining. Day 3 (**Figure 5.6**) was the only time point that showed any signs of inflammation. This time point demonstrated signs of inflammation. In a normal injection, there would be no signs of this at day 3 but it is hypothesized because heparin was part of the injected formulation, clotting and wound healing was hindered. On days 7 and 12, no signs of inflammation were seen. Further investigation is needed to support the hypothesis for inflammation at day 3. If this hypothesis holds true, then this preliminary study gives optimism that PAGS is biocompatible for *in vivo* applications.

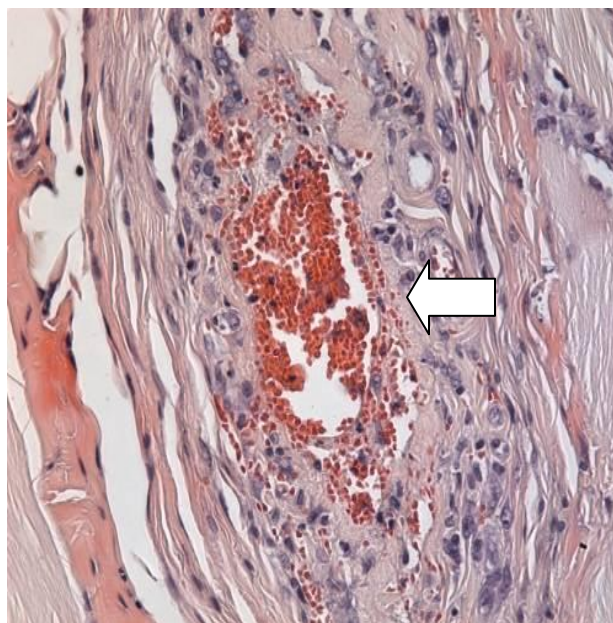


Figure 5.6. Preliminary *in vivo* biocompatibility of [PAGS:heparin] complexes. Rats were injected subcutaneously in hind-limbs with [PAGS:heparin] suspension. Day 3 shows inflammation (arrow) that is most likely due to the injection and the presence of heparin. Magnification of presented image was taken at 40x.

There have been many attempts to deliver growth factors in a safe, efficient manner. The delivery strategy reported here is the first to deliver growth factors by a system composed of only a polycation and a polysaccharide. In trying to mimic the *in vivo* neovascularization response, the goal of this strategy was to have the aptitude to bind a multitude of growth factors, maintain their bioactivity, and deliver them under controllable release kinetics. The resultant [polycation:heparin] complex demonstrated that it can deliver bioactive FGF-2 for at least 28 days, and that the release rate of FGF-2 can be controlled through the molecular weight of the polycation.

The use of a synthetic polycation as a component of this delivery strategy was a difficult proposition. Without a polycation in this strategy, heparin will not precipitate out of solution. Conversely, synthetic polycations are well-known for being cytotoxic. The use of PAGS meets the requirements of being both a polycation and biocompatible. This claim of PAGS's biocompatibility is explained in detail in prior chapter. In this system, the polycation served as an anchoring mechanism for heparin and ultimately FGF-2. This then allowed for the controllable release kinetics of FGF-2 through PAGS's molecular weight. By using a lower molecular weight species of PAGS, the release rate of FGF-2 was dramatically slower and the amount of growth factor released was considerably less when compared to the higher molecular weight species of PAGS. It is

hypothesized that the difference between release kinetics is due to a change in binding affinities between the [PAGS:heparin:FGF-2] ternary complex (**Figure 5.7**). The higher molecular weight PAGS has a higher binding affinity (k_{on2}) for heparin. This leads to an increase in formation of C_3 , thus there is more heparin and FGF-2 present in the ternary complex. It is assumed that FGF-2 and heparin interact with PAGS as a complex (C_1) because of its prior incubation together. To explain why k_{on4} does not have a larger role in the loading efficiency is because the ternary complex has not reached equilibrium at the time the loaded FGF-2 was measured, thus it is more kinetically regulated. This explains the increased loading efficiency of the high molecular weight PAGS. This higher molecular weight PAGS also leads to a higher dissociation constant (k_{off3}) with FGF-2 and the [PAGS:heparin:FGF-2] complex (C_3). The higher molecular weight PAGS competes with FGF-2 for binding sites, thus making it more difficult for FGF-2 to stably interact with heparin. This results in the equilibrium shifting to [PAGS:heparin] (C_2) quicker than in the lower molecular weight species. This ultimately results in more FGF-2 released from the high molecular weight PAGS complexes.

Moreover, the release mechanism of the [PAGS:heparin] complex has yet to be completely elucidated. It is hypothesized that FGF-2 release kinetics are a function of

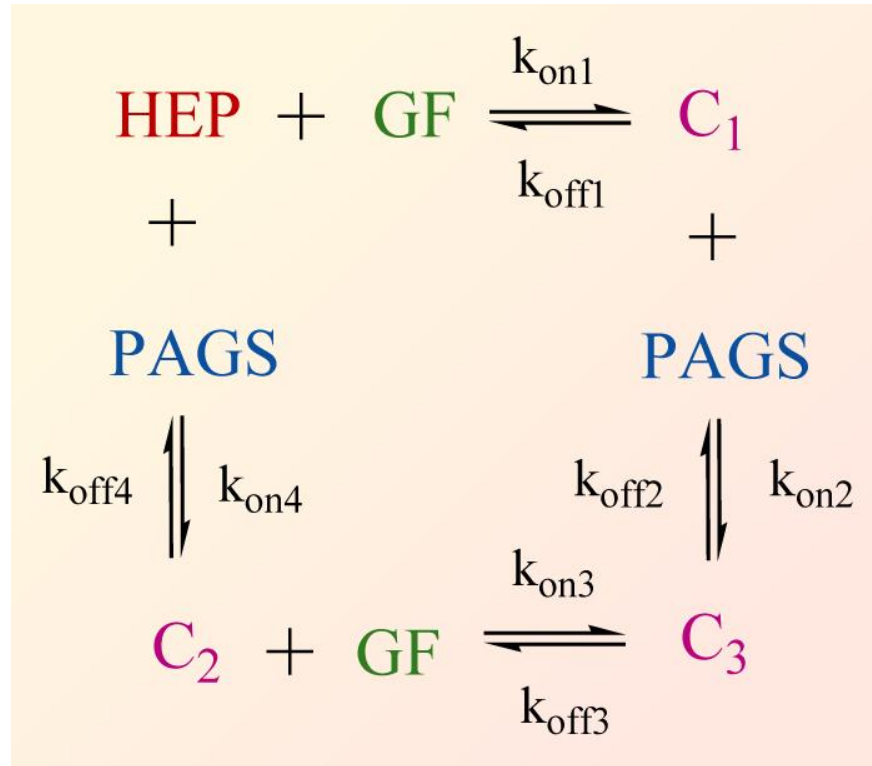


Figure 5.7. [PAGS:heparin:FGF-2] binding model. This model is used to explain the release kinetics and loading efficiency between the 3 different components of the ternary complex. Heparin is denoted as HEP and FGF-2 is denoted as GF.

dissolution of [PAGS:heparin:FGF-2], [PAGS:heparin], and [heparin:FGF-2], the degradation of PAGS and subsequent release of [heparin:FGF-2], and the diffusion of FGF-2 from the complex. Preliminary experiments investigating the degradation profile of PAGS exhibit the PAGS's half-life in aqueous solution at 37°C is approximately 70 days. This observation makes a claim that the release mechanism could be influenced by polymer degradation. This degradation rate would likely be slower when PAGS is complexed with heparin because the complex is no longer in solution, but degradation might still have a small role in the release of FGF-2. This then points to the release of

FGF-2 as a function of dissolution and diffusion mechanisms. Further investigation of the affinities of PAGES for heparin as well as a more in depth PAGES degradation profile will help characterize the release mechanism. It should be noted that the degradability of PAGES may have a more intricate role for *in vivo* applications in determining release kinetics.

The [PAGES:heparin] delivery system takes advantage of heparin's ability to interact with FGF-2. Heparin is known to have a high affinity to FGF-2 (K_d : 8.6×10^{-9} M) (161). Heparin's high affinity for FGF-2 allows the [PAGES:heparin] system to sequester FGF-2 and have a high loading capacity and efficiency of this growth factor. This is an improvement compared to most synthetic growth factor delivery systems that have little or no affinity to the target growth factor. Heparin is also known to prolong the biological half-life of FGF-2 as well as increase the stimulatory effects of the FGF-2 signaling pathway. These effects are demonstrated in the bioactivity experiments reported here as well in other reported bioactivity assays (138). Moreover, the formation of the [PAGES:heparin] complex is carried out in aqueous solution and does not use any harsh organic solvents. Some delivery systems lose bioactivity of growth factor because they have to use these organic solvents.

This maintained bioactivity of FGF-2 was confirmed in two different examinations and cell types. The first assay and cell type was a proliferative examination using HUVECs. HUVEC cells numbers were slightly lower in the day 1 release media compared to the day 3 release media. The opposite would be the intuitive because the concentration of FGF-2 is higher than the day 3 release media. A possible explanation of these results is the passage number of the HUVECs used for the day 1 release media

experiment was higher than the HUVECs in the day 3 release media experiments. The stimulatory effects of FGF-2 could elicit a stronger response in lower passage HUVECs.

The second assay was a functional examination of both HUVECS and HAECs cultured on Matrigel[®]. The amount of tube formation from the [heparin:FGF-2] media was slightly more than the release media in the HUVEC samples. In the HAEC samples, the opposite was true with the release media exhibiting higher results than the [heparin:FGF-2] media. This same trend between these experimental groups was also displayed in the potency assay using HUVECs. It should be noted, the results between these two experimental groups were not statistically significant. A possible reason for this result is that HUVECs are more sensitive than HAECs to the concentration of heparin present in the media. In the samples incubated with [heparin:FGF-2], the amount of heparin is known. In the samples incubated with [PAGS:heparin:FGF-2] release media, the amount of heparin is unknown. For example, if the concentration of heparin in the release media is higher than the [heparin:FGF-2] media, then HUVECs could be over stimulated and experience receptor down regulation. This in turn could result in a lower stimulatory effect of FGF-2. The amount of heparin present in the release media is an experiment that should be investigated in the future. The donor age of the cells also could also have a role in this observation. A younger donor would most likely have a more robust result relative to an older donor. The donor age of these cell were unknown at the time of their use.

The [PAGS:heparin] delivery strategy has demonstrated it can deliver bioactive growth factors with release kinetics that are governed by the molecular weight of the polycation. These experiments were initially done with FGF-2 as a proof of concept

evaluation, but this delivery system theoretically could work with any growth factor that binds heparin. Heparin and heparin derivatives are known to bind growth factor and cytokines including fibroblast growth factors, hepatocytes growth factor, vascular endothelial growth factor, heparin-binding epidermal growth factor, platelet derived growth factor, transforming growth factor β , macrophage-colony stimulating factor, interleukins (examples: IL-1,2,3,4,6,7,8), interferon γ , among others (134). From this list, it can be seen there are multitude of potential applications. It is expected that release kinetics will vary from growth factor to growth factor due to their varied affinities for heparin. It should also be noted that the [PAGS:heparin] delivery strategy is not strictly limited to applications involving therapeutic neovascularization. The scope of applications could potentially include nerve regeneration to orthopedics and is essentially dictated by the growth factors that bind to heparin. Additionally, the [PAGS:heparin] delivery strategy is an injectable suspension that could serve a non-invasive alternative to therapeutic treatments.

Conclusion

The PAGS-based delivery strategy has the ability to deliver bioactive FGF-2 with controllable release kinetics. The release of FGF-2 correlates directly to the molecular weight of the polycation in the complex. The [PAGS:heparin] complex is able to deliver FGF-2 for at least 28 days and likely over 100 days if the observed trend continues. The FGF-2 released from [PAGS:heparin] complexes has bioactivity similar to bolus FGF-2 and comparable to heparin stabilized FGF-2. The bioactivity of FGF-2 is preserved by

using heparin in its native form. Finally, this deliver strategy has the potential to deliver numerous therapeutic growth factors for a multitude of different applications.

Materials and Methods

General Materials and Methods

Succinic acid (TCI, Tokyo, Japan), arginine ethyl ester dihydrochloride (Research Organics, Cleveland, OH), 1,3 meta chloro peroxy benzoic acid (Acros Organics, Morris Plains, NJ), and all other chemicals (Alfa Aesar, Medford, MA) were used without purification, except for 1,3 meta chloro peroxy benzoic acid. This compound was lyophilized overnight to remove water. Flash chromatography was performed on a Buchi Fraction Collector C-660 equipped with a UV photometer C-635 (Flawil, Switzerland). FGF-2 was purchased from R&D Systems (Minneapolis, MN) and is an N terminally truncated form of human FGF-2 that contains the amino acid residues Proline 10 to Serine 155. It is a carrier free product that was reconstituted in PBS with 1mM DTT.

Synthesis of PAGS

PAGS was synthesized via polycondensation reaction of a 1:1 molar ratio of diglycidyl succinate and arginine ethyl ester in anhydrous N,N-dimethylformamide under N₂. The reaction mixture was stirred and kept at 60°C for 7 days. The resultant polymer was placed under vacuum and heated to 60°C for 24 hours to remove solvent. The residual dimethylformamide was removed by dissolving the polymer in methanol, precipitating polymer out with ethyl acetate, and dried under vacuum at 60°C overnight. Ethyl acetate wash is used to remove unreacted product and oligomers. The polymer

was characterized by FTNMR, FTIR, differential scanning calorimetry, and gel permeation chromatography.

Preparation of [PAGS:heparin:FGF-2] Complexes

PAGS (8 mg/ml) and heparin (10 mg/ml) were prepared in a solution of D-PBS (with $\text{Ca}^{+2}/\text{Mg}^{+2}$). A solution FGF-2 (100 $\mu\text{g}/\text{ml}$) was prepared as described by the manufacturer. Heparin and FGF-2 were combined at a mass ratio of 1:0.002 (114 $\mu\text{g}/200$ ng). This solution was incubated at room temperature for 15 minutes with mild agitation. PAGS solution was then added drop-wise to the aforementioned solution and allowed to incubate at room temperature for 30 minutes with mild agitation. The amount of PAGS used relative to heparin was a mass ratio of 35:1 (4 mg:114 μg). After this incubation time, samples were centrifuged for 10 minutes at 12,100 x g (2x). Supernatant was removed and fresh D-PBS was added to pellet. Each sample contained 4 mg PAGS, 114 μg , and 200 ng FGF-2 (mass ratio of [35:1:0.002]). Other ratios of [PAGS:heparin:FGF-2] were also used and noted as such.

[PAGS:heparin] Charge

[PAGS:heparin] complexes were prepared as detailed in sample preparation section with the exception of using molecular grade water in place of PBS and without the presence of FGF-2. From prepared samples, 750 μl was diluted to a final volume of 1.5 ml and analyzed via for zeta potential measurements.

[PAGS:heparin] Morphology

[PAGS:heparin] complexes were prepared as detailed in the sample preparation section with the exception of using molecular grade water in place of PBS. A volume of 100 μ l of matrix suspension was then added to a 1 mm aluminum stub and lyophilized for 4 h. All samples were sputtered with gold, and viewed with a Hitachi S-800 SEM (15 kV, 3–5 nm spot size) or a Leo 1530 SEM (10 kV, 3 nm spot size).

[PAGS:heparin] Complex Loading Analysis

[PAGS:heparin:FGF-2] complexes were prepared as detailed in sample preparation section. Radiolabeled ^{125}I -FGF-2 (0.1% of FGF-2 was hot, Perkin Elmer) was incorporated into the complex. After the supernatant had been removed (after final centrifugation), a 5% solution of acetic acid in PBS (with $\text{Ca}^{+2}/\text{Mg}^{+2}$) was added to pellet. The pellet was then agitated overnight and read on a gamma counter the following day. The amount of FGF-2 loaded was measured relative to a control of ^{125}I labeled FGF-2 alone in solution. Mass ratios of [PAGS:heparin:FGF-2] include [35:1:0.002], [21:1:0.002], [7:1:0.002], [35:1:0.0002], and [35:1:0.02].

[PAGS:heparin:FGF-2] Complex Release Kinetics

[PAGS:heparin:FGF-2] complexes were prepared as detailed in sample preparation section. Radiolabeled ^{125}I -FGF-2 (0.1% of total FGF-2 was “hot”) was incorporated into the complex. Supernatant was removed, replaced with 1 ml fresh PBS (with $\text{Ca}^{+2}/\text{Mg}^{+2}$), and incubated at 37°C. At specified time points (1, 3, 7, 10, 14, 17, 21, 24, and 28 days) the complex was centrifuged for 10 minutes at 12,100 x g.

Supernatant was removed and analyzed on a gamma counter. After each time point, 1 ml of fresh PBS (with $\text{Ca}^{+2}/\text{Mg}^{+2}$) was added to complex. Mass ratios of PAGES/heparin/FGF-2 used in these experiments include: [35:1:0.002] and [7:1:0.002].

Potency of Released FGF-2

[PAGES:heparin:FGF-2] complexes were formed in the same manner as described in prior section. Supernatant was removed, replaced with 1 ml M-199 medium, and incubated at 37 °C. After 1 and 3 days, the suspension was centrifuged for 10 minutes at 12,100 x g. The FGF-2-containing supernatant was removed and the tube was replenished with 1 ml of fresh M-199 medium. Human umbilical vein endothelial cells (HUVEC, P5-10) were seeded in a 24-well tissue culture treated polystyrene plate at a density of approximately 10,000 cells/well with 1 ml of M-199 growth media. HUVECs were incubated overnight at 37 °C, 5% CO_2 , washed with PBS, and then incubated with different media conditions. After the different media conditions had been added, cells were incubated at 37 °C, 5% CO_2 for 48 hours. After the 48 hour incubation period, HUVECs were detached via trypsinization and counted on a Coulter cell counter (Coulter Multisizer II). The biological activity of FGF-2 released from [PAGES:heparin] complexes was determined by comparing the stimulatory effects observed in wells containing basal M-199 Media (no FBS or growth factors), M-199 media supplemented with bolus FGF-2 (11 ng/ml for day 1 and 7 ng/ml for day 3), release media from [PAGES:heparin] matrices, and media supplemented with [heparin:FGF-2]. The amount of heparin used is 1:25 weight ratio of heparin:FGF-2 (138). Concentration of FGF-2 in

bolus and [heparin:FGF-2] media is identical to the amount of FGF-2 in [PAGS:heparin] release media.

Functional Analysis of Released FGF-2

HAECs (Human Aortic Endothelial Cells) (P4-6) and HUVECs (P4-6) were seeded on ECMatrix (Chemicon In Vitro Angiogenesis Assay Kit) in a 96-well tissue culture treated polystyrene (TCPS) plate at a density of 10,000 cells/well. HAECs were exposed to different media conditions for a period of 8 hours. [PAGS:heparin:FGF-2] complexes were formed in the same manner as described prior. Supernatant was removed, replaced with 1 ml EGM-2 medium, and incubated at 37°C. After 1 day, the suspension was centrifuged for 10 minutes at 12,100 x g. Supernatant (150 µl) containing the released FGF-2 was added to the HAECs cultured on a 96-well TCPS plate with ECMatrix. Endothelial tube formation was quantified by measuring total capillary tube length. Capillary tubes were measured from representative images using Image Pro. These values were averaged and tested for significance against HAECs incubated with the EGM-2 medium, FGF-2- and [heparin:FGF-2]-supplemented EGM-2 media under the same conditions. Each experimental set contained n = 4.

Preliminary *In Vivo* Investigation of [PAGS:heparin]

PAGS was dissolved in D-PBS (with Ca⁺²/Mg⁺²) at a concentration of 15 mg/ml. Heparin (10 mg/ml) was then added to PAGS solution drop-wise and allowed to incubate at room temperature for 15 minutes. The [PAGS:heparin] suspension was then injected (300 µl) subcutaneously in the hind limbs of Sprague–Dawley (Harlan, Indianapolis, IN)

rats (Female, 9-10 weeks old, 280g, n=3). Animals were cared for in compliance with protocols approved by the Committee on Animal Care of the Georgia Institute of Technology following NIH guidelines for the care and use of laboratory animals (NIH publication No. 85-23 rev. 1985). Rats were anesthetized with isoflurane during the injections. Acute tissue toxicity in rats were examined on days 3, 7, and 12. On each respective day, rats were sacrificed by carbon dioxide. Skin and muscle samples from the site of injection were collected, and dehydrated in a graded series of ethanol and xylenes and embedded in paraffin. All paraffin molds were sectioned into 10 μ m slices and subsequently stained with hematoxylin and eosin. Sections were analyzed for the degree of inflammation and fibrosis. The inflammatory response to each implant was assessed by rating the levels of lymphocytic and histiocytic infiltrate, and fibrosis was identified by collagen deposition

Statistics

For each variable group tested there were at least three replicates for the experimental and control samples. Multicomparisons ANOVA, Tukey Method, was used to statistically compare the different experimental values; $p < 0.05$ was considered statistically significant. The results are reported as mean values with standard deviations.

CHAPTER 6

Future Considerations and Conclusion

A Biocompatible, Synthetic Polycation

The synthesis of a biocompatible polycation is a far from trivial accomplishment. Presently, there is no reported polycation that has demonstrated to be biocompatible in both *in vitro* and *in vivo* environments. PAGES has exhibited unprecedented biocompatibility in both of these settings, thus making it a ground breaking discovery. Now that a biocompatible polycation has been synthesized, it can now be reverse engineered to discover what exactly makes PAGES non-toxic. We have hypothesized that a biodegradable polycation composed of naturally derived compounds or derivatives of these components would result in a biocompatible polycation. This hypothesis needs to be tested in a more rigorous manner to be completely validated. A more in depth investigation of PAGES's interactions with the cell membrane, how it gets into the cell, and where it is trafficked within the cell needs to be done. Also, a study of PAGES's degradation products should be examined. Ultimately, it is PAGES's degradation product that the cell will finally interact with in both *in vitro* and *in vivo*. A characterization of PAGES's degradation products would help elucidate whether our hypothesis is valid. Currently, the Wang Lab is in the process of synthesizing these degradation products.

Other than further characterizing PAGES's biocompatibility, the synthesis itself could be optimized. PAGES was the first successful generation of biocompatible polycations from the Wang Lab and optimization of the polymer synthesis was not the initial goal of the project. The synthesis of PAGES could be improved as far as yield,

control of molecular weight, and overall polymerization. Presently, the Wang Lab is working on the synthesis of other biocompatible polycations that follow the same template that proved successful with PAGES, and these polycations should be synthesized more efficiently as well as yield higher molecular weight species.

Overall, I believe the synthesis of PAGES has introduced a novel biomaterial that holds the potential to be the stepping stone to unlocking the biocompatibility concerns of synthetic polycations. I say stepping stone because PAGES is far from perfect. It takes an extremely long time to synthesize and the control of molecular weight is poor at best. I also say stepping stone because PAGES has introduced increased biocompatibility but the exact mechanism behind it is unknown. PAGES interactions with cells can be studied and this mechanism can hopefully be elucidated. When and if this can be accomplished, PAGES will serve as another example of a biomaterial that provided a breakthrough by rationale design.

In conclusion, the field of synthetic polycations has yet to produce a polycation that has exhibited both *in vitro* and *in vivo* biocompatibility. To circumvent this problem, we chose to mimic a polycation that is biocompatible and present in the body, a cationic peptide. The essential traits of a cationic peptide we incorporated into our polycation were biodegradability and a cationic species originated from a biological molecule that is found in the body. We also used a synthetic linker that was a derivative of a biomolecule found in the body. Through a rational design template we synthesized a polycation that exhibited both *in vitro* and *in vivo* biocompatibility. This is the first reported polycation to demonstrate these characteristics. For decades research has attempted to synthesize a polycation with this type of biocompatibility, and this research is the first to report one.

[PAGS:heparin] Delivery Strategy

The [PAGS:heparin] delivery strategy presented in this research was a proof of concept study. The aptitude to deliver bioactive growth factor under a controllable release was investigated with FGF-2. The [PAGS:heparin] complex demonstrated that it was capable of releasing a growth factor for a period of at least 28 days with a controllable release rate. The FGF-2 released from the complex also maintained its bioactivity.

From this point there are a couple of different paths that could be pursued. A more extensive *in vitro* assessment of the complex can be investigated and will be need to be done at some point. This would include characterizing the interactions and affinity between PAGS and heparin. Other growth factors will also have to be examined for release kinetics. In order to truly mimic the *in vivo* neovascularization response a number of different growth factors will need to be used. An example of two other growth factors that will be needed is VEGF and PDGF. The release kinetics of these growth factors would have to be determined accurately to mimic the *in vivo* response.

The other path that will eventually be examined is the *in vivo* potential of the [PAGS:heparin] complex. Its potential could initially be tested with FGF-2 alone or with a combination of growth factors. Regardless if it is using FGF-2 alone or as a combination of different growth factors, there are a number of different disease models that could be used. The initial disease model would be a hind limb ischemia model, which is a standard model of peripheral ischemia that mimics some aspects of human atherosclerosis. In this animal model, mice would be subjected to femoral artery and vein ligation. Mice would then be injected with the [PAGS:heparin:FGF-2] suspension. Hind

limb tissue would then be retrieved at specified time points and analyzed via immunohistochemistry, perfusion levels, and quantitatively by the appearance of hind limb. The growth factors incorporated into complexes could also be radiolabelled to examine the growth factor's diffusion radius. The hind limb recovery results would most likely be more positive if a combination of different growth factors were used, as in neovascularization. If done with a combination of different growth factors, these will have to be investigated *in vitro* first. One aspect that concerns myself is how the stability of these complexes will hold up *in vivo*. The complex is regulated by non-covalent interactions and has been studied in simplified *in vitro* environments. When exposed to the more complicated *in vivo* environment it is hard to predict how it will exhibit the same *in vitro* characteristics. The future of the [PAGS:heparin] delivery strategy hinges on its success in these *in vivo* experiments, as it does with most biomaterials.

The [PAGS:heparin] delivery vehicle is not limited to cardiovascular diseases, but can be used for any therapy that would benefit from the presence of exogenous growth factors. This is true as long as the potential growth factor has some affinity for heparin. A potential application of the [PAGS:heparin] complex in the Wang Lab would be for nerve tissue engineering. Presently, the Wang Lab is attempting to produce a scaffold fabrication method for nerve guidance channels made from PGS. These nerve guidance channels could be coated with the [PAGS:heparin:growth factor] suspension prior to being implanted. The delivery of growth factors (example: NGF) could potentially aid in nerve regeneration.

In conclusion, the [PAGS:heparin] delivery system has provided a novel strategy for the delivery of growth factors. There have been other heparin-based delivery

systems, but none that have accomplished an efficient delivery through the type of simplicity. In this situation, simple is good because it could potentially cut down on cost by only having 3 components. Other systems that bind heparin either use covalent modifications of heparin or bind heparin through non-covalent interactions. Covalent modifications of heparin can potentially alter the bioactivity of heparin and its target growth factor. Systems that use non-covalent interactions to bind heparin use heparin-binding domains (amino acid sequences). The PAGES system uses only 1 amino acid, arginine, and this should not only maintain heparin's bioactivity but also cut the cost of the delivery vehicle. This strategy not only accomplished controllable release kinetics of bioactive FGF-2, but did it in a more efficient manner than other reported systems. Another characteristic of the PAGES system is it is injectable through 25G needles, thus it is expected that a minimally invasive delivery system is feasible. Further development of this growth factor delivery system may lead to tangible clinical benefits in a variety of medical fields.

Appendix A

Gene Delivery Applications

Introduction

The field of gene therapy holds the potential to treat a broad array of diseases that are considered incurable including: cystic fibrosis, hemophilia, and severe combined immunodeficiency disease. This potential has yet to be realized due to the remaining obstacle of a safe and efficient approach to deliver genetic material. Presently, viral vectors, such as recombinant viral and adenoviral vectors, are the most efficient gene delivery vehicles but this efficiency is burdened with certain concerns. Some of these drawbacks including immunogenicity, size limitations of inserted genetic material, and potential of oncogenicity have impeded its clinical impact. Conversely, non-viral gene delivery agents offer a safer alternative compared to their viral counterparts. They can potentially allow for greater control of their molecular architecture, flexibility in the size of the genetic information delivered, and lower immunogenicity (29, 115, 154, 162-164). Consequently, this has led to an increased interest in non-viral vectors as gene delivery agents, specifically cationic polymers.

Cationic polymers have been widely used as vectors because of their ability to protect DNA from nucleases and to condense DNA through electrostatic interactions. Examples of cationic polymers currently being used are: polyethyleneimine (PEI), poly-L-lysine (PLL), and polyamidoamine dendrimers (165, 166). These cationic polymers have been the most successful cationic vectors but there still remains the problem of having a low transfection efficiency and being cytotoxic. This issue of cytotoxicity is due

mostly to the poor biocompatibility of these non-degradable polymers (57). Due to the latter complication, our research has been concentrated on the advancement of biocompatible polymers for gene delivery.

Arginine is one of the most positively charged biomolecules found in nature and it is an amino acid that has been associated with important cellular functions such as transmembrane and nuclear transport (167). This is why arginine has generated interest in the scope of gene delivery with the hope of harnessing its abilities and exploiting them for gene delivery applications. Arginine has been incorporated into vectors as a single molecule, part of a peptide (homo and hetero peptides), and as its functional group (guanidinium side chain). These methods have met with varying degrees of success and these vectors have also been saddled with cytotoxicity concerns (67, 168-170).

In this study we designed an arginine-based polymer that is biocompatible. The biocompatibility of PAGES ensures that it will not be cytotoxic to cells and the positively-charged arginine incorporated into the polymer backbone will allow PAGES to compact genetic information efficiently and transport it across the cell membrane. Furthermore, the versatile design of this polymer allows for the incorporation of different modalities for varied applications.

Results and Discussion

[Polycation:DNA] Complex Characterization

The ability of a polymer to compact DNA through electrostatic interactions between the positively charged nitrogens of the polymer and the negatively charged phosphates in the DNA backbone is critical to its success in gene delivery. This ability of

PAGS and PAHGS to self-assemble with plasmid DNA was demonstrated through an agarose gel retardation assay, particle size analysis, and zeta potential measurements.

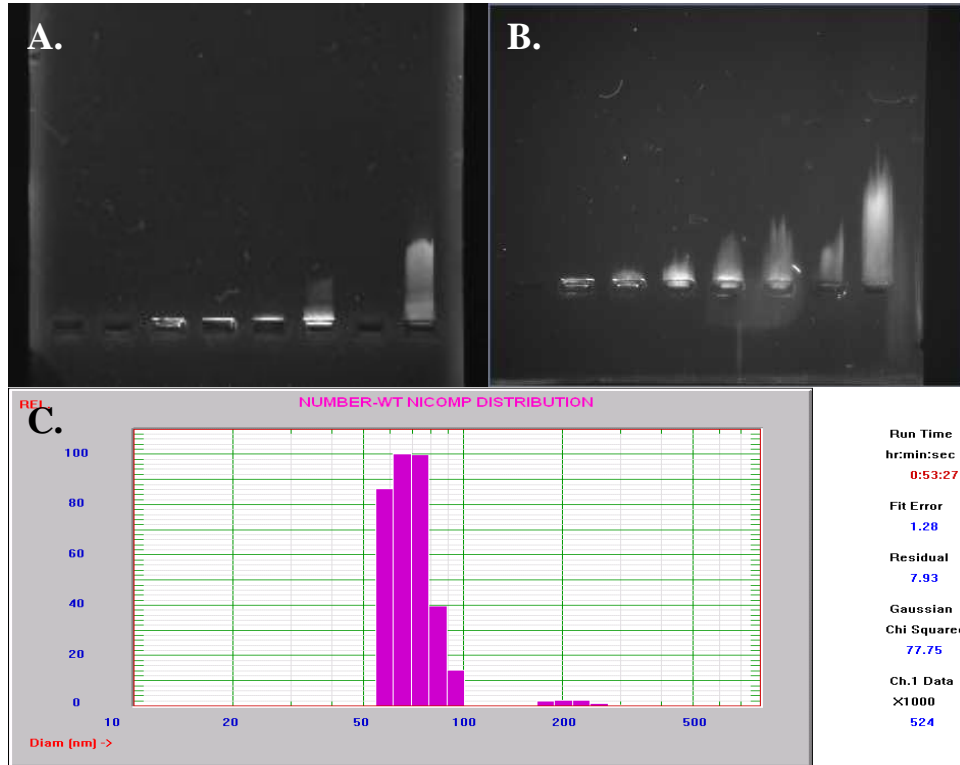


Figure A.1. Potential of polycation as gene delivery vectors. (A) Gel electrophoresis was used to investigate the N/P ratio need to completely neutralize plasmid DNA. Gel retardation assay displays complete retardation of plasmid DNA at N/P ratio between 4/1-8/1 for PAGS. From right to left: naked DNA, 4/1, 8/1, 12/1, and 16/1 N/P ratios. (B) PAHGS retarded plasmid DNA at a ratio between 10/1-15/1. From right to left: naked DNA, 1/1, 5/1, 10/1, 20/1 and 40/1 N/P ratios. The second well from the right was supposed to be empty but a small amount of sample had fallen into it while placing samples in the other lanes. (C) [PAGS:DNA] complexes were characterized by dynamic

light scattering experiments. [PAGS:DNA] complexes averaged a diameter of approximately 70 nm and carried a charge of + 41 mV.

Agarose gel electrophoresis separates molecules based on charge and size. Complete retardation of the DNA/polymer complex is a result of charge neutralization, and is one way to measure a polymer's capability to complex DNA. Polymer and DNA were both placed in solutions of molecular grade water, with the concentration of polymer changing to the desired N/P ratios. Polyplexes were formed by adding the DNA solution drop-wise to the polymer solution and then gently vortexing for 15 minutes. The resulting polyplex solution was then run on an agarose gel by gel electrophoresis (**Figure A.1A, B**). DNA was completely retarded at a N/P ratio between 4/1-8/1 for PAGS and 10/1-12/1 for PAHGS. From these results a N/P ratio of 10/1 for PAGS and 15/1 for PAHGS was used for all further experiments to ensure the plasmid was completely neutralized and compacted.

Agarose gel retardation assays can be helpful in determining the necessary polymer concentration to completely complex DNA, but it cannot determine the actual size of the [polycation:DNA] complexes formed nor the actual zeta potential carried by these complexes. Particle size and the charge of the polymer can be measured through dynamic light scattering (DLS). These measurements revealed that the complexes averaged approximately 70 nm (**Figure A.1C**) in diameter and carried a charge of +41 mV. Particles under 200 nm in diameter are capable of entering cells through the endocytotic pathway, so [PAGS:DNA] complexes should not experience difficulty entering the cell. Also, the positive charge on the particles could improve interactions

with negatively charged proteoglycans present on the cell surface. PAHGS was not characterized by dynamic light scattering experiments.

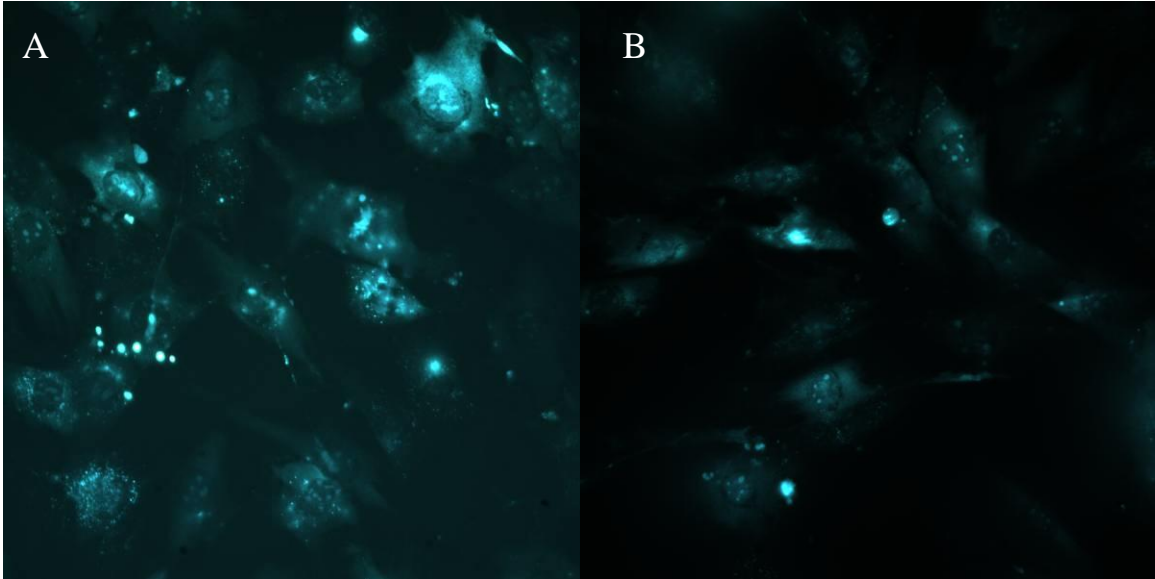


Figure A.2. *In vitro* transfection. Plasmid DNA labeled with YOYO-1 was complexed with PAGS and was used to transfect lung epithelial cells. Polyplexes appeared to be present in vesicles instead of evenly distributed in cytosol. Figure 3.A is using PAGS, 3.B is using linear PEI (25kD).

***In Vitro* Transfection**

A gene delivery agent may be able to condense genetic information on a nanoscale level, but it will not be viewed as a successful delivery vehicle unless it can also transport this genetic material across the cell membrane. There are different techniques employed to measure a vector's ability to transport material into cells, and these methods can either be qualitative or quantitative. Some examples of these

techniques include transfecting cells with a reporter plasmid (luciferase, fluorescent proteins) or fluorescently labeling the nucleic acid that is being delivered. Both green fluorescent protein and the fluorescent dye were used to track the movement of plasmid DNA in this series of transfections.

Plasmid DNA (enhanced green fluorescent protein, eGFP) was selected as a reporter vector for transfection experiments. GFP is a well-characterized reporter system and has been used in applications from gene delivery to tissue engineering. Plasmid DNA encoding for eGFP was complexed with polycation for a period of 30 minutes at room temperature. Complex solution was then added to lung epithelial cells and allowed to incubate at 37°C for 4-5 hours. Polyplex solution was then either replaced with full growth medium or basal media. Epithelial cells were then incubated overnight at 37°C and analyzed for fluorescence. There was no fluorescence visualized, demonstrating an unsuccessful transfection.

To further investigate the path of the polyplex, plasmid DNA was intercalated with YOYO-1. This was then complexed with PAGES to examine its capacity to deliver nucleic acids across the cell membrane. The polyplexes were observed inside of the cells 4-5 hours after transfection (**Figure A.2A**), similar to the positive control, linear PEI (25kD) at a N/P ratio of 7/1 (**Figure A.2B**). The ratio of 7/1 was used for PEI because it appeared from the literature to be a good compromise between transfection efficiency and cytotoxicity (29). The complexes appeared to be contained within endosomes, as indicated by the punctated fluorescent signals. This demonstrated that PAGES can transport genetic information into cells. From these transfection experiments, it was hypothesized that PAGES could transfect cells but could not escape the endosome. To

overcome the inability to escape endosomes, histamine was incorporated into the PAGES polymer. Histamine was incorporated because of its lower pKa, thus having greater buffering capacity. A theory on why PEI is so successful at gene delivery is because of its buffering capacity, which ultimately bursts endosome through the proton sponge effect. The incorporation of histamine should allow PAGES the ability to escape endosomes. This addition of histamine into the polymer, PAHGS, did not increase the transfection efficiency of the complexes. It is possible that not enough histamine was incorporated into the polycation, thus the buffering capacity was not great enough. Also, there may be more obstacles that PAGES could be facing during gene delivery other than escape from the endosome.

Conclusion

Two biodegradable poly(aminoglycerol esters) that contain a positive charged supplied by arginine to compact genetic material efficiently has been developed in this study. PAGES proved to condense plasmid DNA and transport it across the cell membrane. This transport did not result in successful gene delivery, so histamine was incorporated into the polymer for enhanced buffering capacity. This did not result in increased gene delivery. These arginine-based vectors are less cytotoxic than PEI and other synthetic polycations used for gene delivery, thus offering a biocompatible alternative. With further investigation, these polymers could prove to be successful gene delivery agents.

Materials and Methods

Chemicals and General Methods

Succinic acid was purchased from TCI (Wellesley Hills, MA). Arginine ethyl ester dihydrochloride was purchased from Research Organics (Cleveland, OH). 1,3 meta chloro peroxy benzoic acid was purchased from Acros (Morris Plains, NJ). All other chemical were purchased from Alfa Aesar (Ward Hill, MA) and used without purification. Flash chromatography was done on a Buchi Fraction Collector C-660 w/ UV photometer C-635. Nuclear magnetic resonance (NMR) spectra were recorded on a 400 MHz Mercury-400BB NMR. Fourier transform infrared (FTIR) spectra were recorded on a ThermoNicolet IR-100 spectrometer. Dynamic light scattering measurements were recorded on a Zeta Potential/Particle Sizer NICOMP 380 ZLS.

Polymer Synthesis

The arginine/histamine-based polymer (PAHGS) was synthesized via polycondensation reaction of a 1:1 molar ratio between diglycidyl succinate and the combination of arginine ethyl ester and histamine in anhydrous N,N-dimethylformamide under N₂. The reaction mixture was stirred and kept at 60°C for 7 days. The resultant polymer was placed under vacuum and heated to 60°C for 24 hours to remove solvent. The residual dimethylformamide was removed by dissolving the polymer in methanol, precipitating polymer out with ethyl acetate, and dried under vacuum at 60°C overnight. Ethyl acetate wash is used to remove unreacted product and oligomers. The polymer was characterized by NMR and FTIR. PAGES and polymer precursors were synthesized as mentioned in earlier experimental sections.

Plasmid DNA

The 4.7 kb plasmid DNA, pEYFP-N1, contains the enhanced yellow fluorescent protein driven by the human CMV promoter and was obtained as a gift from Dr. Bao's Lab. Plasmid DNA was amplified by insertion into JM-109 E. coli, and was purified using the Maxi-Prep DNA Purification kit from Qiagen (Germantown, MD). The purity of the plasmid DNA was measured by UV/Vis (TECAN Safire) with A_{260}/A_{280} in the range of 1.7-1.85.

Agarose Gel Retardation Assay

DNA/Polymer complexes were formed by adding 50 μ L of plasmid DNA solution (0.04 μ g/ μ L) in molecular grade water to 50 μ L of polymer solution in molecular grade water. Polymer concentrations were altered to yield the desired nitrogen/phosphate (N/P) ratios. These mixtures were gently vortexed and allowed to incubate at room temperature for 45 minutes. From these mixtures, 10 μ L was loaded on a 0.6% agarose gel with a 10% Ficoll 400 loading buffer (without bromophenol blue) in a 20 mM HEPES buffer. The gel was run at 108 V for 60 minutes and visualized by ethidium bromide staining. Two different polymers were used for gel retardation assays: PAGES and PAHGS.

Particle Sizing and ζ -Potential Measurements

DNA/Polymer complexes were formed in the same manner as explained in the agarose gel retardation assay. Samples were diluted with 900 μ L of molecular grade water and average particle sizes and ζ -potential measurements were carried out at 25°C.

Three measurements were made on each sample, and the average diameter and ζ -potential were reported.

***In Vitro* Transfection**

L2 (mouse lung epithelial) cells were cultured in 24-well tissue culture treated polystyrene plates for at a seeding density of approximately 40,000 cells per well in 1 mL of Ham's F12k growth medium. Ham's F12k growth media consisted of Ham's F12k balanced salt solution, 10% fetal bovine serum, 1mM glutamine, and antibiotics. Cells were grown overnight up to 70% confluency then growth medium was replaced with transfections medium. Complexes were prepared in the same manner as the agarose gel retardation assay. To further characterize the path of the complexes, they were prepared in the same manner as the agarose gel retardation assay, but prior to adding the plasmid DNA to the polymer solution it was complexed with a DNA intercalating agent, YOYO-1 (gift from Dr. Bao's Lab). The concentration of YOYO-1 used for 2 μg DNA was 4×10^{-6} M and they were incubated at room temperature for 30 min before adding them to PAGES solution (171). The complex solution was then diluted with serum free medium to a volume of 1 mL to form the transfection medium. Various N/P ratios were used with 2 μg of DNA for each well. Linear PEI (25kD) was used as a positive control and was prepared in the same manner as the other polymer solutions. Other controls included naked plasmid DNA with and without YOYO-1, YOYO-1, and PAGES:YOYO-1. Cells were incubated at 37°C, 5% CO₂ for 4-5 hours and visualized by fluoresce using a Nikon Eclipse TE 2000-U microscope equipped with a FITC filter.

Appendix B

Release Kinetics of FGF-2 via ELISA

Introduction

A critical characteristic of any delivery system is the release of its cargo over time. The resultant release profile of a delivery system is essential in elucidating the appropriate applications it can be used for. This property of delivery systems is also important in ensuring it will be delivering therapeutics in a safe and efficient manner. The release kinetics of the [PAGS:heparin] complex, along with bioactivity of released FGF-2, was one of the first properties investigated.

The first attempt to investigate the release kinetics of FGF-2 from the [PAGS:heparin] complex were done using an enzyme-linked immunosorbent assay (ELISA). It was hypothesized that ELISA would serve a dual function for the characterization of the [PAGS:heparin] complex. The two properties that could be assayed were release kinetics and bioactivity. ELISA could measure the concentration of FGF-2 in the release solution. Also, bioactivity could be quantitatively assessed because the released FGF-2 would still have to be in its native form to interact with the antibodies present in the ELISA.

Results and Discussion

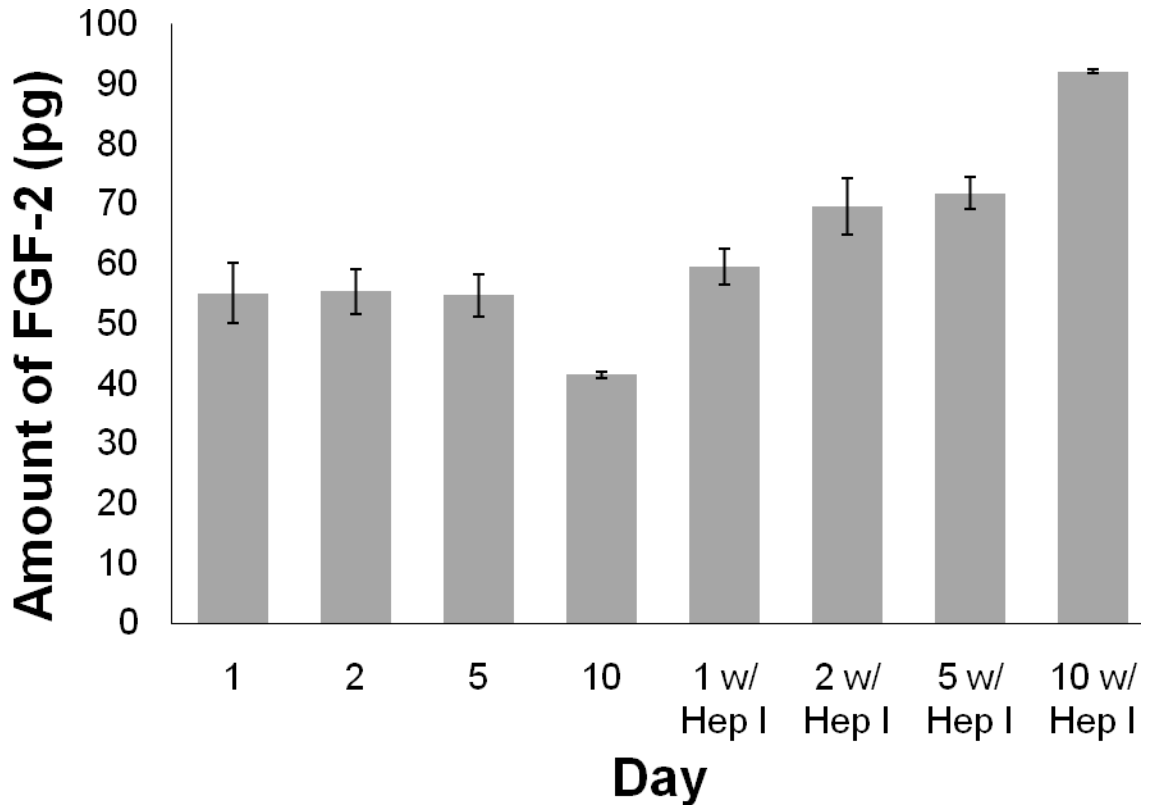


Figure B.1. Release kinetics of FGF-2 from [PAGS:heparin] complexes. The concentration of FGF-2 in the supernatant of [PAGS:heparin] complexes was examined by ELISA. The concentration of FGF-2 released remained constant for the first 5 days and decreased in the next 5 days. After exposing the release solution to heparinase I, the concentration of FGF-2 demonstrated an increase through 10 days.

The release dynamics of FGF-2 from [PAGS:heparin] complexes were first characterized by ELISA. ELISA was chosen to evaluate the concentration of FGF-2 release solution because of its accuracy and because it could qualitatively investigate

bioactivity of released FGF-2. The concentration of FGF-2 exhibited in **Figure B.1** is an individual daily assessment, not a cumulative amount. The rate of FGF-2 released was constant at approximately 55 pg/day from days 1-5, and then decreased to 41 pg/day on day 10. It was hypothesized that FGF-2 is released in conjunction with heparin. This hypothesis could potentially explain why on day 10 the amount of FGF-2 in the release solution had decreased. Both heparin and the ELISA antibody for FGF-2 will have a high affinity for FGF-2. The amount of heparin present in the [PAGS:heparin] release solution is unknown, and it is possible that the amount of heparin in the release solution is greater than days 1-5. This would present a situation where two molecules are in competition for the binding of FGF-2, and ultimately an underestimate of the concentration of FGF-2.

To validate the prior hypothesis, [PAGS:heparin] release solution underwent a heparinase digest. Heparinase I is an enzyme that digests heparin, and should remove heparin from competing with the ELISA antibodies for FGF-2. When the supernatant underwent a heparinase digest, the concentration of FGF-2 increased to 92 pg/day on day 10. It should also be noted that the concentration of FGF-2 on each day increased when exposed to the enzyme digest, compared to the concentration of FGF-2 without digest. This is demonstrated on day one FGF-2 concentrations of 55 pg/day (without digest) and 60 pg/day (with digest). The other time points have more extreme differences when exposed to the heparinase digest. The release kinetics of FGF-2 involving heparinase demonstrated heparin was competing with antibodies within the ELISA. This competition then resulted in a lower measured concentration. As mentioned earlier, the day 10 concentration of FGF-2 was approximately 15 pg/day less relative to days 1-5. A

possible explanation of these results could be related to PAGES. If this batch of PAGES had a molecular weight lower than later reported batches, it is possible that polymer degradation could have an effect. The degradation of PAGES could potentially lead to an increase in the [heparin:FGF-2] concentration, thus more competition with the ELISA antibodies.

Conclusion

ELISA is a useful tool in determining the concentration of FGF-2 in solution. It also has the added benefit of examining if the FGF-2 is bioactive after being released from the [PAGES:heparin] complex. In this experiment, ELISA demonstrated a release of FGF-2 from [PAGES:heparin] complexes at an increasing rate. This conclusion came after FGF-2 release solution was exposed to a heparinase digest. This digest was necessary because soluble heparin was also present in the release solution, and this most likely created a situation where heparin was competing with the ELISA antibodies for the binding of FGF-2.

The use of an ELISA resulted in an accurate determination of FGF-2 concentration, but there were complications that led to the pursuit of another method for FGF-2 detection. The initial problem was attempting to find the total amount of loaded FGF-2 in the [PAGES:heparin] complexes. To determine the loading efficiency, [PAGES:heparin] complexes had to undergo a heparinase digest at 37°C. The [PAGES:heparin] complexes contained 114 µg of heparin. To digest this amount of heparin without using large amounts of heparinase I, which can be very costly, would take on the order of days. The temperature could have been increased but that could lead

to increased denaturation of FGF-2. Also, it is well understood that FGF-2 has a short-half life *in vitro*, and this can lead to loss of bioactivity in a short amount of time. Given these circumstances, total loading experiments never concluded accurate results. This then creates problems in characterizing the release kinetics of FGF-2. Another issue with using the ELISA was realized in the release experiments. It is believed the release results were never accurate to what was measured in the release solution. As mentioned prior, the heparinase digest resulted in FGF-2 being denatured because of the time it took to digest the heparin. The concentration of heparin in the release solution was unknown, thus an estimate of time or amount of heparinase needed could have been misjudged. With these difficult circumstances, it was decided to pursue a different option to characterize the release kinetics of FGF-2. The option we pursued was radiolabelled FGF-2.

Materials and Methods

PAGS (8 mg/ml), heparin (10 mg/ml), and FGF-2 (2 µg/ml) were prepared in solutions of PBS. Each sample contained 4 mg PAGS, 114 µg, and 10 ng of FGF-2. The FGF-2 solution was added to the PAGS solution then the heparin solution was added to this mixture. The resultant suspension was agitated and allowed to incubate at room temperature for 10 min. The [PAGS:heparin:FGF-2] complex was then centrifuged for 10 minutes at 12,100 x g, supernatant was removed, and 1ml of PBS was added to resuspend the pellet. [PAGS:heparin:FGF-2] complexes were incubated at 37°C for 1, 2, 5, and 10 days. At the specified time, samples were centrifuged for 10 minutes at 12,100 x g. Then the supernatant was removed and stored at -80°C. After supernatant was

removed, fresh PBS 1 ml) was added to complex. The FGF-2 concentration in each supernatant was quantified using a FGF-2 ELISA kit. Supernatant was also incubated with 0.001 IU of Heparinase I (IBEX Pharmaceuticals) at 37°C for 48 h and then FGF-2 concentration was quantified using a FGF-2 ELISA kit (R and D Systems).

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