APTAMER-FUNCTIONALIZED HYDROGELS FOR THE PROGRAMMABLE
RELEASE OF GROWTH FACTORS

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by
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Abstract

Growth factors are potent signaling molecules that regulate numerous physiological processes. However, the safe and efficient delivery of growth factors remains an unmet goal when growth factors are applied to treat various pathologies. To control the spatiotemporal delivery of growth factors, material carriers such as hydrogels have been investigated. Hydrogels are promising growth factor delivery systems for their high water content and similarities to native tissue. However, the high permeability of hydrogel releases the loaded growth factors rapidly. In addition, the release rate of multiple growth factors cannot be controlled individually when multiple growth factors are incorporated. In order to better control the release rates of growth factors from hydrogels, functionalization strategies using affinity ligands should be explored.

Nucleic acid aptamers are synthetic oligonucleotides that bind to target molecules with high specificity and high affinity. In addition, these aptamers can be chemically modified with various functional groups and conjugated to a variety of biomaterials. For these reasons, hydrogels functionalized with aptamers could be a valuable tool for controlling the release of growth factors. Two main objectives were pursued in this work: 1) characterizing the molecular interactions of aptamers with cognate biomolecules, and 2) develop aptamer-functionalized delivery systems for the controlled release of growth factors. Modulation of the binding affinity can occur through the introduction or removal of steric hindrance or the use base substitutions. In addition, the use of an oligonucleotide complementary to the aptamer can be used to inactivate the aptamer. The aptamers were then used to develop an aptamer-functionalized system for the high retention and regulated release of growth factors. The work described herein presents a promising method to control growth factor delivery for the treatment of many human diseases.
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Chapter 1

Introduction

1.1. Endogenous Growth Factors

Growth factors are potent biomolecules that stimulate cellular processes. These chemical signals function as part of a large communications network that activates cellular differentiation, growth, proliferation, and migration [1]. Growth factors are secreted by cells and act on target cell receptors through three possible modes of action: 1) autocrine, in which a growth factor acts on the cell of its origin; 2) paracrine, in which a growth factor acts on a neighboring cell after diffusing a short distance from the cell of origin; and 3) endocrine, in which a growth factor acts on a remote target after diffusing a far distance [2-5]. Growth factors typically act in either an autocrine or a paracrine mechanism as their slow diffusion and short half-lives limit the distance in which the growth factor can exert its function [6]. Intracellular signal transduction cascades are activated when a growth factor binds to its target transmembrane receptor to modulate gene expression within the responding cell and create a biological response [7] (Figure 1.1).

Growth factors have diverse effects on growth, development, and metabolism [8]. For example, angiogenesis is strongly regulated by growth factors. Angiogenesis is the formation of new blood vessels from preexisting vasculature [9, 10] and is critical in the postnatal development of tissue [11, 12]. By adulthood, the blood vessels become quiescent, except in a few biological processes [13, 14]. The cells in a normal adult blood vessel undergo cell division very infrequently, perhaps to maintain tissue homeostasis [15, 16]. Despite their inactive state, vessels retain the ability to rapidly branch in response to certain growth factors [13, 14]. Angiogenesis is reactivated in
Growth factors are important signaling biomacromolecules that regulate cellular function. A growth factor binds to a specific transmembrane receptor on the target cell to result in certain biological responses through the activation of signal transduction cascades and subsequent alteration of gene expression. Examples of biological responses include cell migration, proliferation, and cellular differentiation.

Figure 1.1. Growth factors are important signaling biomacromolecules that regulate cellular function. A growth factor binds to a specific transmembrane receptor on the target cell to result in certain biological responses through the activation of signal transduction cascades and subsequent alteration of gene expression. Examples of biological responses include cell migration, proliferation, and cellular differentiation.
physiological processes, such as reproduction (in the corpus luteum during ovulation and in the placenta during pregnancy) and during wound healing [9, 10, 13, 17-22]. During these processes, angiogenesis is briefly but strongly regulated (i.e., activated for a period of days and then completely inactivated) by growth factors [10, 17]. Dysregulation of vessel growth or excessive angiogenesis due to the uncontrolled delivery of endogenous growth factors are associated with numerous pathologies, including cancer, psoriasis, rheumatoid arthritis, proliferative retinopathy and age-related macular degeneration, obesity, asthma, atherosclerosis, and infectious diseases [12, 23]. In addition to angiogenesis, growth factors are involved in the development of the central nervous system and the regulation of interstitial fluid pressure [24, 25].

1.2. Delivery of Exogenous Growth Factors

The role and delivery of growth factors for the development and homeostasis of physiological systems has inspired much interest in utilizing exogenous growth factors to control cellular behavior for the treatment of numerous diseases [5, 6]. Advances in recombinant protein technology (bacterial and eukaryotic expression systems) have led to the possibility of producing large quantities of protein for this goal. In fact, the percentage of new proteinaceous drugs approved by the US Food and Drug Administration (FDA) increased from 17% in 2005 to 32% in 2011 [26, 27]. Though the number of protein drugs has steadily increased throughout the years, only a small percentage represents the number of growth factors that have been commercialized for therapy [28-31]. The delivery of growth factors and other protein drugs has primarily been through the intramuscular, intravenous, or subcutaneous injections of liquid formulations since parenteral administration are fast and inexpensive [32]. Despite promising in vitro and in vivo data, many clinical trials using growth factors have yielded poor results [33, 34]. For example, recombinant human vascular endothelial growth factor (rhVEGF) was delivered by a single intracoronary infusion that was followed by three intravenous infusions in a placebo-controlled phase II study to
stimulate vascularization in ischemia [33]. While the VEGF was well tolerated by most patients at most of the dosages used [35], it failed to improve conditions beyond the placebo [33]. Patients who could not tolerate VEGF experienced severe side effects including hypotension [33].

The failure of the systemic administration of the growth factor could be attributed to many reasons. Systemic administration transports the growth factor not only to the target site, but also to all the tissues in the body. The amount of the growth factor that would reach the target site would be substantially less than that administered due to the widespread distribution in the body, necessitating a concentration of the growth factor be injected into the body that is higher than what is needed to treat the disease. However, increasing the dosage of the growth factor would come with increased risks of side effects and may not improve the therapeutic benefit. For example, supraphysiological concentrations of VEGF should be avoided as VEGF would accumulate in nontarget tissue and could result in the pathological formation of blood vessels (e.g., tumors) [36]. In addition, growth factors have short circulation half-lives \textit{in vivo}. Vascular endothelial growth factor and other growth factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) have circulation half-lives of a few minutes due to degradation (denaturation, aggregation, oxidation, proteolysis, and interfacial damage) and renal excretion [12, 37-39]. Therefore, it is possible that the transient exposure to VEGF was insufficient to stimulate therapeutic activity, suggesting that a higher concentration or a persistent exposure is needed [20].

Thus, alternative methods of growth factor delivery that avoid the problems associated with systemic administration (e.g., fast clearance, degradation, and distribution to nontarget tissue) need to be investigated for the safe and efficient treatment of numerous diseases.

To ensure that a sufficient concentration of growth factors is administered to the target site, local delivery of exogenous growth factors is being explored. Bolus injections at the target site allow the growth factors to remain localized and without affecting remote tissues, thereby increasing its therapeutic benefit and efficacy. The subcutaneous delivery of recombinant human
nerve growth factor (rhNGF) to treat diabetic polyneuropathy was evaluated in clinical trials [34, 40, 41]. In the initial trials, supraphysiological concentrations of NGF resulted in adverse side effects, such as myalgia, thereby limiting the amount of NGF that could be delivered in a single dose [40]. In the phase III study, a lower concentration of NGF was administered subcutaneously 3 times a week for 12 months. Unlike in the previous trials, treatment of the disease with the growth factor failed to show significant benefits. Furthermore, administration of NGF was halted in 10% of the patients due to hyperalgesia at the injection site or other adverse side effects [34]. While subcutaneous injections allow growth factors to act locally, they are still rapidly cleared from the injection site and the tissue is not exposed to the growth factors for times needed to activate biological responses [42]. To overcome these problems, new delivery methods are being investigated to replace traditional bolus injections to increase therapeutic efficiency by protecting the growth factor and prolonging its delivery.

1.3. Biomacromolecule Delivery Systems

The idea of prolonging the delivery or activity of biomacromolecules to increase their efficacy in vivo is not a novel concept. Research in the late 1930s investigated the possibility of the increased and prolonged action of insulin administered subcutaneously in the form of a solid material [43]. Unfortunately, the hypoglycemic action of the insulin from the solid tablet was prolonged only slightly in comparison to that of the free insulin. Moreover, the solid tablet triggered severe inflammation and the formation of a cyst around the tablet [43].

The prolonged action or continuous delivery of insulin from an implant was investigated again in 1972 [44]. Implants of various concentrations of polyacrylamide with insulin admixed were evaluated in diabetic rats. Polyacrylamide forms a porous material (pore size ~10-20 Å) through which the entrapped insulin can diffuse and exit the material. The pore size and ability of insulin to diffuse out of the polyacrylamide carrier was dependent on the concentration of
acrylamide. The higher concentrations of acrylamide resulted in smaller pore sizes in the polyacrylamide carrier and subsequently slowed insulin diffusion from the carrier to the animal. Diabetic rats receiving insulin implants were able to regain lost weight and had nonglycosuria urine. Diabetes in rats receiving a slowly releasing implant was controlled for a longer period than in rats receiving a fast-release implant. When the insulin-loaded implants were excised from the rats, the rats became emaciated with elevated urinary sugar levels. Taken together, this suggests the implant was able to regulate insulin delivery to control diabetes [44]. This study, which demonstrated that a porous, polymeric material could be used to slow the release of loaded biomacromolecules, was an important advancement in the field of controlled delivery. Not only can pore size be tuned to control delivery rates of loaded biomacromolecules [44, 45], but theoretically any biomacromolecule can be admixed since the biomacromolecule is retained in the polymer matrix by physical incorporation.

One limitation of using polyacrylamide and other polymers like polyvinylpyrrolidone as carriers for the controlled release of biomacromolecules [44, 45] is that they often inflame animal tissue [46]. To create less inflammatory delivery systems, various polymers were assessed for their ability not to elicit inflammation and to sustain protein delivery [47]. Polyvinylpyrrolidone results in significant inflammation in vivo and polyacrylamide causes inflammation in the majority of animals; washing poly(vinyl alcohol) implants prior to implantation significantly reduces the risk of significant inflammation; washing ethylene-vinyl acetate copolymer implants eliminates the risk of inflammation; and poly(hydroxyethyl methacrylate) inherently does not incite a biological response [47]. The three most biocompatible polymers of the study were chosen to investigate the sustained release of admixed proteins. The delivery rate of the loaded proteins could be controlled to a degree by varying the polymer concentration of the implant. However, all the polymer systems exhibited a significant fraction of the loaded proteins being released within the first hour of incubation (i.e., burst release) [47]. The degree of burst release can cause serious side effects in
various applications, but the possibility of creating systems for the controlled delivery of biomacromolecules has led to the development of many biomaterials (both solid and viscoelastic materials) to better control the release rates of a variety of biomacromolecules.

1.3.1. Solid Materials

A number of mechanically rigid growth factor delivery systems, such as bioactive glasses and bioactive ceramics, have been developed for therapeutic applications. Bioactive glasses and ceramics possess common characteristics such as a high porosity with high interconnectivity and the time-dependent kinetic modification of the surface. Their reactive surfaces allow these delivery systems to bind with tissues [48, 49].

1.3.1.1. Mesoporous Bioactive Glass

Mesoporous bioactive glasses have been investigated as growth factor delivery systems. They are silicon dioxide-based and calcium oxide-based materials, many of which the FDA has approved for biomedical applications [49]. These glasses represent a group of surface-reactive biomaterials known for their ability to interact with the physiological environment. The glass network is disrupted through hydrolysis of the Si-O-Si bridges, converting silicon dioxide into silanol and subsequently, converting the glassy material into a gel-like material. Furthermore, calcium phosphate can deposit onto the gel and can mineralize to resemble natural bone [49]. For these reasons, bioactive glasses have been applied to bone regeneration. To accelerate bone healing, growth factors can be loaded into and delivered from a bioactive glass.

Mesoporous bioactive glass with transforming growth factor beta (TGF-β) added during its processing was evaluated for its potential to deliver bioactive concentrations of the growth factor [50]. This growth factor was chosen for its biological functions in tissue regeneration and its ability to tolerate acidic reaction conditions. The release profiles of TGF-β show a rapid release in the first
three hours of incubation and 0.5% of the loaded 500 ng was released in the first week. In addition, the released TGF-β retained its bioactivity [50]. However, it is not clear if the harsh synthesis conditions led to the denaturation of the majority of the growth factor and is the reason a low release rate of TGF-β is achieved.

One promising strategy to prevent the denaturation of growth factors is to decouple the loading of a growth factor from the synthesis of the bioactive glass. In this method, the growth factor would be loaded into the carrier postsynthesis through physical adsorption. Incorporation of VEGF into a mesoporous bioactive glass material through physical adsorption was investigated for its potential to enhance the vascularization of bone tissue in tissue regeneration applications [51]. Decoupling these steps allowed the carrier to be synthesized at harsh reaction conditions (e.g., acidic conditions and long incubation times at extremely high temperatures) without denaturing the growth factor. Postsynthesis of the carrier, VEGF could be adsorbed into the bioactive glass with a loading efficiency of 95% [51]. The high loading efficiency can be attributed to the mesoporous structure and the formation of hydrogen bonds and electrostatic interactions between VEGF and silanol. The interactions led to a lower burst release and a more sustained release of VEGF from the bioactive glass [51].

While bioactive glass can be used as a carrier for growth factor delivery in tissue regeneration, the range of therapeutic applications is limited. Bioactive glass can bind to both soft and hard tissues [52]. However, the mineralization and osteogenesis of the bioactive glass makes it suitable for only bone regeneration applications.

1.3.1.2. Bioceramics and Bioceramic Cements

Bioceramics and bioceramic cements also have been investigated for the delivery of growth factors to stimulate angiogenesis and promote wound healing. Both are calcium phosphate-based materials and are inherently osteoconductive [53]. Bioceramic cements have advantages over
traditional bioceramics because, unlike bioceramics that are preformed solids, cements are self-setting at low temperatures and form a highly interconnected, microporous structure in *in vivo* conditions [54]. In addition, the ability of bioceramic cements to set *in vivo* also permits the injection of the liquid formulation to fill defects of complex geometries [54]. Because the cement solution (*e.g.*, a putty or a paste) is easily handled and sets at physiological temperature, growth factors and/or other biomolecules can be readily added to the solution to incorporate them into the resulting scaffold [55-58]. With this approach, the growth factors can be loaded into the scaffold with high efficiency without the risk of denaturation [59]. However, the mechanical properties of bioceramic cements are poor [58, 60]. In particular, the low mechanical performance limits the application of bioceramic cements to no-load bearing situations [61]. To increase the mechanical strength of bioceramic cements, changes in the chemical composition of reactants and/or the presence of nucleating agents are needed to tune the porosity and subsequently, the mechanical properties [54]. However, these changes will alter the release kinetics of the loaded growth factors, making the selection of optimal scaffold properties difficult. In addition, like bioactive glass, bioceramics and bioceramic cement are only feasible for use in hard tissues due to its stiffness. The limitations of rigid scaffolds have prompted the investigation of soft materials whose mechanical properties are easily controlled and can be applied to all tissue types.

1.3.2. Hydrogels

Hydrogels are three-dimensional networks comprising crosslinked hydrophilic polymer chains. The hydrophilic property of hydrogels allows them to absorb a relatively large volume of water [62]. As a result, they closely resemble native tissue and are more biocompatible than rigid systems [62]. Hydrogels can be synthesized from a wide variety of materials. Broadly speaking, these monomeric or oligomeric materials can be natural or synthetic. Natural materials are derived from a variety of biological sources. For example, collagen [63-65] and its derivative gelatin [66-
69], hyaluronic acid [70-73], chitosan [74-78], alginate [79-85], fibrin [86-89], and dextran [90-93] are biologically derived materials that have been used as hydrogels for the delivery of growth factors. Collagen and gelatin are derived from cartilage and other tissues [94]; hyaluronic acid can be derived from rooster combs and umbilical cords [95]; chitosan is synthesized through deacetylation of the chitin from fungi and the shells of crustaceans [96, 97]; alginate is extracted from algae [98]; fibrin is obtained through the fibrinogen in plasma [94]; and dextran is synthesized from sucrose [96]. The benefit of using biological materials in hydrogels for tissue regeneration applications is that they are naturally biodegradable and the byproducts are well metabolized [99]. This property allows the hydrogel to serve as a provisional scaffold so the regenerating tissue can replace the hydrogel as the hydrogel degrades. In early 2000, the FDA approved absorbable collagen sponges loaded with bone morphogenetic protein 2 (BMP-2) as an alternative to bone grafts for the clinical treatment of spinal fusion and long bone fractures [59]. The use of loaded collagen sponges for these applications allows for a local delivery of BMP-2 and eliminates the need of autologous bone harvesting, which is commonly associated with donor site morbidity and postoperative pain [59].

However, there are many disadvantages in using natural materials to develop growth factor delivery systems. One disadvantage is that natural materials are extracted from biological sources. Little can be obtained from one source and there are batch-to-batch variations among lots of the same material [100, 101]. In addition, biologically derived materials typically have poor mechanical properties and degrade too quickly to be of appreciable use [100-102]. Lastly, materials derived from animal tissue pose risks of immunogenic reactions and transmission of infectious agents [59, 97, 103, 104].

Synthetic polymers, on the other hand, are well-defined materials and are typically inert [105]. Examples of synthetic polymers used in the development of hydrogels for growth factor delivery include poly(hydroxyethyl methacrylate) [106-110], poly(vinyl alcohol) [111-114],
poly(acrylic acid) [115-117], polyacrylamide [118], and poly(isopropylacrylamide) [119-123], poly(ethylene oxide) and poly(ethylene glycol) [124-130], and poly(lactic-co-glycolic acid) [69, 111, 131, 132]. Because there is little to no batch variation with synthetic materials, the hydrogels formed from these materials have predictable physicochemical properties and the composition, degradation rate, and mechanical and chemical properties are easily tailored by altering the polymer and its concentration [133].

Hydrogels can be formed through chemical or physical crosslinking of the polymers. Chemical means of crosslinking polymers include the use of free radicals and reactions among complementary chemical groups. The first utilizes a free radical to chain polymers bearing free vinyl groups together. Free radical polymerization can be achieved through the use of a redox reaction of an initiator-catalyst pair [134], photoinitiators with ultraviolet light [135, 136], or high-energy irradiation [137]. These polymerization schemes are efficient and form hydrogels quickly in mild condition [138, 139]. Growth factors can be loaded into the hydrogel by admixing the growth factors with the prepolymer solution, which is then followed by free radical polymerization of the prepolymer solution. However, unreacted products have to be removed from the hydrogel before its use in biomedical applications [140]. While this can be achieved by washing the hydrogel to remove the unwanted chemical specials, the desired growth factors would elute the hydrogel, resulting in a lower concentration of growth factors within the hydrogel and possibly a lower therapeutic benefit [65]. In addition, the free radicals formed during polymerization are highly reactive and often will damage the growth factors [141], though some proteins are more resistant to oxidative damage than other proteins [142].

In addition to free radical polymerization, reactions among complementary functional groups or with small molecules (e.g., glutaraldehyde to crosslink polymers bearing hydroxyl groups) can be used. However, unlike with free radical polymerization, reaction conditions such as a low pH or a high temperature, or solvents are needed to synthesize the hydrogels [143]. These
conditions will denature any growth factor loaded in the prepolymer solutions. Furthermore, molecules like glutaraldehyde are toxic, even at low concentrations, making them undesirable for use in biomedical applications [144-146].

Because of the harsh synthesis and denaturing conditions found in the covalent crosslinking of polymers, physical crosslinking methods that allow the growth factors to be loaded into the hydrogel in mild reaction conditions have been explored to maintain the activity of growth factors. The many methods of physically crosslinking polymers to form hydrogels include ionic interactions, electrostatic interactions, hydrogen bonds, hydrophobic interactions, protein interactions, or a combination thereof. Ionic interactions utilize free divalent cations to crosslink polyanions. One well-known example is the crosslinking of alginate, a copolymer of mannuronic and glucuronic acid residues that can be crosslinked with calcium ions at standard conditions [147]. Electrostatic interactions between polycations and polyanions polymer have been exploited for the development of stratified hydrogels [148-151]. This is achieved by alternating the deposition of oppositely charged polymers through the immersion of a substrate into polyelectrolyte solutions [152]. Hydrogels formed through hydrogen bonds utilize the interactions between hydrogen donors and hydrogen acceptors. Hydrogels created through hydrogen bonds with polyacids [153, 154], peptides the formation of β-sheets [155-157], and DNA through the hybridization of complementary oligonucleotides [158-160] have been explored as protein delivery systems. Due to the weak hydrogen bonds and the subsequent weak shear strength of the formed hydrogels, these growth factor delivery systems can be used as injectable materials [161]. Hydrophobic interactions utilize the aggregation of hydrophobic blocks on polymer chains to form micellular domains. These micelles assume the role of a crosslinker and can form a hydrogel for growth factor delivery [162, 163]. Unlike in chemical crosslinking to form hydrogels, many physical hydrogels are easier to synthesize and do not require postprocessing to remove toxic molecules. However, using physical hydrogels for growth factor delivery presents many challenges, most notably, physical hydrogels
have poor mechanical integrity that leads to the rapid release of loaded growth factors with little means to regulate release kinetics [161].

1.3.3. Regulated Delivery of Growth Factors

The delivery of growth factors from hydrogels has been mediated primarily through the diffusion of physically entrapped growth factors through the hydrogel. However, the rapid release of loaded growth factors because of the high water content and the high permeability of the hydrogel is one of the major limitations facing growth factor delivery systems. To control the release rate of the growth factor, the physical properties (e.g., pore size) of the hydrogel can be tuned. However, tuning the release kinetics may compromise the physical properties of the hydrogel such as decreasing the matrix hydrophilicity and decreasing the loading capacity for the growth factor. Despite being able to slow the release kinetics of the growth factors or other biomacromolecules to some degree, the release kinetics often remain too fast (e.g., large burst release or premature depletion of loaded growth factors) and/or lack regulatory control after implantation [75, 125, 127, 164-173]. To increase the utility of hydrogels, functionalization is needed to impart new properties to the hydrogel to better control the release of growth factors. Hydrogel functionalization is the addition of chemical moieties or ligands into the polymer network a hydrogel to impart properties of the additive to the hydrogel. Such modifications can be addition of affinity ligands to slow the release of growth factors further and/or reactive elements to accelerate growth factor delivery.

1.3.3.1. Affinity Hydrogels

Many hydrogels are inert and do not interact with the loaded growth factors, leading to a delivery mechanism based solely on diffusion. Affinity hydrogels exploit molecular recognition properties between pairs of chemical groups or between ligands and their binding cognates to slow
the release of growth factors. In these systems, one of the binding partners is covalently immobilized on the hydrogel that can interact with the corresponding chemical group or the binding site of the diffusible growth factor. Such strategies can be achieved by functionalizing the hydrogel with charged monomers, metal ions, heparin, and peptides.

By introducing electrostatic interactions through modifying the polymer backbone or the pendant groups on the backbone, weak interactions between chemical groups of the hydrogel and complementary chemical groups on the growth factor can be utilized to slow the release of loaded growth factors. For example, increasing the methacrylic acid content in chemically crosslinked dextran hydrogels showed responses in the release kinetics of variously charged proteins [174]. Charged proteins could be strongly retained within the hydrogel through oppositely charged electrostatic interactions if the charge density is sufficiently high [174]. Conversely, the release rate of a protein can be significantly increased if the protein is loaded into a hydrogel bearing a charge similar to the protein [174].

Metal ions also can be used to control the release of growth factors by forming coordination complexes with the growth factor [175, 176] (Figure 1.2) with bond strengths that can reach 400 pN as determined by single-molecule atomic force microscopy [177]. Modulation of protein release kinetics can be achieved by varying the species of the divalent metal ion and/or its concentration [175]. For example, replacing low-affinity nickel ions with higher-affinity copper ions can decrease the release rate of growth factors [175].

Hydrogels can be functionalized with heparin to slow the release of growth factors. Biologically, heparin binds directly to more than 50 growth factors [178], protects them from denaturation and degradation [179-184], and activates them by increasing their affinity for their receptors [185]. Furthermore, heparin acts as a storage depot for proteases in mast cell granules [186, 187] and can enhance the binding of growth factors to the extracellular matrix through the catalytic activation of fibronectin [188]. Because of its natural ability to bind to growth factors,
heparin is a logical reagent to functionalized biomaterials to control the delivery of growth factors [130, 189-193].

Affinity peptides that bind various domains or binding sites of growth factors also are useful in slowing growth factors release [129, 194-197]. Many of the peptides developed for controlled growth factor release are derived from the receptors of the respective growth factor [198]. Therefore, unlike the affinity hydrogels mentioned previously, affinity peptide-functionalized hydrogels are able to bind growth factors specifically [194]. In addition, the affinity peptide-functionalized hydrogels are highly customizable and versatile systems, for the release rate of the binding growth factor can be tailored by altering the sequence/architecture of the affinity peptide, the valency of the peptide, and the ratio of the peptide to the growth factor [129].

Despite the ability of these affinity hydrogels to slow the release of growth factors, there are many issues concerning the use of the various strategies mentioned. While the method of utilizing electrostatic interactions to slow protein release certainly works in low ionic strength solutions, it does not translate easily to physiological systems where the ionic strengths are higher. The higher ionic strength of physiological systems would weaken the interactions between the polymer and the protein. Therefore, it would be necessary to greatly increase the charge density of the polymer to compensate for the altered release kinetics [174], but this also may increase the inflammatory response to the hydrogel in \textit{in vivo} applications [199, 200]. Controlled growth factor delivery based on the formation of coordination complexes between divalent metal ions and the growth factor requires that the growth factor be labeled with a polyhistidine tag [175, 176], which may influence the bioactivity of the growth factor. Furthermore, metal ions can be toxic \textit{in vivo} [201-208]. Though heparin is widely investigated for the controlled delivery of numerous growth factors and other cationic biomacromolecules, numerous issues remain concerning the use of heparin. Purification of heparin is difficult and time-consuming [196]. Moreover, heparin is inherently heterogeneous with a varying number of saccharides and sulfation pattern, which makes
Figure 1.2. Schematic of protein binding to the hydrogel through metal ions. The protein is chemically modified with a polyhistidine tag that allows it to interact with hydrogels through coordination with metal ions (e.g., nickel) and a chelator, such as nitrilotriacetic acid.
heparin difficult to reproduce and use [197]. To control the delivery of growth factors, an excessive amount of heparin is needed for binding [209], which increases the risk of thrombocytopenia and other physiological complications [210, 211]. Moreover, varying the release rates among multiple growth factors that have similar isoelectric points remains challenging because heparin binds growth factors nonspecifically. To overcome these challenges, new affinity ligands that can bind growth factors specifically and are nontoxic and nonimmunogenic should be explored. Affinity peptides can address these challenges, but there is no mechanism by which the release of growth factors can be accelerated on demand to meet the requirements of the body. To control the delivery of growth factors postimplantation, hydrogels that respond to stimuli have been investigated.

1.3.3.2. Responsive Hydrogels

There have been many advances in developing hydrogels with biologically responsive feedback mechanisms or with user-controlled properties to regulate growth factor delivery. These responsive hydrogels change their mechanical properties in response to stimuli such as temperature, pH, and light.

Thermoresponsive hydrogels that change their mechanical properties in response to the application of heat are useful carriers for growth factors [123, 212-218]. The most commonly investigated thermosensitive materials are poly(N-isopropylacrylamide) and poly(lactic acid)/poly(ethylene glycol) block copolymers, which can have a lower critical solution temperature that is below body temperature and will exhibit a solution-to-gel (sol-gel) transition in physiological conditions [6, 219, 220]. This allows growth factors to be admixed with a prepolymer solution and upon increasing the temperature of the environment to above the lower critical solution temperature, to become physically entrapped in the hydrogel [220]. In addition, the in situ formation of the hydrogel is minimally invasive and allows the implant to fill voids of complex geometries. Other thermosensitive hydrogel systems have been developed that exhibits a gel-sol
transition upon the addition of heat [221, 222]. However, the need of an external heat source to activate growth factor delivery thermally may not be feasible for *in vivo* applications because of the risk of damaging tissues thermally. Therefore, alternative methods such as using the dissipative properties of a hydrogel as an internal heat source [223] or other stimuli to accelerate growth factor delivery have been explored.

Hydrogels that respond to changes in local pH can be valuable in the treatment of diseases resulting in areas of acidosis, such as infections and ischemia [122, 224]. Carboxylic acid-derived monomers are commonly used to create pH-responsive hydrogels for protein delivery [116, 122, 225]. In most of these systems, the hydrogels are stable at physiological pH, but protonation of the polymer side chains due to acidification destabilizes the hydrogel, leading to growth factor release [6]. Poly(acrylic acid) and poly(methacrylic acid) hydrogels have a pKₐ of approximately 4.28 [226] and 5 [227], respectively. However, these low pKₐ values limit the responsiveness of these hydrogels to low pH systems like the stomach, where the pH of gastric acid is approximately two [228]. Increases of the pKₐ of the hydrogels can be achieved through the addition of propylacrylic acid into the polymer [153]. This allows phase transitions to occur at pH values 6 or greater [229]. Hydrogels based on ureido-pyrimidinone and poly(ethylene glycol) exist in solution form at pH ≥ 8.5 and exhibit a sol-gel transition at physiological pH [230]. This transition has been used to create a hepatocyte growth factor and an insulin-like growth factor delivery system for the treatment of ischemic heart disease in pigs [230].

By combining pH-responsive and temperature-elements into a single delivery system, polymer solutions that are in a liquid state at physiological pH and room temperature can undergo a sol-gel transformation in low pH, high temperature environments (*e.g.*, ischemic tissue) [122]. Furthermore, as the damage is healed and the environment is restored to physiological pH, the delivery system will undergo gradual dissolution, eliminating the need to excise the hydrogel [122].
Temperature and pH are two example stimuli that could be used to regulate the delivery of growth factors. Other examples of stimuli that can alter hydrogel properties include electric fields [231-236], magnetic fields [237-241], and ultrasound [242]. While their proof-of-concept has been demonstrated in vitro, it is difficult to translate these mechanisms to trigger growth factor release in in vivo applications [6]. Moreover, the mechanisms rely on changing the bulk properties of the hydrogels to release the loaded growth factor. That is, they do not trigger the delivery of growth factors specifically. If more than one growth factor were loaded into the hydrogel, then these mechanisms would trigger the delivery of all the growth factors. Therefore, it is difficult to achieve the delivery of multiple growth factors with individualized release kinetics.

To trigger the delivery of multiple growth factors individually, hydrogels functionalized with photocleavable linkages have been explored [243]. By incorporating various wavelength-selective units into the systems, lasers can be used to cleave certain linkages. For example, isoforms of bone morphogenetic proteins, BMP-2 and BMP-7, were covalently bonded to a hydrogel network through photocleavable units based on nitrobenzyl ether and coumarin methylester, respectively. The nitrobenzyl ether unit degrades upon exposure to light of 365 nm while the coumarin methylester unit degrades at 405 nm. Selective and sequential release of the growth factors could be achieved by alternating the light from one wavelength to the other. The release kinetics of each growth factor could be regulated through manipulation of the wavelength, the intensity of the light, the light exposure time, and when the light is applied [243]. Such a method could prove important in the development of programmable delivery systems of multiple growth factors. However, the use of light to trigger growth factor release in vivo will be difficult to achieve because light does not penetrate tissue easily, especially at low wavelengths and intensities [244-246].

Though significant advancements have been made in the development of controlled growth factor delivery systems (e.g., affinity and responsive hydrogels), the current systems have many
shortcomings. Many are unable to slow and sustain the release of growth factors (*i.e.*, large burst release) and many are unable to control the delivery of multiple growth factors with release rates that are regulated individually. Lastly, the release rates of growth factors cannot be tuned once implanted; that is, the release kinetics are preprogrammed by the mechanical properties of the hydrogel (*e.g.*, pore size). The ability to regulate the release of growth factors postimplantation is desirable for personalized medicine, in which the release of therapeutics can be adjusted in accordance with the progress of treatment. To create a release system capable of delivering multiple growth factors with individualized release kinetics and of responding to a stimulus, new affinity ligands should be investigated. These would bind to the growth factors with a high binding affinity and specificity, and the binding functionality can be reversed by a stimulus. To control the delivery of growth factors from a hydrogel, nucleic acid aptamers can be used to functionalize hydrogels.

1.4. Nucleic Acid Aptamers

Nucleic acid aptamers are a unique class of oligonucleotides. They are synthetic RNA or DNA that fold into well-defined nanostructures. Such nanostructures include stems, loops, bulges, hairpins, pseudoknots, and G-quadruplexes [247, 248]. They are unlike other oligonucleotides (*e.g.*, natural DNA that encodes genetic information) because these nanostructures bind a cognate partner with a high affinity and specificity. For example, an RNA aptamer screened against theophylline binds theophylline with an equilibrium dissociation constant (*K*<sub>d</sub>) of ~0.1 µM [249]. In contrast, this aptamer binds caffeine, which differs structurally by one methyl group, with a binding affinity that is ~10,000-fold less [249]. The high binding affinity and specificity of nucleic acid aptamers for their targets rivals that of antibodies for their antigens and thus, have been regarded as synthetic antibodies [250]. However, unlike antibodies, aptamers are robust biomolecules that can withstand harsh chemical and thermal conditions and therefore are amenable to a wide array of chemical reactions and environments [251]. This allows aptamers to be used in numerous applications to
achieve unique goals. Two stages are used to identify an aptamer for a target. The first is upstream selection to identify binding aptamers, and the second is downstream truncation to optimize the binding affinity of an aptamer to its target.

1.4.1. Upstream Selection

Nucleic acid aptamers are screened against a target through an iterative process called the systematic evolution of ligands through exponential enrichment (SELEX) [252, 253] (Figure 1.3). Unlike identifying antibodies for an antigen, aptamer screening occurs in vitro. One advantage of using an in vitro selection process over an in vivo method is that a wider variety of targets can be screened (e.g., toxins that cannot be tolerated by the host during in vivo selection) [254-256]. The SELEX process begins with incubating a library of randomized RNA or DNA sequences with the target in controlled binding conditions. A typical library consists of $10^{14}$-$10^{15}$ oligonucleotides, with each oligonucleotide composed of 80-100 bases [257]. Molecular recognition through hydrophobic interactions, hydrogen bonds, van der Waals and electrostatic interactions, pi stacking, and steric hindrances, govern the association of the target with the oligonucleotides of the library [258, 259]. Oligonucleotides with a high affinity will associate with the target whereas oligonucleotides with a low binding affinity will remain unbound and are removed from the library pool. After partitioning the unbound oligonucleotides, the bound oligonucleotides are dissociated from the target and amplified to create a second library pool. The processes of incubation, partition, elution, and amplification are repeated for several cycles. After several iterations, oligonucleotides of high affinity for the target will be isolated and then will be called aptamers. Through SELEX, aptamers have been identified for a wide range of targets including small molecules (e.g., metal ions [260-263], dyes [252, 264], biochemicals [265-268], and molecular drugs [249, 269-274]), amino acids [275-278] and proteins (e.g., growth factors [279-282], cell receptors [283-286], enzymes
Figure 1.3. Aptamer selection through the SELEX process. An initial library of $10^{14}$-$10^{15}$ oligonucleotides is incubated with the target molecule. After association, nonbinding oligonucleotides are removed from the library and binding oligonucleotides are eluted from the target molecule. Isolated oligonucleotides are amplified through PCR to create an enriched library. The SELEX process is repeated using the enriched library for several iterations with increasing stringency until oligonucleotides that bind the target with a high affinity are found.
[253, 287], and others [288, 289]), and other targets (e.g., Escherichia coli [290] and viruses [291-293]).

Since the introduction of the SELEX process, many modifications and improvements were implemented to identify aptamers of greater stability, greater specificity, or to increase the ease of isolating an aptamer. Like growth factors and other biomacromolecules, aptamers have short in vivo half-lives of only a few minutes, due to enzymatic degradation and renal filtration [294]. However, unlike other biomacromolecules, the chemical properties of aptamers can be modified without the aptamer losing its functionality. To increase the therapeutic potential of aptamers, libraries of modified oligonucleotides with increased endonuclease resistance have been used in SELEX to yield aptamers that are more durable. Any modification of oligonucleotides must be chosen with care so they are still recognized by the polymerases during the amplification step of SELEX. Substitutions in RNA libraries of 2'-hydroxypirimidines with 2'-aminopyrimidines [279, 295-298] or 2'-fluoropyrimidines [297, 299] have been reported to be compatible with polymerases and the resulting aptamers have increased endonuclease resistance. In addition, substitutions of the phosphate backbone with phosphorothioate prior to SELEX can yield aptamers with increased endonuclease resistance [300]. It is possible to screen aptamers from a pool of unmodified oligonucleotides and introduce chemical groups that confer endonuclease resistance post-SELEX (e.g., 2'-methoxypurines in RNA aptamers), but this may decrease the binding affinity of the aptamer for its target [299]. Capping strategies (modifications of the 3’ or 5’ positions after identifying an aptamer through SELEX) have also been investigated to increase in vivo half-lives by increasing exonuclease resistance and/or decreasing renal clearance [301-303].

1.4.2. Downstream Truncation

Aptamers screened from SELEX are typically composed of 80-100 nucleotides [257]. However, not all the nucleotides contribute to the high binding affinity for the target. An aptamer
can be divided into three regions: a region of essential nucleotides, supporting nucleotides, and nonessential nucleotides. The essential region is composed of nucleotides that are directly involved in the interaction of the aptamer with the target. Any base substitutions or removal of a base in this region will result in a significant loss of binding affinity. The second region comprises supporting nucleotides that indirectly contribute to the binding affinity by stabilizing the secondary structure of the aptamer. Stabilization occurs through the formation of stems by intramolecular base pairing of complementary nucleotides. Base substitutions or decreases in the stem length result in modest decreases of the binding affinity. The third region is the nonessential region and is composed of nucleotides that can be substituted or removed without a loss in the binding affinity for the target. It typically is composed of the primers used in polymerase chain reaction for aptamer amplification during SELEX and nucleotides that do not participate in intra- or intermolecular binding. Because the nonessential nucleotides are irrelevant in aptamer binding, they often are removed from the aptamer. Truncation of an aptamer is desirable because shortened aptamers are easier and less expensive to synthesize. While the chemical synthesis process is well established, sequences of greater than 60 nucleotides or with complex secondary structures (e.g., G-quadruplexes) are difficult to synthesize. In addition, shortened aptamers often have increased binding affinities in comparison to the original full-length oligonucleotide because the nonessential region can destabilize the aptamer structure or interfere with aptamer-target association. Methods to identify the essential nucleotides include sequence alignments, in silico comparisons [304-307], chemical synthesis of shorter sequences [308], enzymatic footprinting [309, 310], and partial fragmentation [281] of the full-length aptamers.

1.4.3. Aptamer Applications

The ability of aptamers to bind their targets strongly and selectively have led to the use of aptamers in a number of applications including therapeutics, purification and biotechnology, and
diagnostics and biosensing [311]. Aptamers can fold into branches or arms, creating extensions that can fit into pockets of macromolecules or allow the macromolecule to bind, resulting in tight and specific binding [311]. The high binding affinity of aptamers for their targets has shown them to be capable of preventing the binding of the target for its natural receptor, giving rise to using aptamers for therapeutic applications. Theoretically, antagonist aptamers with a high binding affinity for their target molecule should allow for dosing at submicromolar levels. Such a concentration would lessen side effects by limiting nonspecific activity [312]. In addition, aptamers are nonimmunogenic and are better tolerated than antibody-based therapeutics [312]. An aptamer selected against factor IXa was selected to selectively block the conversion of factor X to its activated form [313], a prerequisite in the generation of thrombin and fibrin and subsequent formation of fibrin clots. Therefore, the aptamer has a possible application as an anticoagulant. The aptamer was able to increase the activated partial thromboplastin time in human plasma in a dose-dependent manner to that of plasma deficient of factor IX [313]. Excessive prevention of coagulation can result in hemorrhages, a major cause of morbidity associated with anticoagulation therapy [314-316]. Therefore, the strict control of anticoagulation is necessary for safe therapy. To this end, an oligonucleotide complementing the aptamer was developed that could hybridize with the aptamer to neutralize it [313]. Administration of both the aptamer and its complementary oligonucleotide in clinical trials revealed that both the drug and its antidote were well tolerated, with dose-dependent control of anticoagulation and reversal of anticoagulation, and stands to replace poorly controlled unfractionated heparin as the standard of care [317-321]. Numerous other aptamer therapeutics have been tested recently in clinical trials [322-329]. To date, the FDA has approved one aptamer for therapy [330], an aptamer targeting VEGF for the treatment of age-related macular degeneration [299]. Clearly, aptamers can be useful therapeutic agents.

Because they are amenable to functionalization, aptamers are useful as the immobilized ligands in affinity chromatography to concentrate a substance from a biochemical mixture. For
example, a human L-selectin aptamer was used as the initial purification step to purify L-selectin from Chinese hamster ovary cell-conditioned medium with a 1500-fold purification and an 83% single step recovery [331]. In addition, aptamers can bind to the natural form of the desired molecule. Therefore, modifications with glutathione S-transferase or polyhistidine tags that could affect the bioactivity of the molecule are no longer necessary to mediate binding [311].

Aptamers also are reagents in diagnostic applications. While antibodies have high binding affinities for their antigen, it is too difficult to raise antibodies against toxins or drug molecules (e.g., cocaine), which limits the feasibility of using antibodies to develop high-affinity sensors for the detection of toxins or drugs. In principle, aptamers can be selected for any target, such as toxins and small molecule drugs, and the resulting aptamers are expected to have a high binding affinity. Thus, sensors utilizing aptamers, also known as aptasensors, can be developed to detect a wider variety of targets. An aptamer can be used as the recognition element in aptasensors in various modes. In one method, analytes bind to immobilized aptamers on the sensor surface and the accumulation of mass can be detected by monitoring changes in surface plasmon resonance or the frequency of a quartz resonator [332-334]. The second method relies on ligand-induced conformation changes of the aptamer structure to produce a signal. These “aptamer beacons” utilize fluorescence quenching or fluorescence resonance energy transfer (FRET) that changes in response to target binding as their output signal [311, 335-337]. Other modes of analyte detection include the assembly or disassembly of gold nanoparticle aggregates [338] or hydrogels [158, 339] upon the target binding to the aptamer. Because aptamers are able to detect low concentrations of analytes and to incorporate reporters, aptasensors are gaining interest for use in food safety control, environmental pollution monitoring, and chemical threat detection applications.
1.5. Objectives

Nucleic acid aptamers are synthetic oligonucleotides that can bind target molecules with high affinity and specificity. Moreover, they are amenable to conjugation to various substrates for many applications. For these reasons, aptamers are promising ligands that can be used to functionalize biomaterials for the controlled delivery of growth factors. It is expected that the high binding affinity of the aptamer will slowly release the growth factor from the biomaterials. Moreover, as the binding affinity and selectivity of the aptamers is rooted in their sequence and secondary structure, unique complementary oligonucleotides could be used as molecular competitors to regulate the binding between the growth factor and the aptamer. To develop aptamer-functionalized growth factor delivery systems, the molecular interactions among aptamers, target growth factors, and complementary oligonucleotides were first studied. Based on the interactions between these cognate biomacromolecules, hydrogels were then functionalized with aptamers and evaluated for their ability to bind and retain the growth factors within the hydrogel. Finally, the ability of the functionalized systems to slow the delivery of growth factors and for complementary oligonucleotides to control aptamer activity was investigated for the programmable release of growth factors.
Chapter 2

Molecular Interactions between Nucleic Acid Aptamers and Growth Factors
and Complementary Oligonucleotides

2.1. Introduction

Many delivery systems have been created to control the release of growth factors or other proteins into tissues for the treatment of various diseases. Hydrogels created from polymers such as polypeptides, polysaccharides, and oligonucleotides have been created that can store, protect, and release growth factors locally because they are easily synthesized and can have similar mechanical properties to natural tissues [340-343]. Despite the ability of the delivery system to protect growth factors from enzymatic degradation and to release them into the local environment [344], the release of loaded growth factors often remains too fast due to the high permeability of the carrier. The rapid release of growth factors into the local environment can be deleterious in treating the disease, as a high growth factor concentration can cause severe side effects [345]. To slow the release of growth factors, modifications to the hydrogel network can be introduced, such as incorporating charged monomers or biological molecules (e.g., heparin) into the hydrogel to change the physiochemical properties of the hydrogel. Though the electrostatic interactions between the growth factor and the polymer matrix employed by these functionalization strategies can be used to slow the release of a growth factor, the release of the growth factors is not strongly regulated. For example, the release rate of a growth factor from the hydrogel is predetermined by the physiochemical properties of the hydrogels. That is, the release rate of the growth factor from the hydrogel is governed by the attractive forces between the hydrogel and the growth factor, which
is predicated by the isoelectric point of the growth factor and the charge type and charge density in the hydrogel. Because of this, the use of electrostatic interactions does no translate well to the controlled delivery of multiple growth factors. If the electronegativity of multiple growth factors are similar, then the release rate of these growth factors would be similar, which would be problematic if different release rates are desired. In addition, it is difficult to control the release of growth factors once the release of growth factors has begun. This is important to consider in applications needing a programmable mechanism to adjust the delivery of growth factors in accordance with the progression of the disease or treatment or for the pulsatile release of therapeutics. Many hydrogels that react the to the application of heat, electric potentials, magnetic fields, ultrasound, or irradiation to accelerate growth factor delivery have been developed to deliver growth factors upon stimulation [346]. While these strategies have shown promise in triggering the delivery of a growth factor, they lack specificity and cannot regulate the delivery of multiple proteins in distinct stages, which is needed to treat complex diseases [23, 347, 348]. Therefore, new functionalized polymeric delivery systems that can regulate the delivery of multiple growth factors individually are of great interest.

As many functionalization strategies are limited in their ability to regulate the delivery of a variety of growth factors and/or growth factors of similar properties, a new functionalization strategy utilizing nucleic acid aptamers will be investigated. Aptamers are synthetic, high-affinity ligands screened from a library of random oligonucleotide sequences against a desired target [252, 253]. They are partitioned from the library through a cyclic, directed elimination process where oligonucleotides from the library that do not bind or weakly bind to the target are removed with increasing stringency. After several rounds of subdivision, oligonucleotides that bind the target with a high affinity and high specificity are isolated and are called aptamers. Aptamers have several notable properties that make them unlike other ligands. They are tolerant of harsh chemical environments and are amenable to many chemical reactions [158]. Aptamers are also small in size
and present little to no immunogenicity when used *in vivo* [349]. Lastly, the binding function of aptamers can be modulated or inactivated using complementary oligonucleotides, which is difficult to achieve with other ligands [350, 351].

It is not well understood how to utilize nucleic acid aptamers as effectors for the controlled release of growth factors. To understand how nucleic acid aptamers can be used to functionalize hydrogels, systematic studies of the molecular interactions between aptamers and various binding partners need to be conducted. In this chapter, the ability of two aptamers to bind their target growth factor will first be characterized. Second, the ability of complementary oligonucleotides to inactivate these aptamers to liberate the bound growth factor will be evaluated. Lastly, modifications of the aptamers and their complementary oligonucleotides will be rationally introduced to enhance intermolecular hybridization and subsequently, aptamer inactivation.
2.2. Experimental Procedure

2.2.1. Materials

Streptavidin-coated polystyrene microparticles (1.3 μm) were purchased from Spherotech (Lake Forest, IL). Phosphate buffered saline (PBS), Tween 20, sodium azide (NaN₃), and a premixed solution of acrylamide and bisacrylamide (40% solution, 29:1 acrylamide:bisacrylamide) were purchased from Fisher Scientific (Suwanee, GA). Biotinylated aptamers and their complementary sequences (CSs) were purchased from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2.1. Bovine serum albumin (BSA) was purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF) and platelet-derived growth factor-BB (PDGF-BB) enzyme-linked immunosorbent assays kits were purchased from PeproTech (Rocky Hill, NJ). The molecular weight of VEGF and PDGF-BB are 38.2 and 24.3 kDa, respectively.
Table 2.1. List of aptamers and their complementary sequences to study aptamer interactions.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' → 3')</th>
<th>Length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AntiVEGF aptamers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apt-V1</td>
<td>CCCGT CTTCG AGACA AGAGT GCAGG G</td>
<td>26</td>
</tr>
<tr>
<td>Apt-V2</td>
<td>A&lt;sub&gt;10&lt;/sub&gt; CCCGT CTTCG AGACA AGAGT GCAGG GTCCA TTCGT C</td>
<td>46</td>
</tr>
<tr>
<td><strong>Complementary sequences to antiVEGF aptamers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS-V1</td>
<td>CCCTG CACTC T</td>
<td>11</td>
</tr>
<tr>
<td>CS-V2</td>
<td>CTCTT GTCTG G</td>
<td>11</td>
</tr>
<tr>
<td>CS-V3</td>
<td>TGGAA GACGG G</td>
<td>11</td>
</tr>
<tr>
<td>CS-V4</td>
<td>CCCTG CACTC TTGTC T</td>
<td>16</td>
</tr>
<tr>
<td>CS-V5</td>
<td>CCCTG CACTC TTGTC TGGAA G</td>
<td>21</td>
</tr>
<tr>
<td>CS-V6</td>
<td>CCCTG CACTC TTGTC TGGAA GACGG G</td>
<td>26</td>
</tr>
<tr>
<td>CS-V7</td>
<td>GACGA ATGGA CCCTG CACTC TTGTC TGGAA GACGG G</td>
<td>36</td>
</tr>
<tr>
<td><strong>AntiPDGF-BB aptamers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apt-P1</td>
<td>ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT G</td>
<td>36</td>
</tr>
<tr>
<td>Apt-P2</td>
<td>ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT GTGAC T</td>
<td>41</td>
</tr>
<tr>
<td>Apt-P3</td>
<td>ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT GTGAC TTGAC C</td>
<td>46</td>
</tr>
<tr>
<td>Apt-P4</td>
<td>A&lt;sub&gt;10&lt;/sub&gt; ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT G</td>
<td>46</td>
</tr>
<tr>
<td>Apt-P5</td>
<td>A&lt;sub&gt;10&lt;/sub&gt; ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT GTGAC T</td>
<td>51</td>
</tr>
<tr>
<td>Apt-P6</td>
<td>A&lt;sub&gt;10&lt;/sub&gt; ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT GTGAC TTGAC C</td>
<td>56</td>
</tr>
<tr>
<td>Apt-P7</td>
<td>GCGAT ACTCC ACAGG CTACG GCACG TAGAG CATCA CCATG ATTCCT G</td>
<td>46</td>
</tr>
<tr>
<td><strong>Complementary sequences to antiPDGF-BB aptamers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS-P1</td>
<td>CAGGA TCATG GTGAT G</td>
<td>16</td>
</tr>
<tr>
<td>CS-P2</td>
<td>GGTGA TGCTC TACGT G</td>
<td>16</td>
</tr>
<tr>
<td>CS-P3</td>
<td>ACGTG CCGTA GCCTG T</td>
<td>16</td>
</tr>
<tr>
<td>CS-P4</td>
<td>GTGAT GCTCT ACGTG CCGTA GCCTG T</td>
<td>26</td>
</tr>
<tr>
<td>CS-P5</td>
<td>CAGGA TCATG GTGAT GCTCT ACGTG CCGTA GCCTG T</td>
<td>36</td>
</tr>
<tr>
<td>CS-P6</td>
<td>AGTCA CAGGA TCATG GTGAT GCTCT ACGTG CCGTA GCCTG T</td>
<td>41</td>
</tr>
<tr>
<td>CS-P7</td>
<td>GCTCA AGTCA CAGGA TCATG GTGAT GCTCT ACGTG CCGTA GCCTG T</td>
<td>46</td>
</tr>
<tr>
<td>CS-P8</td>
<td>CAGCA TCATG GTGAT GCTCT ACGTG CCGTA GCCTG TGGAG TATCG C</td>
<td>46</td>
</tr>
<tr>
<td>CS-PC</td>
<td>ACTGG TGGCC AGTGG CATGG TACTA TGCTA TGCTG TGGCC TGAAG CGGCT C</td>
<td>46</td>
</tr>
</tbody>
</table>

Apt: aptamer; CS: complementary sequence; nt: nucleotides; V: VEGF; P: PDGF-BB.
2.2.2. Methods

2.2.2.1. Preparation of Aptamer-functionalized Particles

One hundred sixty picomoles of biotinylated aptamers were incubated with 80 μg streptavidin-coated microparticles in 46.4 μL PBS for 30 min. The microparticles were washed with PBS four times. Following washing, 4 ng VEGF or PDGF-BB was added to the microparticle suspension and incubated at room temperature for 2 h. An illustration of preparing the microparticles is shown in Figure 2.1. After immobilizing the proteins, 140 μL of PBS was added to the suspension and aliquoted into 50 μL volumes. After incubating the aptamer-functionalized microparticles in the growth factor solution, the amount of growth factor remaining in the supernatant was quantified using a VEGF or PDGF-BB ELISA kit. All samples were performed in triplicate and were doubly referenced by subtracting the optical density of the reference wavelength and of the zero concentration samples.

2.2.2.2. Examination of Aptamer Inactivation

Microparticle suspensions were incubated in 250 μL release medium (0.1 % BSA, 0.05 % Tween 20, and 0.09 % NaN₃ in PBS) at 37 °C with a shaking rate of 70 rpm. At predetermined time points, the release medium was totally collected and replenished with fresh release medium or triggering medium containing 100 nM CSs. Samples were incubated in the triggering medium for 1 h before the solution was collected. Collected supernatants were stored at -20 °C until analysis. Quantification of VEGF and PDGF-BB in the supernatants was performed using VEGF and PDGF-BB ELISA kits following the provided instructions. All samples were performed in triplicate and were doubly referenced by subtracting the optical density of the reference wavelength and of the zero concentration samples.
Figure 2.1. Illustration of binding a growth factor onto the surface of aptamer-functionalized microparticles. A nucleic acid aptamer is immobilized onto streptavidin-coated microparticles through the nearly covalent interactions between biotin and streptavidin, which is followed by binding of a growth factor to the aptamer.
2.2.2.3. Secondary Structure Prediction

The secondary structures of the aptamers were generated by using the program RNAstructure (http://rna.urmc.rochester.edu/rnastructure.html), which is capable of predicting single- and double-stranded RNA or DNA structures [305]. The predicted structures with the lowest free energies were presented.

2.2.2.4. Gel Electrophoresis

Hybridization of aptamers and complementary oligonucleotides was examined using 12% native polyacrylamide gel electrophoresis. Aptamers were mixed with CSs at a 1:2 molar ratio. The polyacrylamide gels were electrophoresed using a Bio-Rad Mini PROTEAN 3 cell equipped with a PowerPac Basic power supply and were stained with ethidium bromide. The stained gels were analyzed using a Bio-Rad GelDoc XR system (Hercules, CA).

2.2.2.5. Flow Cytometry

Microparticles were prepared with the method previously described. To examine the capability of the aptamer in binding CS, 6-FAM-labeled CS-P8 and CS-PC (ex. = 495 nm; em. = 520 nm) and TYE 665-labeled CS-P5 (ex. = 645 nm; em. = 665 nm) were used to treat the microparticles. The FAM and TYE fluorophores were excited with an argon ion blue laser (488 nm) and a HeNe red laser (633 nm), respectively, using a Coulter FC500 (Beckman Coulter, Brea, California). Ten thousand events were collected.

2.2.2.6. Statistics

Quantitative data is presented as the mean ± one standard deviation of triplicate samples. Statistically significance was evaluated using Student’s t-test via MATLAB (v. 7.11.0.584, The Mathworks, Inc., Natick, MA).
2.3. Results

2.3.1. Growth Factors can Bind to Aptamers Immobilized onto Polymeric Microparticles

Two DNA aptamers selected against VEGF [352, 353] and PDGF-BB [281] were used as models in this study. The secondary structure of the antiVEGF aptamer and the antiPDGF-BB aptamer (herein called Apt-V1 and Apt-P1, respectively) as predicted by RNAstructure are shown in Figure 2.2A. Apt-V1 and the Apt-P1 were used to functionalize microparticles that bind VEGF and PDGF-BB, respectively. Both aptamers could be physically incorporated onto the polymeric microparticles through the nearly covalent interaction between the immobilized streptavidin and the biotin end-group on the aptamer. Microparticles functionalized with Apt-V1 were able to decrease the amount of VEGF in the supernatant to 41.3% ± 2.5% of the amount decreased by the nonfunctionalized microparticles (Figure 2.2B). Similarly, the amount of PDGF-BB in solution decreased to 42.2% ± 2.2% of the nonfunctionalized case when the microparticles were functionalized with Apt-P1 (Figure 2.2B). Thus, aptamers can be immobilized onto polymeric microparticles and bind their target growth factors.

2.3.2. Complementary Oligonucleotides of Sufficient Length can Inactivate Aptamers

After showing the aptamers can tether their target growth factor to the surface of polymeric microparticles, the use of complementary sequences (CSs) was systematically investigated for trigger growth factor release via aptamer inactivation. Short CSs that hybridize to various regions of Apt-V1 were chosen to determine if the location of hybridization is a factor in disassembling the growth factor-aptamer complex. Oligonucleotides CS-V1, CS-V2, and CS-V3 are each 11 nucleotides in length. These CSs bind approximately 40% of Apt-V1, and their hybridizing region is shown in Figure 2.3A. The image of the polyacrylamide gel stained with ethidium bromide after electrophoresis shows that every CS can hybridize with Apt-V1, as evidenced by a decrease in
Figure 2.2. Analysis of aptamer functionality. (A) Predicted secondary structure from RNAstructure of the Apt-V1 antiVEGF aptamer (left) and the Apt-P1 antiPDGF-BB aptamer (right). (B) Ability of microparticles functionalized with Apt-V1 to bind VEGF in solution (left) and microparticles functionalized with Apt-P1 to bind PDGF-BB in solution (right). All values are normalized to the respective amount of free growth factor in solution after incubating the growth factor solution with nonfunctionalized microparticles for 1 h. *** denotes a statistical significance of $p \leq 0.001$. 
Figure 2.3. Examination of using short CSs to inactivate Apt-V1 and displace VEGF. (A) Location of hybridization of various CSs with Apt-V1, which is denoted with green, filled circles. (B) Gel electrophoresis of Apt-V1 aptamer hybridization with the short CSs. (C) Apt-V1 inactivation by CSs for VEGF displacement. The microparticle suspensions were treated with 100 nM of the various CSs for 1 h. The amount of free VEGF was normalized to the unbound VEGF of the microparticles not treated with the CS.
the migration distance (Figure 2.3B). Oligonucleotides CS-V1, CS-V2, and CS-V3 bear no intramolecular base pairing and cannot be stained with ethidium bromide. After showing that the CSs can hybridize with Apt-V1, microparticles were functionalized with Apt-V1 and loaded with VEGF to test the ability of the CSs to displace VEGF from the antiVEGF aptamer. Despite the ability of each CSs to hybridize with free Apt-V1, none of microparticles treated with 100 nM of the various CSs exhibits a significant difference from the microparticles that were not treated with a CS (Figure 2.3C). Thus, the results show that a CS of 11 nucleotides in length is unable to trigger VEGF release from microparticles functionalized with Apt-V1, regardless of the location of hybridization.

A similar procedure was applied to investigate the ability of short CSs to triggered PDGF-BB release from microparticles functionalized with Apt-P1. Three CSs that hybridize with various 16-nucleotide regions of Apt-P1 (corresponding to 40% of the aptamer) were chosen: CS-P1, CS-P2, and CS-P3. The hybridization regions of the CS with the Apt-P1 are shown in Figure 2.4A. As shown in the image of the electrophoresed gel, each of these three CSs can hybridize with free Apt-P1, as indicated by the upward shift in the band position (Figure 2.4B). When 100 nM of these CSs were applied to trigger PDGF-BB release from microparticles functionalized with Apt-P1, no statistically significant difference was found between the various samples (Figure 2.4C), indicating that 16-nucleotide CSs are unable to trigger PDGF-BB release from microparticles, regardless of the hybridization region of the aptamer.

As short CSs were unable to trigger VEGF or PDGF-BB release from their respective functionalized microparticles (Figure 2.3 and Figure 2.4), experiments were perform to investigate the systematic increase of hybridization length on triggered growth factor release. Complementary oligonucleotides for Apt-V1 were increased lengthwise in steps of 5 nucleotides, from 11 nucleotides (CS-V1) to 16 nucleotides (CS-V4), 21 nucleotides (CS-V5), and 26 nucleotides (CS-V6) in length. These CSs hybridize with approximately 40%, 60%, 80%, and 100% of Apt-V1.
**Figure 2.4.** Examination of using short CSs to inactivate Apt-P1 and displace PDGF-BB. (A) Location of hybridization of various CSs with Apt-P1, which is denoted with the red, filled circles. (B) Gel electrophoresis of Apt-P1 aptamer hybridization with the short CSs. (C) Apt-P1 inactivation by CSs for VEGF displacement. The microparticle suspensions were treated with 100 nM of the various CSs for 1 h. The amount of free PDGF-BB was normalized to the unbound PDGF-BB of the microparticles not treated with the CS.
respectively (Figure 2.5A). When these sequences were applied to microparticles functionalized with Apt-V1 and loaded with VEGF, only CS-V1 was unable to trigger VEGF release (Figure 2.5A). When the length of the CS increased from 11 nucleotides to 16 nucleotides (increasing the hybridization of the aptamer to 60%), the amount of VEGF in the supernatant increased by 1 order of magnitude. Any further increase in the hybridization length of the aptamer did not result in any further increase of VEGF in the supernatant (Figure 2.5A). A similar procedure was applied to the CSs for Apt-P1. The length of CS-P1 was increased stepwise from 16 nucleotides to 26 nucleotides (CS-P4) and 36 nucleotides (CS-P5). The hybridization length of CS-P1, CS-P4, and CS-P5 corresponds to approximately 40%, 70%, and 100% of Apt-P1 (Figure 2.5B). When 40% of Apt-P1 was hybridized, there was no increase in the amount of PDGF-BB in the supernatant due to the inactivation of the antiPDGF-BB aptamer. When the length of the CS was increased from 16 nucleotides to 26 nucleotides, there was an increase in the amount of PDGF-BB that moved into the supernatant. When the length of the CS was increased to hybridize with 100% of the aptamer by using CS-P5, there was a further increase in the amount of PDGF-BB in the supernatant in comparison to using CS-P4 (Figure 2.5B).

The ability of CSs to trigger growth factor release varies between the VEGF and the PDGF-BB systems. When the microparticles are functionalized with Apt-V1 and loaded with VEGF, a CS that hybridizes with 60% of the aptamer is capable of releasing VEGF 11-fold over the sample receiving no CS treatment and further increases in the hybridization length does not increase the amount of VEGF release. On the other hand, when microparticles functionalized with Apt-P1 and loaded with PDGF-BB are treated with a CS that hybridizes with 70% of the aptamer, there is a 4-fold increase in the amount of PDGF-BB release. In addition, increasing the hybridizing length to 100% of the aptamer length increased the amount of PDGF-BB released to nearly 6-fold. To begin understanding why these two aptamers and their CSs behave differently regarding the ability of CSs to trigger growth factor release, rational sequence modifications of the Apt-P1 and its CSs
Figure 2.5. Inactivation of aptamers and displacement of growth factor by increasing the length of the CS. (A) Apt-V1 inactivation for VEGF release by treating the aptamer with increasing lengths of its CS. (B) Apt-P1 inactivation for PDGF-BB release by treating the aptamer with increasing lengths of its CS. The colored nucleotides denote the location of hybridization for the various CSs. * denotes a statistical significance of $p \leq 0.05$ between the compared data, ** denotes $p \leq 0.01$, and *** denotes $p \leq 0.001$. 
were introduced to improve growth factor binding and aptamer inactivation.

2.3.3. Rational Sequence Design Can Improve Growth Factor Binding and Aptamer Inactivation

To enhance triggered PDGF-BB release through the inactivation of Apt-P1 by hybridization, a linear oligonucleotide tail was added to the aptamer. Five and ten nucleotides were added to the 3’-end of Apt-P1 to create Apt-P2 and Apt-P3, respectively. The addition of these tails is predicted by RNAstructure to not change the secondary structure of Apt-P1 (Figure 2.6A). To test the ability of Apt-P1, Apt-P2, and Apt-P3 to bind PDGF-BB, microparticles were functionalized with the aptamers and incubated in a PDGF-BB solution. As the length of the tail increased, there was a proportional decrease in the ability of the aptamer to bind PDGF-BB (Figure 2.6B). When five nucleotides were added to the 3’-end of the aptamer, twice the concentration of PDGF-BB was found in the supernatant in comparison to the concentration seen when microparticles functionalized with no tail were used. When 10 nucleotides were added as the tail, the concentration of PDGF-BB that did not bind to the aptamer increased three-fold. Despite the addition of either tail to promote hybridization, the ability to an oligonucleotide complementary to the aptamer with the tail to release the bound PDGF-BB was not enhanced (Figure 2.6C).

As the addition of the tail to the 3’-end of the Apt-P1 decreased the binding ability of the aptamer for PDGF-BB, a polyadenosine spacer was added to the 5’-end of the aptamer to distance the aptamer from the surface of the microparticle. The software RNAstructure predicted the secondary structures of the aptamer would not change when the spacer is added (Figure 2.7A). The ability of the aptamer with and without the spacer to bind PDGF-BB was tested by incubating microparticles functionalized with Apt-P1 and Apt-P4 in a PDGF-BB solution. Quantification of the PDGF-BB concentration in the supernatant revealed that these aptamers have an equal ability
Figure 2.6. Influence of a tail on antiPDGF-BB binding and inactivation. (A) Predicted secondary structure of Apt-P1 with various tails added to the 3’-end of the aptamer. (B) The ability of the aptamer to bind PDGF-BB decreased with increasing tail length. The amount of free PDGF-BB in the supernatant was normalized to the amount of PDGF-BB left in the supernatant from the aptamer with no tail. (C) Hybridization of the various aptamers with a fully complementary sequence (i.e., binding to the aptamer and the tail) showed no enhancement in aptamer inactivation. * denotes a statistical significant of \( p \leq 0.05 \) in comparison to any sample.
Figure 2.7. Effect of adding a spacer to the 5’-end of Apt-P1. (A) Predicted secondary structures. The polyadenosine in Apt-P4 is marked red. (B) The addition of the polyadenosine tail does not affect the ability of the antiPDGF-BB aptamer in binding PDGF-BB. (C) No difference is observed in the activation of the aptamer region between Apt-P1 and Apt-P4.
to sequester PDGF-BB from solution (Figure 2.7B). The ability of a CS to trigger PDGF-BB release from the microparticles was also evaluated. Because the role of the spacer is solely to distance the aptamer from the microparticle and not participate in intermolecular hybridization, CS-P5 was chosen to hybridize with both Apt-P1 and Apt-P4. As shown in Figure 2.7C, the amount of PDGF-BB released into the supernatant did not change due to the addition of the spacer.

As the addition of the spacer did not affect the ability of the aptamer to bind PDGF-BB or hybridization between the aptamer and its CS, the effect of adding a spacer and a tail to the aptamer was investigated. RNAstructure predicted the addition of the polyadenosine spacer to the 5’-end and the addition of the 5-nucleotide and 10-nucleotide tail to the 3’-end would not alter the secondary structure of the aptamer (Figure 2.8A). Incubating microparticles functionalized with Apt-P4, Apt-P5, and Apt-P6 in a PDGF-BB solution showed that these aptamers have an equal capability in binding PDGF-BB, indicating that the addition of the tail did not deter PDGF-BB binding (Figure 2.8B). The effect of adding the various tails with the spacer present on triggering PDGF-BB release was also evaluated. In comparison to inactivating Apt-P4 which does not have a tail, hybridization of the aptamer with a 5-nucleotide (Apt-P5 with CS-P6) resulted in only a small increase of PDGF-BB in the supernatant (Figure 2.8C). In comparison to microparticles functionalized with Apt-P4 and receiving no CS treatment, this resulted in nearly a 7-fold increase in the amount of PDGF-BB released. When a 10-nucleotide tail was added to the aptamer (i.e., Apt-P6) and hybridized with CS-P7, there was a significant improvement in the ability of the CS to inactivate the aptamer and free PDGF-BB, as seen from the increase 7-fold of Apt-P5 hybridized with CS-P6, to nearly 10-fold in comparison to the amount of PDGF-BB measured when no CS is applied (Figure 2.8C). Through the addition of the linear spacer and tail to the aptamer, the ability of a CS to hybridize with the aptamer to inactivate its function was improved.

As the addition of a spacer and a tail to the antiPDGF-BB enhanced its hybridization with its CS, a spacer and a tail was added to the antiVEGF aptamer to evaluate any potential increase in
Figure 2.8. The release of PDGF-BB could be further increased when a spacer and a tail was added to the anti-PDGF-BB aptamer and the hybridization length was increased. (A) Predicted secondary structures of polyadenylated anti-PDGF-BB aptamers with various tail lengths. (B) Influence of tail length on PDGF-BB binding to the aptamer. (C) Influence of increasing hybridization length through the addition of a tail on anti-PDGF-BB aptamer inactivation. * denotes a statistical significance of $p \leq 0.05$ between the compared values.
hybridization-based antiVEGF inactivation. The secondary structure of Apt-V1 before and after the addition of the spacer and 10-nucleotide tail at the 5’-end and the 3’-end respectively, is shown in Figure 2.9A. The incorporation of these nucleotides is expected to not alter the architecture of the aptamer. Microparticles functionalized with Apt-V2 showed an ability to bind VEGF in solution seemingly equal to that of microparticles functionalized with Apt-V1 (Figure 2.9B). Specifically, the addition of the spacer did not enhance aptamer binding nor did the tail interfere with binding VEGF. When CS-V7, a CS that hybridizes with the aptamer and the tail, was used to trigger VEGF release from the microparticle, there was no increase in the amount of VEGF released relative to using CS-V6 to trigger VEGF release from the aptamer with no tail added (Figure 2.9C).

In the previous inactivation studies of the antiPDGF-BB aptamer, the roles of the spacer and the tail were delineated as individual segments. The addition of the spacer accommodated the incorporation of the tail for enhanced hybridization (Figure 2.8). To examine the combined roles of the spacer and the tail, one oligonucleotide sequence was added to the 5’-end of the antiPDGF-BB aptamer to function as both (Apt-P7). The secondary structure of the aptamer is shown in Figure 2.10A. Sequestration of PDGF-BB from solution shows that Apt-P7 retains its ability to bind its target when it is immobilized onto polymeric microparticles (Figure 2.10B). To test the ability of the immobilized antiPDGF-BB aptamer to hybridize with CSs, flow cytometry was used. The rightwards shifts of the fluorescence intensity shows that Apt-P7 was able to hybridize with CS-P5 and CS-P8 to fluorescently label the microparticle (Figure 2.10C). In contrast, Apt-P7 would not hybridize with the control CS-PC, which has a randomized nucleotide sequence and no sequence complementarity to Apt-P7. The inset figures show the bulk fluorescence of the microparticle suspension before and after labeling the particles with the fluorescent CSs (Figure 2.10C). After showing that the CSs can hybridize with Apt-P7, the ability of the CSs to hybridize with the aptamer to dissociate bound PDGF-BB was evaluated. The results show that CS-P5, which hybridizes with the aptamer, was less effective in triggering the release of PDGF-BB from the microparticles than
Figure 2.9. Effect of increasing aptamer and hybridization length on antiVEGF aptamer inactivation. (A) Predicted secondary structures of the antiVEGF aptamer before and after adding a spacer and tail. (B) The addition of the spacer and the tail does not effect retention of VEGF on the microparticles. (C) Increasing the hybridization length of the CS through the addition and binding of the tail does not enhance antiVEGF inactivation.
Figure 2.10. Molecular recognition of antiPDGF-BB aptamer. (A) Predicted secondary structure of Apt-P7 aptamer. The combined spacer and tail is denoted with red. (B) PDGF-BB sequestration from the solution onto the surface of microparticles by Apt-P7. (C) Hybridization between Apt-P7 and CS via flow cytometry and fluorescence imaging. CS-P5 (left) and CS-P8 (center) hybridized with Apt-P7 on the surface of microparticles whereas CS-PC (bottom) did not. Inset: bulk fluorescence of microparticle suspension. (D) Dissociation of PDGF-BB from the aptamer-functionalized particles, normalized to No CS. * denotes a statistical significance of $p \leq 0.05$ between the compared data and *** denotes $p \leq 0.001$. 
CS-P8, which hybridizes with the aptamer and the spacer (Figure 2.10D). In contrast, the control CS-PC could not release PDGF-BB from Apt-P7.
2.4. Discussion

In this work, two model aptamers were used to study the molecular interactions between aptamers, their target growth factors, and their CSs. Aptamers are screened from a library of synthetic oligonucleotides comprised of random bases. Despite being comprised of a permutation of five bases (adenine, guanine, cytosine, thymine, and uracil), aptamers can adopt a wide variety of architectures to form unique secondary structures [247, 248]. The antiVEGF used in this work is predicted to be mostly a linear molecule with a stem region comprised of four base pairs and a trinucleotide loop. On the other hand, the antiPDGF-BB aptamer used has a three-way helix junction bearing a trinucleotide loop in the junction (Figure 2.2A). Despite their dissimilar secondary structures, these aptamers bind to their targets with a high affinity. The antiVEGF aptamer and the antiPDGF-BB aptamer are reported to have an equilibrium dissociation constant (K_d) of 0.7 nM and 0.1 nM for their target, respectively [281, 352]. As aptamers are robust molecules, they are amenable to conjugation reactions, such as biotinylation, without losing their function. When the antiVEGF aptamer and the antiPDGF-BB aptamer were immobilized onto the surface of polymeric microparticles, they were able to decrease the solution concentration of their target molecules (Figure 2.2B), indicating that the aptamers were functional and could sequester their targets.

After showing that the microparticles could be functionalized with aptamers and that target growth factors can bind to the aptamer, CSs were characterized to develop a mechanism for the triggered release of growth factors. The triggered release of proteins from a carrier system could have tremendous impact in the treatment of human diseases. For example, most protein delivery systems are not able to control the delivery of proteins once implanted. By creating responsive delivery systems, the release kinetics of the therapeutics can be controlled using a stimulus, such as hybridizing oligonucleotides, to match the progress of disease treatment. A CS comprised of 16 nucleotides binding to the 3’-end of Apt-V1 (CS-V4) was sufficient to trigger a 10-fold release of
VEGF from the aptamer, whereas a full-length CS to Apt-P1 (CP-P5) was able to trigger PDGF-BB release, but at half the efficiency in comparison to CS-V4 triggered VEGF release from Apt-V1 (Figure 2.5). The secondary structure and the degree of intramolecular base pairing may account for the difference seen in the triggering efficiency between these two systems. The secondary structure of aptamers plays an important role in binding its target. Secondary structure features such as bulges and loops create shape-complementarity with the target growth factors, allowing the aptamer to interact with the target through binding pockets [250, 311]. Therefore, eliminating these features will change the binding affinity of the aptamer for the target growth factor, which can be achieved through hybridization of the aptamer. In order for a CS to function as a molecular trigger, the formation of the oligonucleotide duplex needs to be more favorable than the formation or the maintenance of the aptamer-target complex.

To increase the hybridizing strength of the CS with the aptamer, the length of the CS can be increased [354-356]. Therefore, increasing the length of the CS for the antiVEGF aptamer from 11 to 16 nucleotides not only increased the hybridizing strength of the CS, but also allowed the CS to hybridize with the stem of the aptamer, thereby altering the secondary structure and inactivating the aptamer. When the length of the CS for the antiPDGF-BB aptamer was increased, there was a proportional increase in the inactivation of the aptamer. However, the amount of increase was significantly less than the VEGF system. There are many possible reasons for this observed inefficiency. The high degree of intramolecular base pairing within the antiPDGF-BB aptamer makes intermolecular hybridization difficult, as the intermolecular base pairs need to be broken before the CS can hybridize with the aptamer. To increase the hybridization efficiency of the antiPDGF-BB aptamer and its CS, a linear tail and a polyadenosine spacer were added to the 3’-end and the 5’-end, respectively. The tail was added to promote hybridization and the spacer was added to decrease steric hindrance and give the hybridization a more solution-phase behavior [357, 358]. In this study, the role of the spacer and the tail were kept separated to study the effect of their
incorporation individually. Increased hybridization could be achieved with the antiPDGF-BB aptamer by incorporating a linear tail at the end of the aptamer, but only after the distance between the aptamer and the microparticle surface was increased through polyadenylation. Without the spacer, the addition of the tail increased steric effects and decreased the ability of PDGF-BB to bind to the aptamer. The addition of the spacer decreased the steric effects between the aptamer and the growth factor and between the microparticle surface and the CS. The release of PDGF-BB from its aptamer could reach a similar efficiency as triggered VEGF release, but only when the spacer and tail is added. When a spacer and a tail was added to the antiVEGF aptamer, there was no enhancement in VEGF release due to aptamer inactivation, presumably because the antiVEGF aptamer inherently has little resistance to hybridization as the secondary structure is essentially a linear molecule with only a few intramolecular base pairs.

After showing that a spacer and a tail can be incorporated with the antiPDGF-BB aptamer as individual segments, the incorporation of a linear to the antiPDGF-BB aptamer that functions as both a spacer and toehold (Apt-P7) was evaluated for triggered PDGF-BB release. The binding study shows that the aptamer could easily remove PDGF-BB from solution by binding the growth factor to the particle. The efficiency of triggering PDGF-BB release from this aptamer through hybridization was evaluated by treating microparticles with bound PDGF with various CSs. Similar to the previous data (Figure 2.8), a CS that hybridizes with only the aptamer is less efficient in triggering PDGF-BB release from the aptamer in comparison to a CS that hybridizes with the aptamer and the toehold (Figure 2.10). Therefore, the addition of a linear segment to the antiPDGF-BB aptamer can be used as a spacer and as a toehold to facilitate hybridization. In addition, combining the functions of the spacer and the toehold into a single segment is beneficial for a number of other reasons. First, shortening the length of the oligonucleotide will simplify the synthesis of the oligonucleotide and decrease its cost. Second, reducing the number of nucleotides in an aptamer sequence will limit steric hindrance and the formation of metastable secondary
structures, thereby maintaining the high binding affinity of the aptamer for its target. Third, smaller sizes allow for a greater density of aptamers to be immobilized to a substrate [359].
2.5. Conclusions

In this work, the molecular recognition properties of two model aptamers were investigated for their ability to bind their target growth factors and their CSs. Both the antiVEGF and the antiPDGF-BB aptamer could be immobilized to a polymeric support without inactivation of their binding function. A CS that hybridizes with 60% of the antiVEGF aptamer was found sufficient to dissociate bound VEGF from the aptamer. However, a CS that hybridizes with an equal percentage of the antiPDGF-BB aptamer as the antiVEGF aptamer was not able to displace PDGF-BB. To create a CS that was capable of releasing PDGF-BB from the aptamer, the hybridizing strength of the CS was increased by lengthening the oligonucleotide. To prevent steric effects, a spacer was needed to position the aptamer away from the microparticle and allow the aptamer to hybridize with the CS with a more solution-like behavior. In addition, hybridization with a tail or a toehold of the antiPDGF-BB aptamer was able to increase the efficiency of which the CS hybridizes. The characterization of the fundamental interactions between aptamers and their cognate biomolecules will be invaluable for the development of aptamer-functionalized systems for the delivery of growth factors.
Chapter 3
Development of Aptamer-functionalized Systems for the Retention of Growth Factors

3.1. Introduction

The development of growth factors depots that can slowly release growth factors into the target site are promising tools for the treatment of numerous diseases. To this end, hydrogel carriers have been investigated for the controlled release of growth factors. Hydrogels can be synthesized from a variety of natural (peptides, polysaccharides, and oligonucleotides) and synthetic materials [340-343]. Hydrogels are appealing as carriers because their viscoelastic properties are tunable and provide an environment that can preserve the biological activity of growth factors [360]. To a limited extent, the release of growth factors can be controlled through drug loading, the type of polymer material used, and processing conditions [360]. However, the release kinetics of the growth factors often remains too rapid. The rapid elution of growth factors from the hydrogel can cause adverse reactions and can result in the premature depletion of growth factors before treatment is completed [345]. To slow the release of growth factors further, various functionalization strategies have been tested. For example, peptides, metal ions, and heparin have been conjugated to the polymer network of many hydrogels to slow growth factor release. However, issues such as low binding, lack of specificity, or toxicity remain a concern in these systems. Therefore, other ligands should be investigated for the development of growth factor delivery systems.

Nucleic acid aptamers are ligand that can be screened for virtually and target. Because they are discovered through a directed evolution approach, they can have a high binding affinity and
specificity for their target [252, 253]. In addition, aptamers are nonimmunogenic and are amenable to conjugation reactions, making them ideal materials to functionalize hydrogels for controlled growth factor delivery applications [158]. The ability of an antiVEGF aptamer and an antiPDGF-BB aptamer to bind their respective targets was investigated in the preceding chapter. The information gained by studying the interactions between aptamer and their cognate targets will be useful in the development of aptamer-functionalized systems for the controlled delivery of growth factors.

In this chapter, two aptamer-functionalized hydrogels were created, a composite hydrogel and a superporous hydrogel. In the first system, aptamers were attached to polymeric supports through noncovalent binding, which were then loaded into a hydrogel. In the second system, aptamers were chemically conjugated to the hydrogel network itself. The ability of the aptamers to bind their target growth factors were investigated for both hydrogel systems. As the sequence and secondary structure of the aptamer are critical for the binding of the target to the aptamer, alterations to the aptamer were introduced to study their effect on the ability of the aptamer-functionalized hydrogel to retain the growth factors within the hydrogel. Lastly, the therapeutic potential of the developed aptamer-functionalized hydrogels was evaluated by testing the ability of the hydrogels to bind a large concentration of growth factors.
3.2. Experimental Procedure

3.2.1. Materials

Streptavidin-coated polystyrene microparticles (1.3 μm) were purchased from Spherotech (Lake Forest, IL). Agarose was purchased from Promega (Madison, WI). Phosphate buffered saline (PBS), Tween 20, sodium bicarbonate, acetic acid, N,N,N',N'-tetramethylethane-1,2-diamine (TEMED), ammonium persulfate (APS), and sodium azide (NaN₃) were purchased from Fisher Scientific (Suwanee, GA). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 3.1. Poly(ethylene glycol) diacrylate (PEGDA, Mₙ = 700) and Pluronic F-127 were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Invitrogen were purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF, Mₚ = 38.2 kDa) and platelet-derived growth factor BB (PDGF-BB, Mₚ = 24.3 kDa) enzyme-linked immunosorbent assays kits were purchased from PeproTech (Rocky Hill, NJ).
Table 3.1. List of aptamers to for the retention of growth factors within superporous hydrogels.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Length (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AntiPDGF-BB aptamers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apt-PH</td>
<td>CAGCA TCATG GTGAT GCTCT ACGTG CCGTA GCCTG TGGAG TATCG C</td>
<td>46</td>
</tr>
<tr>
<td>Apt-PM</td>
<td>GCGAT ACTCC ACAGG CTACG GCACG TAGAG CATCA CCATG ATCCC A</td>
<td>46</td>
</tr>
<tr>
<td>Apt-PL</td>
<td>CAGAT ATGTG TAGCG AACCC GAGTG GCCAS ATGTT ACCCA GACCC C</td>
<td>46</td>
</tr>
<tr>
<td><strong>AntiVEGF aptamer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apt-V</td>
<td>CCCGT CTTCC AGACA AGAGT GCAGG G</td>
<td>26</td>
</tr>
</tbody>
</table>

*Apt: aptamer; nt: nucleotides; V: VEGF; P: PDGF-BB; H: high affinity; M: moderate affinity; L: low affinity.*
3.2.2. Methods

3.2.2.1. Preparation of Aptamer-functionalized Particles and Composite Hydrogel

One hundred sixty picomoles of biotinylated aptamers were incubated with 80 μg streptavidin-coated microparticles in 46.4 μL PBS for 30 min. The microparticles were washed four times with PBS supplemented with 0.1% BSA and 0.05% Tween 20. Following washing, 4 ng VEGF or PDGF-BB was added to the microparticle suspension and incubated at room temperature for 2 h. After immobilizing the proteins, 140 μL agarose solution at 42 °C was added to the microparticle suspension and 50 μL volumes were quickly aliquoted into cylindrical molds. The final agarose concentration was 0.5 % (w/v).

3.2.2.2. Preparation of Aptamer-functionalized Superporous Hydrogel

Superporous hydrogels were synthesized by using a free radical polymerization reaction coupled with a gas foaming reaction [361]. In brief, Pluronic F-127, acetic acid, TEMED, APS, and PEGDA were mixed with or without aptamers. The prepolymer solution (25 μL) was then added to sodium bicarbonate in a cylindrical mold to initiate the formation of the gas foam and the hydrogel. After the formation, the hydrogels were thoroughly washed in deionized water for three days to remove any unreacted molecules.

To load the growth factor into the hydrogel, lyophilized growth factor was reconstituted by dissolving the powder in PBS with 0.1% BSA. Prior to loading the superporous hydrogel with growth factors, the hydrogel was gently blotted with tissue paper to dehydrate them. A growth factor solution of 120 μL was then added to the hydrogel and the loaded hydrogel was incubated at 4 °C for 24 h. The loaded hydrogel was used directly after incubation without washing for the examination of the functionality of the hydrogel.
3.2.2.3. Scanning Electron Microscopy

Superporous hydrogels were prepared by completely drying the hydrogels at 37 °C for several days in a convective air incubator and sputter coated with gold. Hydrogels were imaged via a JEOL 6335F Field Emission Scanning Electron Microscope. The dimensions of the pore in the hydrogel were calculated using the software ImageJ (http://rsb.info.nih.gov/ij/). The average of the longest and the shortest diameter of the orifice of a pore was calculated and presented as the pore diameter.

3.2.2.4. Ethidium Bromide Staining

Superporous hydrogels were stained with ethidium bromide to assess aptamer incorporation into the hydrogel network. Hydrogels were incubated in an ethidium bromide bath for 1 h and washed for 2 h to remove unbound ethidium bromide. Hydrogels were then imaged under UV light with a Bio-Rad GelDoc XR system (Hercules, CA).

3.2.2.5. Confocal Microscopy

Aptamer-functionalized superporous hydrogels were incubated in the release medium containing a 6-FAM-labeled complementary sequence for 1 h. After thorough washing, the hydrogels were subjected to observation under a Nikon A1R Spectral Confocal Microscope (Nikon Instruments Inc., Melville, NY) to image the porous network at various depths into the hydrogel. The fluorophore was excited at 488 nm with an argon laser. X-y scans were made every 10 µm from the surface of the hydrogel to a depth of 400 µm in the z-direction at an optical resolution of 0.47 µm. Captured images represent a 1280 µm x 1280 µm field.
3.2.2.6. Examination of Growth Factor Binding

Composite hydrogels were incubated in 250 μL collection medium (0.1 % BSA, 0.05 % Tween 20, and 0.09 % NaN₃ in PBS) at 37 °C with a shaking rate of 70 rpm. At predetermined time points, the release medium was totally collected and replenished with fresh release medium. Collected supernatants were stored at -20 °C until analysis. Superporous hydrogels were treated equally as the composite hydrogels, with the exception that the superporous hydrogels were incubated in 1 mL collection medium.

Quantification of VEGF and PDGF-BB in the supernatants was measured using VEGF and PDGF-BB ELISA kits following the provided instructions. Where necessary, supernatant samples were diluted with the diluent to ensure that the growth factor concentration fell within the detectable range of the assay. The absorbance of each sample was measured using a BioTek Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, VT) at 405 nm and doubly referenced by subtracting the absorbance at the reference wavelength (650 nm) and the absorbance of the zero concentration. All experiments were performed in triplicate. The amount of growth factor retained within the hydrogel is calculated as the amount of growth factor loaded into the hydrogel less the total amount of growth factor released.

3.2.2.7. Surface Plasmon Resonance Spectroscopy

The molecular interaction between the PDGF-BB and its aptamers was studied using Surface plasmon resonance (SPR) spectroscopy (SR7500DC; Reichert Analytical Instrument; Depew, NY). A carboxyl group-functionalized sensor chip (Reichert Analytical Instrument; Depew, NY) was used as the substrate for PDGF-BB immobilization. To immobilize PDGF-BB, the sensor chip was activated by flowing 0.0040 g/mL EDC and 0.012 g/mL NHS for 7 min at 20 μL/min. After the activation of the sensor chip, 10 μg/mL PDGF-BB in 10 mM sodium acetate (pH = 5.2) was injected for 10 min at 20 μL/min. The running buffer was PBS containing 0.05% Tween
20. To test aptamer binding to PDGF-BB, 100 nM of aptamers were flowed over the sensor chip at 25 µL/min. The sensor chip was regenerated by washing the sensor chip with 40 mM NaOH for 1 min at 100 µL/min.

3.2.2.8. Rheology

The storage ($G'$) and loss ($G''$) moduli of the superporous hydrogels were measured with an AR-G2 rheometer (TA Instruments, New Castle, DE). A constant normal force of 0.5 N was applied to the superporous hydrogels to prevent slippage during oscillation. An oscillatory stress sweep from 0.01 to 100 Pa at a fixed angular frequency of 1 rad/s was conducted to identify the viscoelastic region. Afterwards, an angular frequency sweep from 0.1 to 100 rad/s at a fixed oscillation stress of 3 Pa was conducted to measure the moduli of the various hydrogels ($n = 4$).

3.2.2.9. Secondary Structure Prediction

The secondary structures of the aptamer were generated by using the program RNAstructure, v5.3 (http://rna.urmc.rochester.edu/rnastructure.html), which is capable of predicting single- and double-stranded RNA or DNA structures [305]. The predicted structures with the lowest free energies were presented. The structure presented with the lowest free energy was presumed to be the predominating structure. Three-dimensional conformations were generated using NUPACK [362].

3.2.2.10. Statistics

Quantitative data is presented as the mean ± one standard deviation of triplicate samples. Statistically significance was evaluated using Student’s t-test via MATLAB (v. 7.11.0.584, The Mathworks, Inc., Natick, MA).
3.3. Results

3.3.1. Aptamer-functionalized Composite Hydrogels can Retain Growth Factors

Composite hydrogels were created by loading aptamer-functionalized microparticles into an agarose solution, which was then allowed to cure and entrap the microparticles within the hydrogel (Figure 3.1A). Aptamers were conjugated onto the polymeric microparticles through the formation of a nearly irreversible noncovalent bond between streptavidin and biotin. Microscopic examination of the microparticles in the agarose hydrogel reveals that the microparticles were well distributed within the hydrogel with no signs of aggregation (Figure 3.1B).

To test the functionality of the composite hydrogel, hydrogels were loaded with microparticles functionalized with either the antiPDGF-BB or the aptamer antiVEGF aptamer, and compared to the nonfunctionalized hydrogel. Microparticles functionalized with the antiPDGF-BB aptamer were tested for their ability to retain PDGF-BB within the hydrogel. Composite hydrogels loaded with nonfunctionalized microparticles and 1000 pg PDGF-BB released 231 pg (23%), 190 pg (19%), and 123 pg (12%) PDGF-BB during the initial 1 h and the next 5 and 18 h of incubation, respectively (Figure 3.2A). The time points chosen correspond to 1 h, 6 h, and 24 h of total incubation time. This corresponds to a total of 544 pg not retained within the hydrogel, or conversely 456 pg (46%) being retained (Figure 3.2A). When microparticles were functionalized with the antiPDGF-BB aptamer, the release of PDGF-BB from the hydrogel was 98 pg (10%) within the first 1 h, 26 pg within the next 5 h (6-h total incubation time), and 12 pg (1%) during the next 19 h (24-h total incubation time) (Figure 3.2A). Therefore, 136 pg PDGF-BB escaped the hydrogel during the 24-hour incubation, leaving 864 pg, (86%) of the loaded PDGF-BB within the hydrogel (Figure 3.2A). Ergo, the antiPDGF-BB aptamer can be used to functionalize microparticles to slow the release of PDGF-BB from a composite hydrogel.

Similarly, when loaded with 1000 pg VEGF, the nonfunctionalized hydrogel released 214
Figure 3.1. Preparation of functionalized composite growth factor system. (A) Illustration of entrapping loaded microparticles into an agarose hydrogel to create the composite system. (B) Micrograph of functionalized microparticles (1.3 µm diameter) embedded in 0.5% agarose hydrogel.
Figure 3.2. Ability of aptamer-functionalized composite systems to retain growth factors within the hydrogel. (A) The amount of PDGF-BB not bound to the nonfunctionalized microparticles (-) and microparticles functionalized with the antiPDGF-BB aptamer (+) were analyzed at various times (left) and the total amount of PDGF-BB retained by the microparticles after 24 h of washing was calculated (right). (C) The amount of VEGF not bound to the nonfunctionalized microparticles (-) and microparticles functionalized with the antiVEGF aptamer (+) were analyzed at various times (left) and the total amount of VEGF retained by the microparticles after 24 h of washing was calculated (right). All systems were loaded with 1000 pg growth factor. *** denotes a statistical significance of $p \leq 0.001$ in comparison to the nonfunctionalized composite system.
pg VEGF into the supernatant within the first 60 min, 194 pg VEGF within the next 5 h, and 175 pg VEGF within the next 18 h. Thus, 583 pg VEGF was released into the supernatant during the first 24 h of incubation (Figure 3.2B). In other words, 417 pg VEGF, corresponding to 42% of the loaded amount, was retained within the composite hydrogel (Figure 3.2B). When the microparticles were functionalized with the antiVEGF aptamer, the hydrogel released 89 pg, 85 pg, and 65 pg VEGF during the first hour and subsequent 5 h and 18 h, respectively. Therefore, 239 pg VEGF eluted the hydrogel during the 24-hour incubation period, corresponding to 76% of the loaded VEGF being retained within the hydrogel (Figure 3.2B). Thus, microparticles functionalized with the antiVEGF aptamer could be used to slow the release of VEGF from the composite hydrogel.

To test the scalability of the composite system to bind a large concentration of growth factors, the amount of microparticles functionalized with Apt-V1 and the amount of VEGF was increased 50-fold. When nonfunctionalized microparticles were loaded into the agarose with the VEGF, the composite system could not retain the VEGF within the hydrogel as effectively as the native hydrogel (i.e., no microparticles added). The amount of VEGF found in the supernatant was greater for the hydrogel loaded with nonfunctionalized microparticles at all the time points analyzed in comparison with the native hydrogel (Figure 3.3A). This led to an amount of unbound VEGF that was 4-fold greater than the amount for the native hydrogel after 24 h (Figure 3.3B). When microparticles functionalized with the antiVEGF aptamer were used, the amount of unbound VEGF in the supernatant was less than the amount VEGF in the supernatant in comparison to using the nonfunctionalized microparticles for all the time points investigated. However, the amount of VEGF that eluted the functionalized composite system was still greater than the amount seen with the native hydrogel (Figure 3.3). After 24-h incubation, the amount of VEGF released from the functionalized composite systems was 2.5-fold greater than the amount release from the native hydrogel (Figure 3.3B).
Figure 3.3. Effect of microparticle density on the retention capacity of the hydrogel and composite systems. Top: The amount of microparticles and aptamers in the hydrogels were scaled up to bind 50 ng VEGF and the amount of VEGF that was not retained by the delivery systems was measured at various times. Bottom: The free VEGF of the composite system with nonfunctionalized microparticles and the composite system with microparticles functionalized with antiVEGF were normalized to the amount of free VEGF from the agarose hydrogel (i.e., the native hydrogel). * denotes a statistical significance of $p \leq 0.05$ in the compared data, ** denotes $p \leq 0.01$, and *** denotes $p \leq 0.001$. 
3.3.2. Aptamer-functionalized Superporous Hydrogels can Retain Growth Factors

The superporous hydrogel developed in this study was formed through the coupled reactions of free radical polymerization and gas formation (Figure 3.4A). To form the superporous hydrogel, the prepolymer solution was mixed with acetic acid and sodium bicarbonate. The acetic acid reacted with the sodium bicarbonate to generate carbon dioxide gas while the prepolymer solution polymerized around the gas bubbles. Though free radical polymerization is sufficient to form a hydrogel, the pore size in the resulting hydrogel is insufficiently large to load growth factors into the hydrogel postsynthesis. Since the concentration of the monomer is critical in the formation of polymer networks, the concentration of PEGDA was varied from 5% to 20% to evaluate its effect on the formation of a superporous hydrogel (Figure 3.4B). At 5% PEGDA, a hydrogel with large pores present did not form, as seen in the SE micrograph. At 10% PEGDA, pore-like structures can be seen in the hydrogel. However, many of these structures were collapsed at this concentration. When the monomer concentration was increased to 15% or 20%, a hydrogel with large pores could be formed. As no difference in the morphology is seen between the 15% or 20% hydrogels, 15% PEGDA was chosen to synthesize the hydrogels in the following experiments. To incorporate the aptamers into the polymer network, the aptamers were chemically functionalized with Acrydite at their 5’-end for copolymerization with PEGDA (Figure 3.4C). To verify that aptamers would be integrated into the superporous hydrogel, the hydrogels were stained with ethidium bromide (Figure 3.4C). The hydrogel with the Acrydited aptamer showed a much stronger fluorescence intensity than the hydrogels with no aptamer or admixed with the nonmodified aptamer. Thus, aptamers can be successfully incorporated into the network of superporous hydrogels via the Acrydite end group.
Figure 3.4. Synthesis and characterization of superporous hydrogels. (A) Reaction scheme of superporous hydrogel synthesis. The hydrogel is synthesized by using free radical polymerization coupled with gas formation. The prepolymer solution with acetic acid reacts with sodium bicarbonate powder to create a gas foam (i) and is quickly cured into a hydrogel (ii). Acrydite-functionalized aptamers are incorporated into the hydrogel network if present during polymerization. (B) Comparison of superporous hydrogels of varying PEG concentrations; scale bars: 100 μm. (C) Illustration of an Acrydite-modified aptamer (top). Images of ethidium bromide-stained hydrogels showing that aptamers were incorporated into the hydrogel through the Acrydite end group (bottom). (D) Comparison between hydrogels synthesized without and with aptamers; scale bars: 100 μm. The pore sizes are normally distributed. (E) Composite of superimposed images captured in 10-μm intervals from confocal microscopy.
Scanning electron micrographs were captured to compare the pore structures of superporous PEG hydrogels without and with aptamers (Figure 3.4D). The morphologies of the functionalized superporous hydrogels are similar to the nonfunctionalized superporous hydrogel. In addition, the aptamer-functionalized superporous hydrogel had a normal distribution of pore sizes, ranging from 20 µm to 100 µm, with an average pore size of 55 ± 16 µm. Along with SEM, scanning laser confocal microscopy was used to visualize the three-dimensional architecture of the superporous hydrogel. The three-dimensional rendering of the superporous hydrogel was created by superimposing images acquired at multiple depths and shows that the gas foam created a tortuous and porous network throughout the hydrogel (Figure 3.4E).

To evaluate the swelling properties of the superporous hydrogel, the hydrogel was dehydrated and allowed to reabsorb water. Using digital photography, images of the superporous hydrogel show that the hydrogel is capable of quickly absorbing the applied solution (Figure 3.5A). To characterize the mechanical properties of the superporous hydrogels, a rheometer was used. The storage modulus (G’) of the nonporous hydrogel was approximately 84,000 Pa and was independent of the angular frequency (Figure 3.5B). Similarly, the storage modulus of the nonfunctionalized and the functionalized superporous hydrogel were virtually independent of the angular frequency. In addition, they exhibited a storage modulus less than the nonporous hydrogel, of approximately 3000 Pa.

To understand the ability of the antiPDGF-BB aptamer to bind and retain PDGF-BB within the superporous hydrogel, the mole ratio of the aptamer to the growth factor was varied. The amount of PDGF-BB loaded into the hydrogel was fixed at 50 ng (i.e., 2.0 pmol). After incubating the loaded hydrogels in medium for 5 min, the amount of PDGF-BB in the supernatant was 37 ng in the 0:1 group (i.e., the nonfunctionalized hydrogel), 23 ng in the 1:1 group, 0.5 ng in the 10:1 group, and 0.5 ng in the 100:1 group (Figure 3.6A). The amount of PDGF-BB retained in these four groups after 24 h of washing was 14%, 53%, 90%, and 96%, respectively (Figure 3.6B). Thus, the
Figure 3.5. Characterization of hydrogels. (A) Photographs of the absorption of water by a dehydrated superporous hydrogel captured in 0.15 s intervals. (B) Rheological properties of nonporous and superporous hydrogels. Triangle symbols (▲) denote the measurement of a nonporous PEG hydrogel, square (□) symbols denote the measurement of a nonfunctionalized superporous hydrogel, and diamond symbols (♦) denote the measurement of aptamer-functionalized superporous hydrogels; filled symbols denote storage modulus (G') and the open symbols denote the loss modulus (G''). n = 4.
Figure 3.6. Effects of the mole ratio of Apt-P7 to PDGF-BB on growth factor retention. Top: The amount of free PDGF-BB in the washing solution after various times of incubation. Bottom: The amount of PDGF-BB retained in the hydrogels after 24-h incubation. The amount of PDGF-BB loaded was 50 ng. ** denotes a statistical significance of $p \leq 0.01$ in comparison to the nonfunctionalized superporous hydrogel and *** denotes $p \leq 0.001$. 
antiPDGF-BB aptamer can be used to retain PDGF-BB within a superporous hydrogel and the degree of PDGF-BB retention increased with increasing mole ratio of the aptamer to the growth factor.

A similar process was applied using the antiVEGF aptamer. The antiVEGF aptamer was used to functionalize superporous hydrogels at mole ratios of 0:1, 1:10, 1:1, and 10:1 antiVEGF to VEGF, with the concentration of VEGF fixed at 200 ng (i.e., 5.2 pmol). After washing the hydrogel for 24 h, the amount of VEGF retained by the various mole ratios were 28%, 38%, 59%, and 97%, respectively (Figure 3.7). Therefore, the antiVEGF aptamer can be used to develop antiVEGF-functionalized superporous hydrogels for the retention of VEGF, and the retention capacity of the hydrogel is correlated to the mole ratio of the aptamer to the growth factor.

### 3.3.3. Binding Affinity of Aptamers can be Modified to Regulate the Retention of Growth Factors

To further investigate the utility of using aptamer-functionalized hydrogels for as growth factor delivery systems, the binding affinity of an aptamer was modified. Two aptamer derivatives were created by modifying the sequence of the antiPDGF-BB aptamer. The first derivative was created by substituting two bases with bases that alter the secondary structure of the antiPDGF-BB aptamer. The second derivative was created by randomizing the sequence of the antiPDGF-BB aptamer. The predicted secondary structures of these oligonucleotides are shown in Figure 3.8A and exhibit very dissimilar secondary structures. Surface plasmon resonance spectroscopy was used to measure the binding affinity of these oligonucleotides (Figure 3.8B and Figure A.1). The nonmodified antiPDGF-BB aptamer has an equilibrium dissociation constant, $K_d$, of 3.2 nM, the oligonucleotide with two base substitutions has a binding affinity of $K_d = 11.3$ nM, and the randomized oligonucleotide has a $K_d = 220$ nM (Figure 3.8C). Because a low equilibrium dissociation constant indicates a strong binding affinity, these oligonucleotides are designated
Figure 3.7. VEGF retention by antiVEGF-functionalized superporous hydrogels. The amount of VEGF retained in the hydrogels after 24-h incubation as function of various mole ratios of the antiVEGF aptamer to VEGF. The amount of VEGF loaded was 50 ng. * denotes a statistical significance of $p \leq 0.05$ in comparison to the nonfunctionalized superporous hydrogel and ** denotes $p \leq 0.01$. 
Figure 3.8. Determination of binding affinity. (A) Secondary structures and three-dimensional configurations of the Apt-H (top), the Apt-M (middle), and the Apt-L (bottom) aptamer. (B) Surface plasmon resonance sensorgram showing the association and dissociation of 100 nM Apt-H, Apt-M, Apt-L. (C) Equilibrium binding plots. Equilibrium dissociation constants ($K_d$) were determined by the software Scrubber2.
Apt-PH, Apt-PM, and Apt-PL, for the high affinity, moderate affinity, and low affinity aptamer, respectively.

The aptamers Apt-PH, Apt-PM, and Apt-PL were used to functionalize superporous hydrogels to characterize how the binding affinity affects the ability of the hydrogel to function as a growth factor delivery system at a fixed mole ratio of ten aptamers to one growth factor. After 5 min of washing, 37 ng of PDGF-BB was detected in the supernatant of the nonfunctionalized superporous hydrogel. In contrast, 14, 4.5, and 0.5 ng of PDGF-BB were detected in the solutions of the Apt-PL, Apt-PM, and Apt-PH groups, respectively (Figure 3.9). The percentages of PDGF-BB retained within the hydrogels after they were incubated in the washing solution for 24 h in the non-, the Apt-PL-, the Apt-PM-, and the Apt-PH-functionalized superporous hydrogels were 14%, 28%, 63%, and 90%, respectively (Figure 3.9). Therefore, the ability of an aptamer-functionalized superporous hydrogel to function as a growth factor delivery system is reliant on the binding affinity of the aptamer for its target, where an aptamer with a strong binding affinity is better able to retain the growth factor within the hydrogel than an aptamer with a lower binding affinity.

3.3.4. Aptamer-functionalized Superporous Hydrogels can Retain a Large Concentration of Growth Factors

To investigate the feasibility of using aptamer-functionalized hydrogels as growth factor delivery systems further, the effect of varying the amount of growth factor loaded into the hydrogel at a fixed mole ratio of ten aptamer to one growth factor was investigated. Superporous hydrogels functionalized with the low affinity antiPDGF-BB aptamer (Apt-PL) and the high affinity aptamer (Apt-PH) were used to study the ability of hydrogel to retain either 50 ng or 2 µg PDGF-BB. After washing the hydrogel for 24 h, the hydrogel functionalized with Apt-PL retained 28% of the loaded 50 ng PDGF-BB and 85% of the PDGF-BB when loaded with 2 µg PDGF-BB (Figure 3.10). The
Figure 3.9. (A) Effect of the binding affinity on PDGF-BB retention within the superporous hydrogel Top: The amount of free PDGF-BB in the washing solution after various times of incubation. Bottom: the amount of PDGF-BB retained in the hydrogels after 24-h incubation. The amount of loaded PDGF-BB was 50 ng. * denotes a statistical significance of $p \leq 0.05$ in comparison to the nonfunctionalized superporous hydrogel and *** denotes $p \leq 0.001$. 
Figure 3.10. Scalability and the relationship between the amount of PDGF-BB loading and growth factor retention. The mole ratio of Apt-PL and Apt-PH to PDGF-BB was fixed at 10:1. Left: Apt-PL; Right: Apt-PH.
Apt-PH-functionalized hydrogels loaded with 50 ng and 2 µg PDGF-BB retained 90% and 99.7% of the loaded amount after the 24-hour wash, respectively (Figure 3.10).
3.4. Discussion

Two methods exist for loading growth factors into a hydrogel. In one method, growth factors are admixed with a prepolymer solution. When polymerization is initiated, the hydrogel will form and entrap the growth factors within its network. With this method, all the growth factors can be loaded into the hydrogel. However, many one-pot syntheses rely on harsh reactions and conditions (e.g., acid-base reactions, free radical polymerization, organic solvents, high-speed mechanical agitation, and/or high temperatures) to polymerize the carriers. These processes lead to the denaturation of the growth factors, thereby decreasing its bioactivity and therapeutic benefit [363]. To prevent the denaturation of growth factors, the carrier can be synthesized first and the growth factors loaded postsynthesis. This method circumvents the harsh polymerization reactions to preserve growth factor bioactivity, but it is often limited by low loading efficiencies [364-366]. Size exclusion by the pores greatly slows the speed by which the growth factors can permeate the hydrogel carrier, as the mesh size of the hydrogel is much smaller than the size of the growth factor, necessitating days or weeks to load the carrier with a sufficient concentration of growth factors.

To load growth factors into the composite hydrogel, aptamer-functionalized microparticles were incubated in a growth factor solution, allowing the growth factors to be tethered to the microparticles. The aptamers were immobilized onto the microparticles utilizing the nearly irreversible noncovalent interaction between the streptavidin-coated microparticles and the biotinylated aptamers. The loaded microparticles were then mixed with an agarose solution. Agarose is a thermosensitive hydrogel that is a semisolid at physiological temperatures and a liquid at elevated temperatures. Therefore, the agarose solution must be heated to allow the microparticles to be incorporated into the hydrogel. This can be problematic as the prolonged exposure of the growth factors to elevated temperatures can denature the growth factor.

To evaluate the ability of a composite system to bind growth factors and slow their release, microparticles were functionalized with various aptamer and loaded into an agarose hydrogel. At
various time points, the amount of growth factors that dissociated from the aptamers and eluted the composite system was measured. Analysis of PDGF-BB binding to the microparticles in the composite system shows that the addition of the antiPDGF-BB aptamer to the microparticles was able to decrease the amount of PDGF-BB that eluted the hydrogel. After washing the microparticles for 24 h, the amount of PDGF-BB that was retained by the composite system increased from 46% when the antiPDGF-BB aptamer was not used, to 86% when the antiPDGF-BB aptamer was added into the system (Figure 3.2). Similarly, the addition of the antiVEGF aptamer to the composite system was able to improve the ability of the composite system to retain VEGF. Analysis of VEGF concentration at various time points shows that the binding of the antiVEGF aptamer with VEGF remained stable over the span of time analyzed as indicated by the lower concentration of VEGF in the supernatant in comparison with the nonfunctionalized composite system. After washing the composite systems for 24 h, the amount of VEGF retained by the nonfunctionalized composite system and the functionalized composite system was 42% and 76%, respectively (Figure 3.2). Thus, the antiVEGF aptamer can be used to functionalize microparticles and they can retain their binding functionality to create affinity composite systems.

To test further the ability of the aptamer-functionalized composite systems to bind growth factors, the amount of VEGF and antiVEGF-functionalize microparticles was increased 50-fold, while keeping the volume of the composite system constant. To compare the effect of microparticle density on VEGF release, a composite system with nonfunctionalized microparticles was created and compared to the native hydrogel (i.e., agarose hydrogel with no microparticles loaded). The results show that loading a large concentration of microparticles into the hydrogel was able to change the ability of the hydrogel to retain VEGF. The use of microparticles as fillers to augment the mechanical properties of bulk hydrogels or other polymeric materials has been widely investigated [367-372]. For example, the addition of calcium chloride microparticles into an alginate hydrogel can increase the strength of the hydrogel [368] and the addition of silica particles
can increase the hydrophilicity and degree of swelling of hyaluronic acid hydrogels [371]. Thus, it is not surprising that greatly increasing the density of microparticles within the hydrogel can affect the ability of the composite to retain VEGF. The decrease in the ability of the composite systems is likely due to increases in the number of network defects and increases in the porosity or the ability of the hydrogel to swell. A similar result has been observed when a low concentration of hydroxyapatite particles enhanced the retention of bovine serum album in a PEG hydrogel, but a large concentration of hydroxyapatite decreased the ability of the hydrogel to retain the protein [372]. To determine if aptamer-functionalized microparticles could retain a large concentration of growth factors within the composite, antiVEGF aptamers were used to functionalize a large concentration of microparticles, which were then loaded into the hydrogel. While the antiVEGF-functionalized composite system was able to retain more VEGF within the composite than the nonfunctionalized composite system, the amount of VEGF retained was still less than the native hydrogel (Figure 3.3). Thus, while it is shown that aptamers can be used to promote the retention of growth factors within a composite system, the use of functionalized composite systems may not be suitable if a large concentration of microparticles is needed.

A superporous hydrogel was investigated as the second possible growth factor delivery system. This system relies on the copolymerization of Acrydited aptamers with PEGDA and the creation of large pores. Unlike the aptamer-functionalized composite hydrogel, growth factors are loaded into the superporous hydrogel postsynthesis. This allows the hydrogel to be washed to remove unreacted monomers and free radicals from the hydrogel, which may be toxic if left in place and the hydrogel implanted in vivo. When the hydrogel was formed without a gas foam, no visible pores were seen in the scanning electron micrographs (Figure 3.4). When a gas foam was added to the polymerization reaction, large pores were created that transverse the hydrogel. These interconnected channels allow the hydrogel to rapidly absorb water through capillary action, rather than just simple diffusion [373]. Thus, the risk of denaturation that is associated with harsh
polymerization conditions can be avoided and it becomes possible to quickly load the growth factors into the hydrogels with a high efficiency and high bioactivity. The rate by which the growth factors are loaded into the carrier can be increased by increasing the pore size, however larger pores would also lead to greater growth factor release rates and would be detrimental to creating a controlled delivery system. By functionalizing a highly porous system, one can take advantage of rapid growth factor loading and a high retention of growth factors, if the binding between the affinity ligands and the growth factors is strong.

Analysis of the absorbency of the superporous hydrogel shows that the absorption rate of water into the hydrogel is nearly instantaneous (Figure 3.5), revealing the superporous hydrogel has superabsorbent properties due to the presence of capillary-sized channels. Because the addition of the channels into the hydrogel created a large volume of void space, it was likely that the mechanical properties of the hydrogel changed. To measure the mechanical properties of the hydrogel, an oscillatory rheometer was used to measure the storage and the loss modulus of the hydrogels. There was a significant loss in the storage modulus of $\Delta G' = 80$ kPa when the channels were added to the hydrogel. This indicates a loss in the bulk mechanical stiffness or an increase of the matrix flexibility. This result is expected since the void of channels decreased the overall density of the hydrogel. It should be cautioned against stating that the mechanical property of the hydrogel material itself changed, as the chemical composition of the hydrogel was not altered. In addition, no difference is observed in comparison of the nonfunctionalized superporous hydrogel with the aptamer-functionalized superporous hydrogel. Taken together with the observations of the scanning electron micrographs, it can be concluded that the incorporation of aptamers directly into the polymeric network of the hydrogel does not change the physical properties of the hydrogel. Thus, the superporous hydrogel offers greater flexibility in the concentration of aptamers than can be incorporated than the composite system, where the latter relies on the increasing the
concentration of microparticles within the hydrogel to increase the concentration of aptamers present.

The ability of an aptamer-functionalized superporous hydrogel to bind and retain target growth factors was first investigated using the antiPDGF-BB aptamer. Various mole ratios of the aptamer to the growth factor were analyzed to find an effective concentration of aptamer that would bind and retain the growth factor within the hydrogel. When PDGF-BB was loaded into the nonfunctionalized superporous hydrogel, the majority of the loaded growth factor (nearly 74%) escaped the hydrogel within the first 5 min of washing. This is attributed to the large channels throughout the hydrogel and lack of growth factor-binding sites. The large pores permitted the rapid transport of the loaded molecules out of the hydrogel. Without the large pores, the size-exclusive pores of the hydrogel would have slowed the diffusion of the loaded molecules. After washing the nonfunctionalized hydrogel for 24 h, the amount of PDGF-BB retained was 14%. Clearly, it would not be practical to use a nonfunctionalized superporous hydrogel for the retention of growth factors. When the superporous hydrogel was functionalized with increasing concentrations of the antiPDGF-BB aptamer, there was an increasing ability of the hydrogel to bind and retain the growth factor. When the mole ratio was increased, the probability for the growth factor to be bound to an aptamer or to rebind with an aptamer after it dissociated increases. At a mole ratio of 10 antiPDGF-BB to 1 PDGF-BB, the hydrogel was able to retain 90% of the loaded 50 ng PDGF-BB after washing the hydrogel for 24 h. In addition, a mole ratio of 100 antiVEGF to 1 VEGF showed a similar efficiency. As the 10:1 mole ratio exhibited the highest efficiency in retaining the growth factor within the hydrogel, it was used for all subsequent experiments.

In addition to the importance of the mole ratio in retaining the growth factor, the binding affinity of the antiPDGF-BB in binding PDGF-BB was also investigated. Two derivatives were created by changing the sequence of the antiPDGF-BB aptamer. Analysis of the equilibrium dissociation constant via SPR spectroscopy revealed that the nonmodified antiPDGF-BB aptamer
had the highest binding affinity, and the two derivatives had a moderate and a low binding affinity. These three oligonucleotides are referred to as Apt-PH, Apt-PM, and Apt-PL, respectively. By decreasing the binding affinity of the aptamer for the target, there was a decreasing ability of the superporous hydrogel to bind and retain the growth factor. Therefore, the binding affinity of the aptamer for the target growth factor is an important parameter in the design of an aptamer-functionalized superporous hydrogel with applications as a growth factor delivery system.

As the density of the aptamer in the hydrogel and the binding affinity of the aptamer are important parameters for the formation of a controlled delivery system, these two parameter were investigated further by scaling the amount of PDGF-BB loaded from 50 ng to 2 µg, while maintaining a mole ratio of 10 aptamers to 1 growth factor. When superporous hydrogels were functionalized with the low-affinity aptamer (Apt-PL), the percentage of PDGF-BB retained by the hydrogel increased by over 50% due to the increase of the aptamer density. The percentage of PDGF-BB retained increased only slightly when the amount of PDGF-BB loaded and the Apt-PH concentration were scaled up. Therefore, though the binding affinity of the aptamer is the strongest determinant of the ability of the superporous hydrogel to bind and retain the growth factor, the retention capabilities of hydrogels functionalized with low-affinity aptamers can be improved by increasing the concentration of the aptamer, thereby increasing the occurrence of rebinding between the aptamer and the growth factor.
3.5. Conclusions

In this chapter, the development of two aptamer-functionalized systems was pursued: a functionalized microparticle-hydrogel composite system and a functionalized superporous hydrogel. Both systems were able to bind and retain more growth factors than their nonfunctionalized counterparts. The results show the composite system is strongly influenced by the concentration of the microparticles. To bind a large concentration of growth factors, a large concentration functionalized microparticles are needed, which unfortunately can decrease the ability of the hydrogel to retain the growth factors. Superporous hydrogels, on the other hand, do not require microparticle supports since the aptamers are directly incorporated into the polymeric network. This allows for a greater flexibility in scaling the aptamer concentration proportionally to the amount of growth factors to be loaded. In addition, the binding affinity of the aptamers can be modified to regulate the retention of the growth factor within the hydrogel. Thus, the use of aptamers to functionalize hydrogels for the retention of growth factors is a promising strategy for the development of growth factor delivery systems that can control the release of growth factors over a long time period.
Chapter 4
Controlled Delivery of Growth Factors from Aptamer-functionalized Delivery Systems

4.1. Introduction

Hydrogels are finding increased attention for their applicability for controlled drug delivery systems for their ability to control the spatiotemporal delivery of drugs. They are formed through the three-dimensional crosslinking of hydrophilic polymers, resulting in a porous material capable of absorbing a relatively large volume of water [62]. To create hydrogel-based drug delivery systems, therapeutics like growth factors can be admixed with a prepolymer solution and entrapped within the hydrogel during polymerization or they can be adsorbed into the hydrogel after polymerization. Despite the many advances in delivery technology and carrier formulations, the high permeability of hydrogels leads to the rapid release of the loaded therapeutics [75, 125, 127, 164-173]. The inability to control the release of the loaded drugs lessens the therapeutic benefit of localized drug delivery and can result in adverse side effects in vivo [345].

To control the delivery of growth factors, many functionalization strategies have been explored [130, 176, 191, 193, 194, 196]. While many of these strategies can be used to control the delivery of one growth factor, they do not extend well into controlling the delivery of multiple growth factors, which is necessary as complex signaling cascades involving the sequential delivery of multiple growth factors regulate many biological functions [374]. Thus, new functionalization strategies should be investigated.
Nucleic acid aptamers are synthetic oligonucleotides with a high binding affinity and specificity for their selected target [252, 253]. Moreover, the activity of the aptamer can be regulated through the application of a complementary sequence (CS) [350, 351]. Thus, they are promising ligands for use in the functionalization of growth factor delivery systems. In a previous chapter, the interactions of aptamers with their target growth factor and their CSs were characterized. Then the functionalization of two systems with aptamers and the ability of the functionalized systems to bind and retain the growth factors were evaluated in the preceding chapter. In this chapter, the ability of aptamer-functionalized systems to control the delivery of growth factors is characterized. Composite systems utilizing aptamer-functionalized microparticles and functionalized superporous hydrogel systems are evaluated for their ability to control the delivery of VEGF, PDGF-BB, and the combination of VEGF and PDGF-BB.
4.2. Experiment Procedure

4.2.1. Materials

Streptavidin-coated polystyrene microparticles (1.3 μm) were purchased from Spherotech (Lake Forest, IL). Agarose was purchased from Promega (Madison, WI). Phosphate buffered saline (PBS), Tween 20, sodium bicarbonate, acetic acid, N,N,N',N'-tetramethylethane-1,2-diamine (TEMED), ammonium persulfate (APS), and sodium azide (NaN₃) were purchased from Fisher Scientific (Suwanee, GA). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 4.1. Poly(ethylene glycol) diacrylate (PEGDA, Mₙ = 700) and Pluronic F-127 were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Invitrogen were purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF, Mₚ = 38.2 kDa) and platelet-derived growth factor BB (PDGF-BB, Mₚ = 24.3 kDa) enzyme-linked immunosorbent assays kits were purchased from PeproTech (Rocky Hill, NJ). Geltrex Reduced Growth Factor Basement Membrane Matrix, Medium 200 basal medium, endothelial growth factor supplement, calcein AM, trypsin, and trypsin inhibitor were obtained from Life Technologies (Grand Island, NY)
Table 4.1. List of aptamers and their complementary sequences for the controlled delivery of growth factors.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ → 3’)</th>
<th>Length (nt)</th>
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</thead>
<tbody>
<tr>
<td><strong>AntiVEGF aptamers</strong></td>
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<td></td>
</tr>
<tr>
<td>Apt-V</td>
<td>GAGGA CGAUG CGGAA UCAGU GAAUG CUUAU ACAUC CG</td>
<td>37</td>
</tr>
<tr>
<td>Complementary sequence to antiVEGF aptamer</td>
<td>CATTC ACTGA TTCCG CATCG TCCTC</td>
<td>25</td>
</tr>
<tr>
<td><strong>AntiPDGF-BB aptamers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apt-PH</td>
<td>GCGAT ACTCC ACAGG CTACG GCACG TAGAG CATCA CCATG ATCCT G</td>
<td>46</td>
</tr>
<tr>
<td>Apt-PL</td>
<td>CAGAT ATGTG TAGCG AACCC GAGTG GCCAC ATGTT ACCCA GACCC C</td>
<td>46</td>
</tr>
<tr>
<td>Complementary sequences to antiPDGF-BB aptamers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CS-P</td>
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<td>46</td>
</tr>
<tr>
<td>CS-P9</td>
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<tr>
<td>CS-P10</td>
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</tr>
<tr>
<td>CS-PC</td>
<td>ACTGG TGGCC AGTTG CATGG TACTA TGTGA TGCCC TGAAG CGGCC T</td>
<td>46</td>
</tr>
</tbody>
</table>

Apt: aptamer; CS: complementary sequence; nt: nucleotides; V: VEGF; P: PDGF-BB; H: high affinity; L: low affinity
4.2.2. Methods

4.2.2.1. Preparation of Aptamer-functionalized Superporous Hydrogel

Superporous hydrogels were synthesized by using a free radical polymerization reaction coupled with a gas foaming reaction [361]. In brief, Pluronic F-127, acetic acid, TEMED, APS, and PEGDA were mixed with or without aptamers. The prepolymer solution (25 µL) was then added to sodium bicarbonate in a cylindrical mold to initiate the formation of the gas foam and the hydrogel. After the formation, the hydrogels were thoroughly washed in deionized water for three days to remove any unreacted molecules.

To load the growth factor into the hydrogel, lyophilized growth factor was reconstituted by dissolving the powder in PBS with 0.1% BSA. Prior to loading the superporous hydrogel with growth factors, the hydrogel was gently blotted with tissue paper to dehydrate them. A growth factor solution of 120 µL was then added to the hydrogel and the loaded hydrogel was incubated at 4 °C for 24 h. The loaded hydrogel was used directly after incubation without washing for the examination of the functionality of the hydrogel.

4.2.2.2. Flow Cytometry

Particles functionalized with the antiVEGF or the antiPDGF-BB aptamer were incubated in solutions contained FAM- or TYE-conjugated CSs. After incubation, the microparticles were washed to removed unbound CSs. Approximately 3.45 µg microparticles in 1 mL PBS were analyzed using a BD FACSCalibur flow cytometer (San Jose, CA).

Affinity particles of the antiVEGF aptamer and the antiPDGF-BB aptamer were incubated with either TYE 665-labeled CS-P5 or FAM-labeled CS-V6. The particles were washed to remove unbound CSs. The FAM and TYE fluorophores were excited with an argon ion blue laser (488 nm)
and a HeNe red laser (633 nm), respectively, using a Coulter FC500 (Beckman Coulter, Brea, California). Ten thousand events were collected.

4.2.2.3. Fluorescence Imaging

Composite and superporous hydrogels were incubated in solutions of fluorescently labeled CSs to assess the ability of the CS to penetrate the hydrogel and hybridize with the aptamer. The fluorophores used were either 6-FAM (ex. = 495 nm, em. = 520 nm) or TYE 665 (ex. = 645 nm, em. = 665 nm). Hydrogels were incubated in a CS solution for various times and extensively washed to remove any unbound CS. Hydrogels were then imaged using a CRI Maestro EX in vivo imaging system (Woburn, MA).

4.2.2.4. Examination of Growth Factor Release

Composite hydrogels were incubated in 250 μL collection medium (0.1 % BSA, 0.05 % Tween 20, and 0.09 % NaN₃ in PBS) at 37 °C with a shaking rate of 70 rpm. At predetermined time points, the release medium was totally collected and replenished with fresh release medium. Collected supernatants were stored at -20 °C until analysis. Superporous hydrogels were treated equally as the composite hydrogels, with the exception that the superporous hydrogels were incubated in 1 mL collection medium.

Quantification of VEGF and PDGF-BB in the supernatants was measured using VEGF and PDGF-BB ELISA kits following the provided instructions. Where necessary, supernatant samples were diluted with the diluent to ensure that the growth factor concentration fell within the detectable range of the assay. The absorbance of each sample was measured using a BioTek Synergy™ HT Multi-Mode Microplate Reader (BioTek, Winooski, VT) at 405 nm and doubly referenced by subtracting the absorbance at the reference wavelength (650 nm) and the absorbance of the zero concentration. All experiments were performed in triplicate. The amount of growth factor retained
within the hydrogel is calculated as the amount of growth factor loaded into the hydrogel less the total amount of growth factor released.

4.2.2.5. Gel Electrophoresis

Hybridization of aptamers and CSs was examined using 12% native polyacrylamide gel electrophoresis. Aptamers were mixed with CSs at a 1:2 molar ratio. The polyacrylamide gels were electrophoresed using a Bio-Rad Mini PROTEAN 3 cell equipped with a PowerPac Basic power supply and were stained with ethidium bromide. The stained gels were analyzed using a Bio-Rad GelDoc XR system (Hercules, CA).

4.2.2.6. Bioactivity of Released VEGF

Two assays were used to determine the bioactivity of released VEGF. A tube formation assay was first used. Geltrex was thawed overnight at 4 °C before use. Eighty microliters of Geltrex was added to the wells of a 48-well culture plate and was incubated at 37 °C for 30 minutes. Human umbilical vein-derived endothelial cells (passage 5) were trypsinized when 80% confluency was reached and were suspended in Medium 200. The VEGF released from the superporous hydrogels was mixed with the cell suspension to a final density of 2 x 10^5 cells/mL. To the wells coated with Geltrex, 600 µL of the VEGF and HUVEC mixture was added and the cells were imaged after 6 h of incubation. To visualize the HUVECs, the cells were stained with calcien AM one hour before imaging. Medium 200 and stock VEGF were used as the negative and positive controls, respectively.

The DNA content of HUVEC treated with VEGF was measured in the second assay, using a Click-iT® EdU Microplate Assay according to the instructions provided (Invitrogen, Grand Island, NY). In brief, 6000 HUVEC were seeded per well in a 96-well tissue culture plate overnight. After incubation, VEGF released from the hydrogels was collected and diluted to 10 ng/mL in
Medium 200. The HUVEC were then treated with the VEGF solutions for 48 h. After the 48-h with VEGF, 5-ethynyl-2´-deoxyuridine (EdU) was added to a final concentration of 10 µM for 6 h before analysis. Fluorescence due to EdU incorporation was measured using a Tecan Infinite M200 Pro (Männedorf, Switzerland) with an excitation wavelength of 500 nm and reading the emission at 585 nm.

4.2.2.7. Secondary Structure Prediction

The secondary structures of the aptamer were generated by using the program RNAstructure, v5.3 (http://rna.urmc.rochester.edu/rnastructure.html), which is capable of predicting single- and double-stranded RNA or DNA structures [305]. The predicted structures with the lowest free energies were presented. The structure presented with the lowest free energy was presumed to be the predominating structure.

4.2.2.8. Statistics

Quantitative data is presented as the mean ± one standard deviation of triplicate samples. Statistically significance was evaluated using Student’s t-test via MATLAB (v. 7.11.0.584, The Mathworks, Inc., Natick, MA).
4.3. Results

4.3.1. Aptamer-functionalized Superporous Hydrogels can Sustain the Release of Growth Factors

In the previous chapter, it was shown that aptamer-functionalized superporous hydrogels could be used to bind and retain a large amount of growth factors. To evaluate the ability of aptamer-functionalized superporous hydrogels to function as growth factor delivery systems, the release of the growth factors was evaluated by measuring the amount of growth factor that eluted the hydrogel periodically. When loaded with 50 ng VEGF, the nonfunctionalized (native) superporous hydrogel released 38 ng (76%) of the amount loaded within the first hour of incubation in medium (Error! Reference source not found. Figure 4.1). In contrast, only 3 ng (6%) of VEGF was released from the superporous hydrogel functionalized with the Apt-V aptamer during the first hour of incubation. By 6 h of incubation, 41 ng (81%) of the VEGF was released from the nonfunctionalized hydrogel and there was a steady release of a low concentration (< 1% each day) of VEGF thereafter. After seven days of incubation, 87% of the loaded VEGF was released from the nonfunctionalized superporous hydrogel (Figure 4.1). The release profile of VEGF from superporous hydrogels shows that by 6 h of incubation, 4 ng (8%) of the loaded 50 ng was released. The release kinetics of VEGF from the functionalized hydrogel was linear, where approximately 1% of the amount loaded was released each day. By Day 7, only 17% of the total VEGF was released. Therefore, functionalization of superporous hydrogels with an antiVEGF aptamer can significantly reduce the release of VEGF from superporous hydrogels.

A similar study was conducted using superporous hydrogels functionalized with an antiPDGF-BB aptamer (Figure 4.2). Nonfunctionalized hydrogels loaded with 50 ng PDGF-BB were unable to control the delivery of the growth factor. After 1 h of incubation, 48 ng (95%) PDGF-BB was released. As nearly all of the loaded growth factor was released within the first hour
Figure 4.1. Sustained release of 50 ng VEGF from superporous hydrogels. Top: The burst release profile showing the amount of VEGF released at each of the early time points. Bottom: The cumulative release profile showing the total amount of VEGF released by each time point.
of incubation, there was little PDGF-BB that could be released in the subsequent time points. After incubating the superporous hydrogel for seven days, the total amount of PDGF-BB released was 98%. In contrast, when the superporous hydrogel was functionalized with Apt-PH, the amount of PDGF-BB released during the first hour decreased from 48 ng to 2 ng (4%). In addition, the release of PDGF-BB from the hydrogel functionalized with Apt-PH was well controlled. The release profile shows that linear release kinetics was achieved, where approximately 0.5% of the 50 ng PDGF-BB was released each day. By Day 7, only 3 ng (7%) of the loaded PDGF-BB was released, which is a total of 46 ng (91%) less than the nonfunctionalized superporous hydrogel. Thus, the addition of the antiPDGF-BB aptamer could significantly reduce the release rate of PDGF-BB from a superporous hydrogel.

To further investigate the ability of functionalized superporous hydrogels to control the delivery of growth factors, the amount of growth factors was increased and the long-term release behavior was studied. When 2 µg of VEGF was loaded into the nonfunctionalized superporous hydrogel, 1.8 µg (91%) of the loaded VEGF was released in the first day on incubation (Figure 4.3). By the third day of incubation, 98% of the loaded VEGF was released, which was followed by the release of a little amount of VEGF each day thereafter. On the other hand, the release of a large concentration of VEGF was significantly reduced through the functionalization of the superporous hydrogel with Apt-V. Only 0.1 µg (5%) of the loaded 2 µg VEGF was released from the functionalized superporous hydrogel in the first day of incubation. After incubation for 3 d, the release rate of VEGF reached a steady state, where only 1% of loaded VEGF was released each day. Cumulatively, the amount of VEGF released by Days 5, 10, and 15 were 0.25 µg (13%), 0.35 µg (18%), and 0.42 µg (21%), respectively (Figure 4.3). Thus, functionalization of superporous hydrogels with Apt-V is a promising method to control the delivery of a large concentration of VEGF for a long period.
Figure 4.2. Sustained release of 50 ng PDGF-BB from superporous hydrogels. Top: The burst release profile showing the amount of PDGF-BB released at each of the early time points. Bottom: The cumulative release profile showing the total amount of PDGF-BB released by each time point.
Figure 4.3. Sustained release of 2 μg VEGF from superporous hydrogels. Top: Daily release profile showing the amount the VEGF released each day. Bottom: Cumulative release profile showing the total amount of VEGF released by each day.
Similarly, a large concentration of PDGF-BB was loaded into the superporous hydrogels functionalized with Apt-PL and Apt-PH. The lower affinity antiPDGF-BB aptamer, Apt-PL, was chosen as a control as it has been well shown that nonfunctionalized hydrogels cannot control the delivery of growth factors, where >90% of the loaded growth factors are immediately released. Previous results showed that functionalization of superporous hydrogels with Apt-PL could be used to retain PDGF-BB within the hydrogel, albeit at a much lower efficiency. Therefore, it is expected that Apt-PL could be used to deliver PDGF-BB at a faster rate than Apt-PH at an equal concentration. Release studies of PDGF-BB from superporous hydrogels functionalized with Apt-PL shows that 0.3 µg (14%) of the 2 µg PDGF-BB loaded was released within the first 24 h of incubation (Figure 4.4). Analysis of the release profile shows that the release rate of PDGF-BB steadily decreased after Day 1. The amount of PDGF-BB released at Day 5 was 0.8 µg (4%), 0.05 µg (3%) at Day 10, and 0.03 µg (2%) on Day 15. The total amount of PDGF-BB released from the superporous hydrogels was 0.8 µg (39%), 1.0 µg (52%), and 1.2 µg (61%) by Days 5, 10, and 15, respectively (Figure 4.4).

In comparison to functionalization with Apt-PL, the release rate of PDGF-BB was drastically slower when the superporous hydrogel was functionalized with the high affinity antiPDGF-BB aptamer, Apt-PH (Figure 4.4). The release of PDGF-BB exhibited linear release kinetics, where 0.006 µg (0.3% of the 2 µg PDGF-BB loaded) was released in the first day, and approximately 0.0034 µg (0.17%) was release each day thereafter. Totally, the amount of PDGF-BB released from the superporous hydrogel functionalized with Apt-PH was 0.054 µg, or less than 3% of the 2 µg PDGF-BB loaded by the end of Day 15 (Figure 4.4). Thus, antiPDGF-BB aptamers can be used to functionalize superporous hydrogels for the controlled delivery of a large
Figure 4.4. Sustained release of 2 µg PDGF-BB from functionalized superporous hydrogels. Top: Daily release profile showing the amount the PDGF-BB released each day. Bottom: Cumulative release profile showing the total amount of PDGF-BB released by each day.
concentration of PDGF-BB. Moreover, the release rate of PDGF-BB from these hydrogels can be modulated by turning the binding affinity of the aptamer.

4.3.2. Complementary Sequences can Easily Penetrate Superporous Hydrogels and Hybridize with Aptamers

To investigate the potential of using aptamer-functionalized superporous hydrogels for the triggered delivery of growth factors, various experiments were performed to characterize how sensitive or responsive these hydrogels are to the application of CSs. Superporous hydrogels were loaded with 50 ng PDGF-BB and functionalized with Apt-PH at a mole ratio of ten aptamers to one growth factor. Incubating the functionalized superporous hydrogels in a solution containing FAM-conjugated CS-P for various lengths of time and imaging the fluorescence shows that with a time as short as 5 min, the CS was able to penetrate the superporous hydrogel and hybridize with the aptamer (Figure 4.5A). Because a molar excess of the antiPDGF-BB aptamer exists within the hydrogel, it was also important to see if the CS can promote PDGF-BB release in addition to hybridizing to the unassociated aptamer. Quantification of PDGF-BB release as a function of CS-treatment time shows that at 30 min of incubation, there was a large increase in the amount of PDGF-BB released in contrast to the shorter incubation times (Figure 4.5B). Thus, the superporous hydrogels can respond quickly to the application of the CS.

In addition to testing the time needed to trigger PDGF-BB from the hydrogel using CSs, the dose-dependent response of PDGF-BB release to the application was evaluated. Mole ratios of 1:4, 1:2, 1:1, and 2:1 CS-P to Apt-PH were tested. Fluorescence images of the functionalized hydrogels after incubation in a solution containing FAM-conjugated CS-P show that a mole ratio of 1:4 CS-P to Apt-PH (corresponding to 5 pmol CS-P) was sufficient to label the hydrogel through hybridization (Figure 4.6A). Treating the hydrogels for 1h with CS-P using a mole ratio of 1:4 showed a modest increase in the amount of PDGF-BB released in comparison to using no CS.
Figure 4.5. CS-regulated PDGF-BB release from the superporous hydrogels. (A) Effect of the time of CS treatment on CS penetration (top) and PDGF-BB release (bottom). The mole ratio of CS to aptamer was fixed at 1:2. The amount of loaded PDGF-BB was 50 ng. *** denotes a statistical significant of $p \leq 0.001$ in comparison to treating the functionalized hydrogel for 5 min.
Figure 4.6. CS-regulated PDGF-BB release from the superporous hydrogels as function of mole ratio. (A) Effect of the mole ratio of CS-P to Apt-PH on CS-P penetration. Fluorescence images were captured with an imaging system. (B) Triggered PDGF-BB release with various mole ratios of CS-P. The time of CS treatment was fixed at 1 h. The amount of loaded PDGF-BB was 50 ng. Top: Amounts of PDGF-BB triggered during the 1-h treatment and during the 23 h following the treatment. Bottom: The total release of PDGF-BB during the 24 h. * denotes a statistical significant of $p \leq 0.05$ in comparison to not adding a CS or using the control CS (CS-PC) and *** denotes $p \leq 0.001$. 
In addition, the amount of PDGF-BB released that was directly proportional to the concentration of CS-P. The amount of PDGF-BB released also increased for the 23 h that followed the 1-h application of CS-P and was proportional to the concentration of CS-P used (Figure 4.6B). Linear regression of the PDGF-BB released during the 1-h treatment, the 23 h following CS-P treatment, and the total amount of PDGF-BB released during the 24-period shows that the triggered response is strongly correlated to the dose of CS-P (Figure A.2). Thus, CSs can be used to quickly and quantitatively regulate the delivery of growth factors. Furthermore, treating the hydrogels with a control CS, CS-PC, resulted in an amount of PDGF-BB released that was equal to not applying CS-P, indicating that the triggered release of PDGF-BB is specific to the oligonucleotide sequence (Figure 4.6B).

4.3.3. Triggered Growth Factor Release is Mediated through Displacement of Growth Factor

As the application of a CS can be used to trigger the release of growth factors from aptamer-functionalized hydrogels, it was worthwhile to investigate the mechanism by which the release rate of the growth factor is accelerated when the CS is applied. The possible mechanisms are shown in Figure 4.7. To determine which mechanism by which triggered growth factor release proceeds, two CSs were chosen to bind to Apt-PH. The first CS, CS-P9, binds to the 16 nucleotides located at the 5’-end of Apt-PH. The second CS, CS-P10, binds to the 16 nucleotides located on the 3’-end of the aptamer (Figure 4.8A). Based on the results of previous triggered PDGF-BB release, it is predicted that CS-P9 would be able to trigger the release of PDGF-BB, whereas CS-P10 would not. Native polyacrylamide gel electrophoresis shows that both CSs are able to hybridize with the aptamer, as indicated by the appearance of higher molecular weight band (Figure 4.8B). Fluorescence imaging of superporous hydrogels functionalized with Apt-PH and loaded with 50 ng PDGF-BB in various FAM-conjugated CSs solutions shows that both CS-P9 and CS-P10 are able to hybridize with the aptamer (Figure 4.8C). The amount of PDGF-BB release due to incubation of the hydrogel in the
Figure 4.7. Possible mechanisms of triggered growth factor release. (A) Initially, the growth factor is bound to the aptamer. (B) After a long time, t, the growth factor passively dissociates from the aptamer, which then can rebind to another aptamer. (C) In the first possible mechanism, the CS can be added, which binds and inactivates only the aptamers not in complex with the growth factor. (D) After the growth factor passively dissociates from the aptamer, the growth factor can easily elute the hydrogel, as the inactivated aptamers cannot rebind the growth factor. (E) In the second possible mechanism, the CS can hybridize with the aptamer that is in complex with the growth factor. Hybridization with this aptamer will result in the inactivation of the aptamer and subsequent displacement of the growth factor.
Figure 4.8. Triggered PDGF-BB release using short CSs that bind to different regions of Apt-PH. (A) Secondary structure of Apt-PH showing the location of hybridization of CS-P9 (red nucleotides) and of CS-P10 (blue nucleotides). (B) Lane assignments and the image of the polyacrylamide gel after electrophoresis showing hybridization of the Apt-PH with the various CSs. (C) Fluorescence imaging of bulk hydrogel after hybridization with fluorophore-labeled CSs. (D) Triggered PDGF-BB release during the 1-h incubation with the CS and during the 23 h following the triggering. * denotes a statistical significance of $p \leq 0.05$ in comparison to the hydrogels not treated with the CS at the corresponding time point and *** denotes $p \leq 0.001$. 
various CS solutions was simultaneously analyzed (Figure 4.8D). With no CS applied, little PDGF-BB could be detected during the 24 h analyzed. When CS-P9 was applied, 3.7 ng PDGF-BB was released during the 1-h treatment and 2.9 ng was released during the 23 h after applying CS-P9. When the hydrogel was treated with CS-P10, much less PDGF-BB was released. Only 0.8 ng PDGF-BB was released during the 1-h treatment with CS-P10 and 2.6 ng in the 23 h thereafter. Thus, as CS-P9 was able to hybridize with Apt-PH and could trigger a significant amount of PDGF-BB during the 1-h treatment, it is believed that the mechanism of aptamer inactivation with growth factor displacement is the predominanting mechanism.

4.3.4. Aptamer-functionalized Superporous Hydrogels can be Triggered to Release Growth Factors

After determining that aptamers in superporous hydrogels could be inactivated by hybridization with CSs to liberate the growth factor, the addition of CSs to regulate growth factor delivery was investigated. First, superporous hydrogels functionalized with Apt-V and loaded with 500 ng VEGF were studied. The release of VEGF was sustained over the span of 28 days. The amount of VEGF release at each time point studied was low and only 48 ng (9.6%) of the loaded VEGF was cumulatively released by Day 28 (Figure 4.9A). After the fourteenth day of incubation, CS-V was applied at a mole ratio of 1 CS to 1 aptamer for 1 h. During the 1-h treatment with CS-V, 48 ng (9.6%) was released, whereas only 2.2 ng (0.4%) was released from the nontriggered hydrogel during the same time point (Figure 4.9B). During the 23 h following the treatment with CS-V, 42 ng (8.5%) was released from the treated hydrogel, whereas only 3.6 ng (0.7%) was released from the hydrogel not treated with CS-V. This corresponds to 91 ng (18%) and 5.9 ng (1.2%) released cumulatively during the 24-h, respectively (Figure 4.9B).
Figure 4.9. Triggered release of VEGF from superporous hydrogels functionalized with Apt-V. (A) Amount of VEGF in the release medium at each time point (top) and the cumulative amount of VEGF released by each time point (bottom). The release of VEGF was triggered at Day 14 by treating the hydrogel with a 1:1 mole ratio of CS-V to Apt-V. (B) The amount of VEGF released during the 1-h treatment with CS-V, during the 23 h following the treatment, and the cumulative amount in the 24 h. * denotes a statistical significance of \( p \leq 0.05 \) in comparison to the sample not receiving the CS, ** denotes \( p \leq 0.01 \), and *** denotes \( p \leq 0.001 \).
Superporous hydrogels functionalized with Apt-PH and loaded with 2 µg PDGF-BB were next characterized. To study the regulatory capacity of CS-P on PDGF-BB, the system was treated twice at different time points using different concentrations of the CS. This is shown schematically in Figure 4.10. After 4 d of sustained PDGF-BB release, the system was treated with CS-P at a mole ratio of 1 CS to 2 aptamer for 1 h. During the 1-h triggering, 0.23 µg (12%) PDGF-BB was released. In comparison, only 0.00076 µg was released from the nontriggered hydrogel during the same time (Figure 4.11A). The total amount of PDGF-BB release during Day 5 was 0.31 µg (15%) PDGF-BB and 0.0038 µg PDGF-BB from the treated and the nontreated hydrogels, respectively (Figure 4.11A). After the ninth day of incubation, the hydrogel was treated again with CS-P, but at twice the concentration as before. During the 1-h treatment with the higher concentration of CS-P, 0.40 µg (20%) PDGF-BB was released. Without applying the CS, only 0.00082 µg was released (Figure 4.11B). The cumulative amount released on Day 10 was 0.72 µg (36%) PDGF-BB from the treated hydrogel and 0.004 µg PDGF-BB from the nontreated hydrogel (Figure 4.11B). The daily release profiles and the cumulative release profiles of the hydrogels treated with CS-P and not treated are shown in Figure 4.11C. In comparing the response of PDGF-BB release to the concentration of CS-P applied, the amount of PDGF-BB released doubled when the concentration of CS-P applied was doubled, showing a strong dose-dependent response. Thus, CS-P can be used to regulate the delivery of PDGF-BB from superporous hydrogels functionalized by triggering the delivery multiple times and in a concentration-dependent manner.

4.3.5. Bioactivity of Growth Factors is Preserved

To evaluate the bioactivity of the released VEGF from antiVEGF-functionalized superporous hydrogels, the ability of the collected supernatant to cause the organization of human umbilical vein-derived endothelial cells into capillary-like structures was assessed. Functionalized
Figure 4.10. Illustration of multiple applications of a CS to trigger the release of a growth factors many times. The amount of growth factor released at each time point can be regulated through the concentration of the CS applied.
Figure 4.11. Regulated release of PDGF-BB from Apt-H-functionalized superporous hydrogels loaded with 2 µg PDGF-BB. (A) Amount of PDGF-BB release on day 5 in the first 1 h (left) and 24 h (right); the molar ratio of CS-2:Apt-1 was 1:2. (B) Amount of PDGF-BB release on day 10 in the first 1 h (left) and 24 h (bottom); the molar ratio of CS-2:Apt-1 was 1:1. (C) Daily release profile of PDGF-BB (left) and cumulative release profile of PDGF-BB when the superporous hydrogel was treated with CS-P multiple times.
superporous hydrogels laden with 500 ng VEGF were incubated in collection medium, where the supernatant was totally collected and replenished with fresh medium periodically (Figure 4.9A). After 28 days of incubation, the functionalized hydrogels were treated with CS-V for 1 h to cause the release of VEGF. During this 1-h treatment, 26.5 ng VEGF/mL was released. In comparison, only 1.8 ng VEGF/mL was release from the hydrogels that were not treated with CS-V (Figure 4.12A). When the supernatant of the nontreated hydrogels was mixed with the HUVEC suspension, there was no organization of the cells after seeding (Figure 4.12B). When the supernatant was mixed with the HUVEC suspension, the cells organized into capillary-like structures within hours of seeding (Figure 4.12C). To confirm that it was the VEGF that was liberated from the hydrogel that stimulated the formation of tubes, VEGF from a stock solution was tested. Application of 0 ng VEGF/mL was not able to cause the organization of HUVEC (Figure 4.12D), whereas the addition of VEGF (10 ng/mL) was able to stimulate organization (Figure 4.12E).

As it is qualitatively shown that the released VEGF retained bioactivity, it became important to determine the percent bioactivity the released VEGF retained in comparison to untreated VEGF. For this, a proliferation assay based on the fluorescence detection of the amount of 5-ethynyl-2′-deoxyuridine (EdU) incorporated into HUVEC DNA was used. The same samples used to test the ability of VEGF to organize HUVEC into capillary-like structures were used to quantitatively measure the ability of VEGF to act as a HUVEC survival factor. The effective dose of VEGF was predetermined to be 5-10 ng/mL, therefore, the samples of released VEGF were diluted to 10 ng/mL. Lyophilized VEGF was reconstituted to a concentration of 10 ng/mL was used at the 100 % bioactivity benchmark. In comparison to the control, the 10 ng/mL of the released VEGF retained 88 % bioactivity (Figure 4.13). Taken together with the previous data, it is clear that functionalized superporous hydrogel are able to maintain the bioactivity of the growth factor for a period of at least 28 days.
Figure 4.12. Bioactivity of VEGF released from antiVEGF-functionalized superporous hydrogels loaded with 500 ng VEGF. Triggered samples were triggered on Day 28 for 1 h. During triggering, the amount of VEGF released into the supernatant became sufficient to organize HUVEC into tube-like structures. (A) 0.9 ng VEGF/mL from the superporous hydrogel not treated with the CS. (B) 23.1 ng VEGF/mL from superporous hydrogel treated with CS. (C) 0 ng VEGF/mL. (D) 10 ng VEGF/mL, stock solution. *** denotes a statistical significance of $p \leq 0.001$ between the compared data.
Figure 4.13. Quantitative analysis of VEGF bioactivity. Survival of HUVEC in the presence of VEGF was determined by fluorescence measurements of Edu incorporation into HUVEC DNA. Samples of released VEGF were collected after treating the antiVEGF-functionalized superporous hydrogels with CS-V for 1 h after incubation for 28 days. The VEGF concentration of the samples were quantified via ELISA then diluted to 10 ng VEGF/mL to be equal to the effective dose concentration. VEGF powder reconstituted at 10 ng/mL and never incubated (i.e., prepared VEGF) served as the 100 % bioactivity benchmark.
4.3.6. Growth Factors can be Sequentially Delivered

To determine the feasibility of aptamer-functionalized superporous hydrogels to regulate the delivery of growth factors in a sequential manner, a superporous hydrogel was functionalized with both Apt-V and Apt-PH and loaded with 200 ng VEGF and 200 ng PDGF-BB. At predetermined time points, the hydrogels were incubated a solution of various CSs for 1 h and a mole ratio of 1 CS to 1 aptamer (Figure 4.14). After the third day, CS-V was added and after the seventh day, CS-P was added. When treated with CS-V, the amount of VEGF released from the hydrogel increased from 0.5 ng (0.3 %) to 15.5 ng (7.8 %) on Day 4 (Figure 4.15A). In contrast, there was no increase in the amount of PDGF-BB released during that time (Figure 4.15B). When CS-P was added into the system, the amount of PDGF-BB released from the hydrogel increased from 1.4 ng (0.7 %) to 26.7 ng (13.4 %) (Figure 4.15A). In contrast, there was no increase in the amount of VEGF released due to the addition of CS-P (Figure 4.15B). The release profiles of VEGF and PDGF-BB from the dually functionalized superporous hydrogels are plotted together in Figure 4.15C.
Figure 4.14. Illustration of sequential applications of a CS to trigger the release of a growth factors specifically.
Figure 4.15. Simultaneous and sequential delivery of VEGF and PDGF-BB. (A) Delivery of VEGF. Left: Daily release of VEGF. Right: Cumulative release of VEGF. (B) Delivery of PDGF-BB. Left: Daily release of PDGF-BB. Right: Cumulative release of PDGF-BB. (C) Comparison of VEGF and PDGF-BB delivery. Left: Daily release; Right: Cumulative release. Aptamer-functionalized superporous hydrogels were loaded with 200 ng VEGF and PDGF-BB with a mole ratio of aptamer to growth factor of 10:1. CS-V was added after Day 3 for 1 h and CS-P was added after Day 7 for 1 h at a mole ratio of 1:1 CS to aptamer.
4.4. Discussion

In the previous chapter, it is shown that the rententive capabilities of two hydrogel systems could be enhanced through functionalization with aptamers. However, microparticle-based composite systems are limited in their ability to bind a large concentration of growth factors. Therefore, only the controlled delivery from aptamer-functionalized superporous hydrogels was analyzed in this chapter. When superporous hydrogels were functionalized with aptamers, there was a significant reduction in the burst release of growth factors in comparion to the release of growth factors from nonfunctionalized superporous hydrogels, where a significant percentage was immediately released. The reduction of the burst release is of note as the large and fast delivery of growth factors could result in side effects and decreased therapeutic efficiency in in vivo applications. In addition, the reduction of the burst release maintains a higher concentration of the growth factors within the composite system, thereby allowing the release of the growth factors to be sustained for a longer period. Currently, a number of resorbable collagen sponges loaded with various therapeutics are approved by the Food and Drug Administration for use in clinical testing or various clinical applications [59]. These sponges are highly porous, exhibit strong biocompatibility, and are easily degraded in vivo. To load the collagen sponge, a collagen matrix is first synthesized than soaked in a solution of the therapeutic to be loaded [375], much like the process of the loading growth factors into the superporous PEG hydrogels presented herein. However, the use of collagen sponges is associated with many problems. While the large porous network facilitates the rapid absorption of therapeutics, it also permits the rapid release of the therapeutics. For example, collagen sponges loaded with the analgesic bupivacaine are being investigated for the management of postoperative pain following major surgeries. The concentration of bupivacaine needed for the localized delivery is far lower than what is delivered by continuous systemic administration and prevents unwanted side effects like difficulty breath, sedation, and nausea and vomiting [376]. However, the delivery system can only provide local
analgesia for up to 96 hours [376]. Resorbable collagen sponges have been loaded with bone morphogenetic protein 2 (BMP-2) for use in spinal fusion and certain oral and maxillofacial surgical procedures [375, 377, 378]. However, the typical release profile shows a large initial burst release of ~30% of the loaded BMP-2 released in the first 24 h, and the release is sustained for less than one week [379]. Despite its approval for clinical use, reports are now forthcoming about the adverse side effects that are the result of the unregulated delivery of BMP-2 from the collagen sponge. Events reported include osteolysis/bone resorption, heterotopic bone, and cancer [380-383]. Therefore, slowing the delivery of therapeutics from highly porous materials is likely to alleviate many of these side effects. In addition, slowing the release of growth factors and other therapeutics is likely to increase the therapeutic efficiency by maintaining a lower but more constant concentration of the therapeutic locally [384]. Thus, aptamer-functionalized superporous hydrogels can address a significant problem facing the clinical use of porous material by significantly slowing the delivery of growth factors or other therapeutics.

In addition to showing that aptamer-functionalized composite systems can slow the release rate of growth factors, investigations into triggering or accelerating the delivery of the growth factors were pursued. The ability to trigger growth factor release has many important applications. For example, the development of blood vessels relies on the temporal delivery of certain growth factors [23, 347, 348]. Growth factors such as VEGF are first delivered to stimulate the proliferation and organization of endothelial cells into nascent vessels. After these premature vessels are formed, growth factors such as PDGF-BB are delivered to stabilize the vessels. Therefore, delivery systems that can be triggered to deliver growth factors at the times they are needed will be useful for the treatment of numerous diseases (e.g., ischemia).

As nucleic acid aptamers are single-stranded oligonucleotides, there exist oligonucleotides that have sequence complementarity to the aptamer. Therefore, it follows that as aptamers bind to their target with high specificity, their complementary sequences (CSs) can hybridize with the
aptamer through intermolecular base pairing to inactivate its binding function with high specificity. Various CSs and incubation conditions were used to test the responsiveness of the hydrogel. It was found that the large pores in the superporous hydrogel allow the aptamers to quickly hybridize with the CSs and to quickly release the bound growth factors. In addition, the amount of growth factor released correlates with the concentration of CS applied and the incubation time with the CS. The ability to regulate the release of a single growth factor species from many has been difficult to achieve in other delivery systems. Many strategies have been explored to trigger or accelerate the release of growth factors from a delivery system. For example, photolysis, mechanical compression, ultrasound, and pH and temperature gradients have been used to change the release kinetics of growth factors or other proteins [6]. These mechanisms change the structural integrity, volume, and/or pore sizes of the carrier to augment the release the loaded therapeutics accordingly. However, these mechanisms act to change the carrier, thereby affecting the release rate of all the loaded therapeutics nonspecifically. The nonspecific nature of these mechanisms makes the controlled delivery of multiple therapeutics with individualized release profiles difficult to achieve. This unsolved challenge may be addressed using CSs to specifically inactivate aptamers for the programmable delivery of multiple growth factors. To test the ability of aptamer-functionalized hydrogels to control the delivery of multiple growth factors, a superporous hydrogel was dually functionalized with the antiVEGF aptamer and the antiPDGF-BB aptamer and loaded with both VEGF and PDGF-BB. Dually functionalized superporous hydrogels were able to slow the delivery of both growth factors, showing that aptamer-functionalized hydrogels can be used for the sustained delivery of multiple growth factors. In addition, treatment of the functionalized delivery system with specific CSs shows that the release of growth factors can be triggered specifically. When a CS specific for the antiVEGF aptamer was applied, only VEGF was released from the hydrogel. Similarly, when a CS specific for the antiPDGF-BB aptamer was applied, only PDGF-BB was released from the hydrogel. Thus, aptamer-functionalized hydrogels can be used to control the
delivery of multiple growth factors, were the delivery of growth factors can be triggered specifically in distinct phases that correlate to the need of the body to cure complex diseases. In addition to the different roles of each growth factor and the time-dependence of each, many growth factors have diminished effects when delivered simultaneously with other growth factors, instead of sequentially [374], further highlighting the importance that a delivery system should release the appropriate growth factors in separate stages.

The nature by which the CSs can trigger growth factor release has not been well elucidated. In the absence of the CS, growth factors passively dissociate from the aptamer to elute the hydrogel or, if the concentration of aptamers in the hydrogel is high, rebind to an aptamer in their diffusion pathway. As there is a molar excess of aptamers in the delivery systems, an aptamer can exist in one of two states: a state where the aptamer is binding the growth factor or one where the aptamer is not in an associated state with the growth factor. This creates two possible hybridization reactions for triggered growth factor release. In the first scenario, if the CS hybridizes with a free aptamer, then the growth factor will not be able to rebind once it passively dissociates from an aptamer. In the second scenario, if the CS hybridizes with an aptamer that is binding a growth factor, the growth factor will be actively displaced due to the inactivation of the aptamer. The determine which mechanism is responsible to for the rapid release of growth factors upon introducing the CS, two shortened CSs were selected that hybridize with various regions of Apt-PH. The first CS, CS-P9, hybridizes at the 5’-end of the aptamer while the second, CS-P10, hybridizes at the 3’-end. Both CSs are able to hybridize with Apt-PH, but CS-P9 is expected to trigger PDGF-BB release, while CS-10 should not be able to trigger PDGF-BB release. Fluorescence imaging shows that both CSs are able to hybridize with aptamers in the hydrogel. Analysis of PDGF-BB release shows that only CS-P9 was able to trigger the release of PDGF-BB during the treatment with the CSs, but both CSs would moderately increase the release rate of PDGF-BB, as seen during the sustained release of PDGF-BB after the free CSs were removed from solution. Taken together, the results suggest that
the two mechanisms are responsible for triggered growth factor release. The least effectively mechanism for accelerating growth factor release occurs when the CS hybridizes with free aptamers to prevent rebinding of a growth factor once the growth factor has passively dissociated from an aptamer. The other mechanism, when the CS hybridizes with an aptamer that is binding a growth factor to actively displace the growth factor, results in a significantly greater growth factor release. The latter mechanism however, can only occur if the CS has sufficient hybridization strength. In this study, the hybridization strength of the CS with the antiPDGF-BB aptamer was increased by adding a linear toehold region to the aptamer sequence.

Growth factors have a low stability in solution. Disruption of the secondary and tertiary structure (denaturation) can occur easily during routine handling and are easily influenced by external conditions like temperature and pH. As denatured growth factors are of little use in the treatment of diseases, it is important that the developed growth factor delivery system maintains the bioactivity of the growth factor for as long as the system is used. The ability of aptamer-functionalized hydrogels to store and protect a growth factor was evaluated after 28 days of incubation in solution. Without treating the hydrogel with a CS, the amount of VEGF released from the hydrogel was low and unsufficient to stimulate HUVEC activity. When the hydrogel was treated with the CS for 1 h, the amount of VEGF liberated from the hydrogel was sufficient to stimulate HUVEC activity. Thus, aptamer-functionalized superporous hydrogels can be long-acting depots of growth factors and be triggered to quickly release an active concentration of growth factors. The ability of the functionalized superporous hydrogel to maintain the bioactivity of the growth factor can be attributed to many factors. First, the loading of growth factors into the hydrogel occurs postsynthesis. This circumvents the harsh reaction conditions used to form the hydrogel. Second, the association and dissociation of the growth factor with the aptamer occurs through noncovalent interactions and the growth factor does not need to be tagged or conjugated to other biochemical moieties. Lastly, the formation of the complex between the aptamer and the growth factor protects
the growth factor from enzymatic degradation. Thus, aptamer-functionalized growth factor delivery systems are possible tools for the long-term delivery of bioactive growth factors and the treatment of diseases.
4.5. Conclusions

In this chapter, a composite system and a superporous hydrogel were functionalized with nucleic acid aptamers for the controlled delivery of growth factors. The models used were an antiVEGF aptamer, an antiPDGF-BB aptamer, and their target growth factors. The addition of the aptamers was able to greatly slow the release rate of growth factors form the superporous hydrogel. Without functionalization, the release of growth factors could not be sustained beyond a period of a few days. With functionalization, the release of growth factors could be sustained for at least 28 days. In addition, as the aptamers are directly incorporated into the hydrogel network and do not rely on rigid supports, the functionalized systems were able to control the delivery of a clinically relevant concentration of growth factors. Unlike other functionalized systems, the release of loaded growth factors can be precisely regulated by using aptamer-functionalized delivery systems and applying desired concentrations of CSs to inactivate the aptamers. In addition, as aptamers have a high specificity for their target, multiple aptamers can be used to functionalize a hydrogel for the delivery of multiple growth factors with individualized release kinetics. As CSs are specific to the aptamers, desired CSs can be applied to trigger the release of growth factors in discrete stages, which has not been achieved with other functionalization strategies. Lastly, as the binding of the growth factor to the aptamer and the release of the growth factor through hybridization occur through noncovalent interactions and nondestructive means, the released growth factor maintains a high percentage of its bioactivity. Thus, aptamer-functionalized growth factor delivery systems are able to control the delivery of a large concentration of multiple growth factors, making them possible tools for the treatment of complex diseases.
5.1. Conclusions

In this work, the development of aptamer-functionalized systems for the delivery of growth factors was pursued. To utilize aptamers as the affinity ligands, the molecular recognition properties between aptamers, growth factors, and complementary sequences (CSs) were first characterized. Microparticles functionalized with aptamer were used to evaluate the ability of immobilized aptamers to bind their target growth factors. Quantification of VEGF and PDGF-BB in solution shows that both the antiVEGF aptamer and the antiPDGF-BB aptamer remain functional and could bind their target. The possibility of inactivating the aptamers to trigger the release of bound growth factors was also investigated. Comparisons between the ability of CSs to hybridize with their aptamer revealed that secondary structure of the aptamer and steric hindrance between the aptamer and the microparticle greatly influence hybridization efficiency. Aptamers that exhibit little or no secondary structure can hybridize with its CS more easily, whereas aptamers with a large number of intramolecular base pairs have a number of hydrogen bonds that must be overcome prior to hybridization. In addition, the close proximity of the aptamer to the surface of the microparticle can hinder the ability of the CS to hybridize with the aptamer. A linear oligonucleotide segment can be added to the aptamer to function as both a spacer to distance the aptamer from the microparticle and impart more “solution-like” behavior and as a toehold to promote aptamer inactivation as hybridization occurs more easily with nonbonded nucleotides. The antiVEGF
aptamer and the antiPDGF-BB aptamer optimized for hybridization were then used for functionalization of various systems for the controlled delivery of VEGF and PDGF-BB.

Composite systems and superporous hydrogels functionalized with the aptamers were able to control the release of the growth factors effectively. In comparison to their nonfunctionalized counterparts, the release rate of the growth factors was much slower for both aptamers. However, the ability to load a large concentration of growth factors into the composite system was limited by the large density of the microparticles. When a large concentration of microparticles were loaded into the composite system, network defects were formed that lead to increased release rates. This problem was not experienced with the superporous hydrogels, which does not rely on microparticles to immobilize to the aptamers. Functionalized composite systems and functionalized superporous hydrogels were treated with the appropriate CS to trigger growth factor release. The composite systems and the superporous hydrogels could be treated with the CS to release a large percentage of the loaded growth factors. When the superporous hydrogels were treated with a CS multiple times, it was found that the release of growth factors could be triggered multiple times and that the amount of growth factor release was proportional to the concentration of the CS used. Lastly, because the CSs hybridize with aptamers in a sequence specific manner, the release of multiple growth factors could be individually triggered. Thus, aptamer-functionalized growth factor delivery systems are versatile tools that could be useful in the treatment of numerous complex diseases.

5.2. Future Work

The work presented herein has provided many insights for the use of aptamer in controlling the delivery of growth factors. However much work remains to fully understand the potential of using aptamer-functionalized delivery systems for the treatment of diseases.
5.2.1. In Vivo Validation

While it was shown that the growth factors would retain their bioactivity to stimulate cellular processes *in vitro*, even after a month of incubation, it remains to be seen if the growth factors delivered from aptamer-functionalized delivery systems can stimulate cellular processes *in vivo* using a murine model. While *in vitro* cell assays may indicate positive results, they are often too simplified and do not reflect the *in vivo* complexities well. For example, commercially sourced endothelial cells are preselected for their proliferative capacity and do not take into account heterospecific cell interactions [385].

To evaluate the bioactivity of the growth factors *in vivo*, an angiogenesis assay will be used. For this assay, various superporous hydrogels will be loaded with VEGF, as this growth factor is a potent stimulator of angiogenesis by activating several key processes needed for the development of new blood vessels (Figure 5.1). To test the delivery of VEGF from the superporous hydrogels, subcutaneous pockets will be made in the dorsa of the mice, into which the hydrogels will be implanted. Three groups will be evaluated: 1) superporous hydrogels loaded with a saline solution, 2) superporous hydrogels loaded with VEGF, and 3) superporous hydrogels functionalized with the antiVEGF aptamer and loaded with VEGF. The first group will test to evaluate if the surgical procedure itself would result in angiogenesis as a result of the inherent healing capabilities of the host. Thus, this group is needed to evaluate any false positives that may result. The second and third group will be compared to evaluate the enhancement of blood vessel growth due to the addition of the aptamer. As the second group is not functionalized with the antiVEGF aptamer, it is expected the will be rapidly released from the hydrogel and inefficient use of VEGF will poorly stimulate the formation of blood vessels, if at all. Only the third group is expected to result in the formation of a large number of blood vessels, as the release of VEGF from the aptamer-functionalized hydrogel is well controlled. If the third group stimulates significant
Figure 5.1. Vascular endothelial growth factor signal transduction.
angiogenesis, then it can be concluded that aptamer-functionalized systems can control the delivery of growth factors \textit{in vivo}.

In addition, as a high concentration of aptamers results in less growth factor released from the hydrogel \textit{in vitro}, it would be interesting to determine if a high antiVEGF concentration can prevent angiogenesis by preventing VEGF delivery \textit{in vivo}. If such a case is possible, then the ability of a CS to stimulate angiogenesis through the inactivation of the aptamer can be evaluated. A CS can be injected into the systemic circulation where the blood flow will distribute the CS throughout the body and to the site of the implant or injected locally at the site of hydrogel implantation. If the CS can inactive aptamers to release the bound VEGF and to stimulate angiogenesis, then it can be concluded that hybridization can occur in complex environments and may be used to specifically trigger the release growth factors \textit{in vivo}.

\textbf{5.2.2. Programmable Extracellular Matrix Mimics}

Native extracellular matrices (ECMs) are intricate biological constructs mediating vital processes in physiological systems. Extracellular matrices are comprised of adhesion molecules, insoluble macromolecules, and soluble signaling molecules (\textit{e.g.}, growth factors). These molecules interact with each other in complex and dynamic manners to regulate cellular and system processes. For example, proteoglycans in the ECM help hydrate the matrix and the attached cells while sequestering and storing signaling molecules. Changes in physiological conditions will initiate proteolytic enzymes to degrade the proteoglycans and release the bound growth factors, which then diffuse to nearby cells for the activation of chemical signal transduction \cite{386, 387}. The process of activating signal transduction pathways is complex as different signaling molecules are released in different stages, depending on the need of the tissue \cite{388}. Thus, aptamer-functionalized growth factor delivery systems can be used as a synthetic analogue of the biochemical signaling aspect of ECMs, where CSs act like the proteolytic enzymes that liberate growth factors.
To create a more biomimetic materials, aptamers can be used to mimic the biophysical aspects of the ECM. Aptamers have been screened against cellular targets to isolate aptamers that bind to cell-surface receptors or other exposed proteins. For example, an aptamer that binds to protein tyrosine kinase 7 of T-cell acute lymphoblastic leukemia cells [389], an aptamer that binds to E-selectin of endothelial cells [390, 391], and an aptamer that binds the αvβ3 integrin [392] have been isolated. If the aptamer for any these targets are immobilized onto a substrate, then cells expressing the target can be specifically bound onto a substrate of which it may have had difficulties adhering otherwise [393]. Aptamer-functionalized cell scaffolds will allow specific cells to grow within an implant to achieve desired cell population. In addition, cell adhesion to an artificial ECM through an immobilized aptamer that binds integrins, for example, may initiate intracellular signaling pathways, such as cell migration. If combined with an appropriate growth factor delivery system, then the growth and other biological processes of the cells can be controlled to achieve various goals (e.g., carrier-tissue integration).
Figure A.1. Concentration-dependent binding sensorgrams. (A) Sensorgrams for Apt-PH. (B) Sensorgrams for Apt-PM. (C) Sensorgrams for Apt-PL.
Figure A.2. Correlation between the mole ratio of the CS to the aptamer applied and the amount of PDGF-BB released in the various times. (A) Amount of PDGF-BB released during the 1-h treatment with the CS. (B) Amount of PDGF-BB released in the 23-h following the treatment with the CS. (C) Cumulative amount of PDGF-BB released during the 1-h treatment with the CS and the 23 h following the treatment. The mole ratios of CS to aptamer tested are 1:4, 1:2, 1:1, and 2:1.
Appendix B

Growth Factor Release from Composite Systems

B.1. Experimental Procedure

B.1.1. Materials

Streptavidin-coated polystyrene microparticles (1.3 μm) were purchased from Spherotech (Lake Forest, IL). Phosphate buffered saline (PBS), Tween 20, sodium azide (NaN₃), and a premixed solution of acrylamide and bisacrylamide (40% solution, 29:1 acrylamide:bisacrylamide) were purchased from Fisher Scientific (Suwanee, GA). Biotinylated aptamers and their complementary sequences (CSs) were purchased from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2.1. Bovine serum albumin (BSA) was purchased from Invitrogen (Grand Island, NY). Vascular endothelial growth factor (VEGF) and platelet-derived growth factor-BB (PDGF-BB) enzyme-linked immunosorbent assays kits were purchased from PeproTech (Rocky Hill, NJ). The molecular weight of VEGF and PDGF-BB are 38.2 and 24.3 kDa, respectively.

B.1.2. Methods

B.1.2.1. Preparation of Aptamer-functionalized Particles and Composite Hydrogel

One hundred sixty picomoles of biotinylated aptamers were incubated with 80 μg streptavidin-coated microparticles in 46.4 μL PBS for 30 min. The microparticles were washed four times with PBS supplemented with 0.1% BSA and 0.05% Tween 20. Following washing, 4 ng
VEGF or PDGF-BB was added to the microparticle suspension and incubated at room temperature for 2 h. After immobilizing the proteins, 140 μL agarose solution at 42 °C was added to the microparticle suspension and 50 μL volumes were quickly aliquoted into cylindrical molds. The final agarose concentration was 0.5 % (w/v).

**B.1.2.2. Confocal Microscopy**

Prior to the microscopy examination, the two microparticles were functionalized Apt-V and Apt-PH, individually. Then the microparticles were mixed together and added to agarose. The composite system was then incubated in a solution of FAM-labeled CS-V and TYE-labeled CS-P, washed, and a thin slice of the composite system was removed and analyzed. The section of the composite hydrogel with fluorescently labeled microparticles was visualized using a Nikon A1R Spectral Confocal Microscope (Nikon Instruments Inc., Melville, NY). The 6-FAM fluorophore was excited at 488 nm with an argon blue laser and the TYE 665 fluorophore was excited at 633 nm with a helium-neon red laser. A volume of 70 μm by 70 μm by 15 μm was scanned.
B.2. Results

B.2.1. Aptamer-functionalized Composite Systems can Slow the Release of Growth Factors

Microparticles functionalized with either the antiVEGF or the antiPDGF-BB aptamer and their respective molecular partner were used to prepare aptamer-functionalized composite system and the ability of these systems to control the delivery of their growth factors over time was evaluated. Analysis of the composite system not functionalized and functionalized with the antiVEGF aptamer (Apt-V) reveals that the immobilization of the aptamer onto the microparticles was able to slow the release of VEGF from the composite system (Figure B.1A). At Day 1, a total of 583 pg VEGF (58%) of the loaded 1000 pg was released from the nonfunctionalized composite sytems, whereas only 239 pg (24%) of the loaded VEGF was released. Analysis of the totals at Day 5 show that 774 (77%) and 334 (33%) of the initial VEGF was released from the nonfunctionalized and the antiVEGF-functionalized composite system, respectively. Approximately 800 pg (80%) and 366 pg (37%) VEGF was cumulatively released during the ten days of release from the nonfunctionalized composite and the functionalized composite system.

The release of PDGF-BB from the various composites system over ten days was similarly studied (Figure B1.B). The quantity of PDGF-BB release by Day 1 was 544 pg (54%) and 136 pg (14%) from the nonfunctionalized composite system and the composite system functionalized with the high affinity antiPDGF-BB aptamer, respectively. At Day 5, 660 pg (66%) PDGF-BB was released from the composite system, whereas 169 pg (17%) was released from the functionalized composite. At the end of the release study 709 pg (71%) and 216 pg (22%) of the initial 1000 pg PDGF-BB was released from the composite system and the functionalized system, respectively.
Figure B.1. Release of growth factors from composite systems. (A) Release of VEGF from composite systems without and with functionalization of Apt-V. (B) Release of PDGF-BB from composite systems without and with functionalization of Apt-P.
B.2.2. Complementary Sequences can Penetrate Composite Systems and Specifically Hybridize with Immobilized Aptamers

In previous studies, it is shown that oligonucleotides with a sequence complementary to the aptamer can be used to inactivate the aptamer to release bound growth factors. Therefore, the ability of a CS to permeate into the composite system and hybridize with the aptamers on the entrapped microparticles was evaluated. Incubation of functionalized composite systems with the appropriate CS conjugated with the FAM fluorophore showed that the CS could penetrate the composite system and remain within the hydrogel after thorough washing (Figure B.2A). To determine if the CS would hybridize with the aptamer to trigger the release of the growth factors, the composite systems functionalized and loaded with the two aptamers and growth factors were incubated in a solution containing 250 nM of CS for 1 h and measured after 24 h (Figure B.2B). Quantification of the amount of VEGF in the supernatant shows that without the treatment of the CS-V, only 18 pg of VEGF eluted the hydrogel. With the application of CS-V, 281 pg of VEGF was found in the supernatant. Therefore, the addition of 250 nM CS-V triggered the release of 263 pg of VEGF into the supernatant.

A parallel study evaluating the ability of CS-P to penetrate the affinity composite system and hybridize with immobilized Apt-PH was also conducted (Figure B.2B). Quantification of PDGF-BB released in 24 h shows 21 pg of PDGF-BB eluted the composite system. In comparison, 208 pg of PDGF-BB was released from the functionalized composite system when treated with 250 nM of CS-P, 187 pg more PDGF-BB over the nontreated composite system. Therefore, the addition of CS-P can trigger the release for PDGF-BB from composite systems functionalized with Apt-PH.

After showing that the CS can permeate the composite systems and hybridize with the immobilized aptamers to accelerate growth factor release into the supernatant, studies evaluating the specificity of hybridization were performed. Native polyacrylamide gel electrophoresis of the hybridized pairs (Apt-V and Apt-PH with CS-V or CS-P) revealed that the hybridization is specific
Figure B.2. Ability of CSs to hybridize with aptamers in functionalized composite systems. (A) FAM-labeled CS-V can penetrate the composite system and hybridize with Apt-V (left) and FAM-labeled CS-P can penetrate the composite system and hybridize with Apt-PH (right). (B) Triggered release of growth factors using 250 nM CS. Left: VEGF. Right: PDGF-BB.
(Figure B.3A). That is, CS-V hybridizes with Apt-V only and CS-P hybridizes with Apt-PH only. Because the presence of the microparticle can affect the ability of the aptamer to hybridize with its CS, flow cytometry was used to evaluate the ability of fluorescently labeled CSs to hybridize with immobilized aptamers. The results show that CS-V conjugated with FAM specifically hybridized with Apt-V and not Apt-PH, as indicated by the large rightward shift in the flow cytometry histograms (Figure B.3B). Similarly, CS-P labeled with TYE 665 labeled the microparticles functionalized with Apt-PH and not the microparticles functionalized with Apt-V (Figure B.3C). Therefore, hybridization of the CS with the aptamer remains specific in the presence of the microparticles. Functionalized composite systems were incubated in solutions of fluorophore-labeled CSs to evaluate the specificity of hybridization when the microparticles are entrapped within a hydrogel. Fluorescence images of the composite systems after incubating the composite systems show that CS-V remains in the system functionalized with Apt-V after thorough washing, but is removed from the system functionalized with Apt-PH (Figure B.3D). Similarly, fluorescence images of composite systems incubated with CS-P show that the system functionalized with Apt-V did not retain CS-P, whereas systems functionalized with Apt-PH did (Figure B.3D). Therefore, CSs are able to permeate the hydrogel of the composite systems and can hybridize with their aptamer specifically.

B.2.3. Aptamer-functionalized Composites Systems can Specifically Trigger Growth Factor Release

After showing that CSs can specifically label functionalized composite systems through hybridization, a functionalized composite system to evaluate the specificity of hybridization in a composite system loaded with two growth factors was sought. To prepare this composite system, one set of microparticles will be functionalized with Apt-V aptamers and loaded with 1 ng VEGF and the second set will be functionalized with Apt-PH and loaded with 1 ng PDGF-BB. After
Figure B.3. Aptamer hybridization with CS. (A) Gel electrophoresis of hybridization. (B) FAM-labeled CS-V was able to bind specifically to Apt-V1 (top), whereas there was little binding of TYE-labeled CS-P5 (bottom). (C) There was no binding of FAM-labeled CS-V (top) to Apt-PH, but there was specific hybridization with TYE-CS-P (bottom). (D) Specific aptamer-CS hybridization in hydrogels.
binding the growth factors to the microparticles, the two microparticle sets will be mixed together and added to an agarose to create a dually functionalized composite system. The composite system will then be treated at one time point with one CS and the amount of VEGF and PDGF-BB released from the hydrogel will be quantified. Then, some time after triggering the release of the first growth factor, the second CS will be applied and the amount of VEGF and PDGF-BB released from the composite system will be quantified. This is shown schematically in Figure B.4A.

Scanning laser confocal microscopy was used to show that the two microparticle systems could be incorporated into agarose and that they retain their ability to hybridize with their CS. The dually functionalized composite system was treated with a solution containing CS-V conjugated with FAM and CS-P conjugated with TYE 665. As the microparticles were functionalized as individual sets, this would allow the emission of the two fluorophores to be easily distinguished. Excitation of the two fluorophores in the removed section of the composite system shows that the microparticles were well distributed in the volume and no aggregates formed (Figure B.4B). In addition, inspection of the merged fluorescence image reveals that the CSs were able to label the microparticles specifically.

To show that multiple functionalized microparticles can be incorporated into a hydrogel without negatively affecting the ability of them to control the delivery of growth factors, the release of growth factors from the dually functionalized composite system was individually investigated. As shown in Figure B.4C, the release of VEGF could still be sustained. There was a small burst release of VEGF, where approximately 15% of the loaded VEGF was released during the first day. The ability to trigger the release of VEGF was also reevaluated. After the fourth day, the release of VEGF was triggered using CS-V for 1 h. The amount of VEGF release during the next day was 14% of the loaded VEGF. In comparison, the amount released from the dually functionalized composite system without CS-V added was only 1% during the same period (Figure B.4C). Thus,
Figure B.4. Programmable release of VEGF and PDGF-BB. Illustration of sequential growth factor delivery. Both loaded microparticles are loaded into agarose to form a dually functionalized composite system. The application of one CS will trigger the release of one growth factor from the microparticles and later the second CS can be applied to trigger the release of the second growth factor. (B) Laser confocal micrograph of the two aptamer-functionalized particles in the hydrogel. Green: Microparticles labeled with FAM-CS-V; Red: Microparticles labeled with TYE665-CS-P. Scale bar: 10 μm. (C) Sustained release and triggered release of VEGF on Day 4. (D) Sustained and triggered release of PDGF-BB on Day 8. (E) Profiles of daily release of VEGF (green) and PDGF-BB (red) regulated via sequence-specific CSs. CS-V and CS-P were added to the release medium on Days 4 and 8, respectively. Each triggering time was 1 h.
the microparticles functionalized with Apt-V can prevent the rapid release of VEGF and retain their ability to release the bound VEGF in response to hybridization with CS-V.

The release of PDGF-BB was examined from the same dually functionalized composite system as mentioned above (Figure B.4D). There was a small burst release of PDGF-BB in the first 24 h of incubation. Approximately 8% of the loaded PDGF-BB was released from the composite system during this time. After the burst release, there was a period where very little PDGF-BB (<1%) was released each day. After the eighth day of release, the composite system was treated with CS-P for 1 h and the amount of PDGF-BB released the next day was quantified. Without the addition of CS-P, only 0.4% of the initial PDGF-BB was released between Days 8 and 9. In contrast, 6% of the PDGF-BB was released when CS-P was added. Thus, the addition of a multiple functionalized microparticles and growth factors did not prevent the sustained release of PDGF-BB and the application of CS-P could trigger the release of PDGF-BB from the microparticles that could then elute the composite system.

To determine the specificity of triggering growth factor release, the triggered release of VEGF and PDGF-BB from the dually functionalized composite system was investigated (Figure B.4E). Analysis of the two release profiles by superimposing the two release profiles shows that on Day 5, 14% of VEGF was released due to the addition of CS-V, whereas there was no increase in the amount of PDGF-B released. The amount of PDGF-BB released on this day was 0.5%. On Day 9 (four days after the addition of CS-V), analysis of the amount of growth factor released due to the treatment of the composite system with CS-P reveals that 6% of the PDGF-BB was release, while only 0.8% of the VEGF was released. Thus, the application of CS-P triggered the release of PDGF-BB and not VEGF. Taken together, CSs can be used to specifically trigger the release of desired growth factors from composites systems functionalized with multiple aptamers.
B.3. Discussion

In this work, the delivery of growth factors from composite systems was characterized. Functionalization of the composite systems individually with Apt-V and Apt-PH proved to be a useful strategy for slowing the delivery of VEGF and PDGF-BB, respectively. By incorporating the aptamers onto the microparticles was able to reduce the burst release significantly. However, the concentration of growth factors used in this was on the order of picograms. In order to apply these systems for in vivo applications, the concentration loaded and delivered should be on the order of nanograms to even milligrams.

As aptamers and their CSs are sequence-specific, it is possible that CSs can be used to trigger growth factor release from aptamer-functionalized growth factor delivery systems specifically. To show that aptamers can be inactivated to release growth factors specifically, a dually functionalized composite system was developed using VEGF and PDGF-BB and their respective aptamers. The composite system was first treated with the CS that is complementary to the Apt-V aptamer. Quantification of the growth factors released from the hydrogel shows that only VEGF release was triggered. Similarly, when the system was treated with the CS complementary to Apt-PH, only PDGF-BB was released. Thus, CSs can be used to regulate the delivery of multiple growth factors with individualized release kinetics (e.g., phasic delivery). However, as the composite systems rely on polymeric supports to immobilize aptamers, the use of these systems for disease treatment may be limited.
References


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