Biodegradable Colloidal Gels as Malleable Tissue Scaffolds and Injectable Drug Carriers

By

Qun Wang

M.S., Environmental Science, Wuhan University, Wuhan, China, 2004
B.S., Environmental Engineering, Zhejiang University of Technology, Hangzhou, China, 2000

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Committee Members:

Dr. Cory Berkland, Committee Chair

Dr. Michael Detamore

Dr. Teruna Siahaan

Dr. Stevin Gehrke

Dr. Jenn-Tai Liang

Sep. 24, 2010
Date Defended

The Dissertation Committee for Qun Wang Certifies that this is the Approved Version of the Following Dissertation:

Biodegradable Colloidal Gels as Malleable Tissue Scaffolds and Injectable Drug Carriers

Committee Members:

Dr. Cory Berkland, Committee Chair

Dr. Michael Detamore

Dr. Teruna Siahaan

Dr. Stevin Gehrke

Dr. Jenn-Tai Liang

Oct. 6, 2010

Date Approved

ABSTRACT

Repair of skeletal defects resulting from traumatic insult, tumor ablation, or congenital deformities remains a formidable challenge for clinicians. From a clinical perspective, the use of injectable materials is an attractive alternative to surgery as it reduces the risk of infection, scar formation, patient discomfort and the cost of treatment. Particularly, injectable scaffolds injected or extruded at low viscosity may be ideal scaffolds for bone repair or for delivery of drugs or cells to injured tissue. Such an approach is minimally invasive and is capable of filling complex 3D defects of irregular size and shape, but achieving a desirable injectable material for these defects is challenging.

Nanotechnology, systems at sizes generally ranging between 1 and 1000 nm, is expected to have an important impact on all industries including semiconductors, manufacturing, pharmaceutics, and biotechnology. Colloidals are a nanostructured system in which the dispersed phase is so small that gravitational force is negligible and interactions are dominated by short-range and temporary forces, such as van der Waals force, electrostatic force, and/or steric force. The unique properties of high concentration, cohesive colloidal gels investigated here make it a potential candidate for injectable filler to repair bone, such as cranial defects.

The objective of this thesis is to use oppositely-charged poly (D,L-lactic-co-glycolic acid) (PLGA) nanoparticles to create a novel cohesive colloidal gel. The relationship between composition of the gels and bulk properties has been explored. Oppositely-charged colloids self-assembled through interparticle

interactions resulting in a stable 3-D network that was easily molded to the desired shape. Rheological tests on colloidal gels showed shear-thinning behavior and reversibility, in that viscosity was recovered. Cell seeding and viability tests with human umbilical cord mesenchymal stem cells (HUCMSCs) indicated excellent biocompatibility with these cells. Drug release from dexamethasone (DEX) loaded colloidal gels followed near-zero order kinetics over two months. These materials were also implanted in rats and histological and histochemical analyses confirmed that the PLGA colloidal gels stimulated bone formation in rat cranial bone defects.

This thesis reports PLGA colloidal gels as novel injectable drug-loaded fillers desirable for promoting reconstruction and regeneration of cranial defects. Similar systems can also be utilized with extended applications in other areas, including repairing different tissue defects and providing long-term, local drug delivery.

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CHAPTER I

Nanostructured Materials for Applications in Tissue Engineering and Drug Delivery

1. Introduction

Nobel Laureate Richard Feynman in his historical lecture "*There is plenty of room at the bottom*"[1] inspired the concepts for the rapidly exploding research topic of nanotechnology. Thereafter, scientists have learned that the manipulation of atoms, molecules, and clusters of the same on surfaces is possible. Also, the new fundamental principles govern the properties of nanomaterials have been studied. Today, nanotechnology has already become to a burgeoning, multidisciplinary scientific field that applies engineering and manufacturing principles at the nano scale.[2] Nanotechnology and nanofabrication have significantly impacted the field of biomedicine where techniques have shifted from microfabrication and micromachining to the nanometer scale.[3] Tissue engineering applications have also aided the development and implementation of nanometer-sized components. In the age of genetic technology, transfection systems with nanostructure are being modified by using polymers or colloids for different applications. An improved understanding of normal physiological processes is being achieved by creating artificial cells or by using nanomaterials to probe physiologic properties. These achievements are only several out of many that have been recently accomplished through nanotechnology. Tissue engineering and drug delivery are two major medical fields which have been deeply influenced by nanotechnology. Since the aim of this thesis is to create a novel injectable nanostructured material with combined features beneficial for tissue scaffolding and for drug delivery, some new nanostructured materials utilized in tissue engineering and drug delivery systems will be reviewed in this chapter.

2. Fabrication of Nanostructured Materials

The building blocks of nanostructured materials are normally oriented from biomolecules, synthetic polymers, silicon-based materials, carbon-based materials, and metal-based materials. These materials are manipulated into nanoscale formats and used for tissue engineering and drug delivery systems. Often, the nanomaterials themselves are integrated or assembled into bulk materials with unique properties. Here, emphasis will be placed on tissue engineering scaffolds and drug delivery systems based on nanostructured materials.

2.1. Nanostructured Materials Made by Biomolecules

The combination of biotechnology and nanotechnology has developed new nanomaterials which incorporate properties of biomolecules and features of nanoparticles.[4-7] Biomolecules are important building materials for nanoparticles. Biomolecules can display specific and strong complementary recognition interactions which result in biomolecule-nanoparticle recognition interactions and self-assembly. Various biomolecules contain several binding sites which allow the multidirectional growth of nanoscale structures. Some small biomolecules, such as protein, can also be genetically engineered and modified with specific anchoring groups which facilitate their aligned binding to nanoparticles, or the site-specific linkage of the biomolecule to particle surface. As a result, the directional growth of nanoparticle structures may be dictated. For example, in Figure 1.1, Chitosan-DNA nanoparticles were synthesized from the complexation of the cationic polymer with a β-gal DNA plasmid

for gene delivery.[8] Furthermore, the application of biocatalysts for the replication of biomolecule-nanoparticle conjugates may provide an effective method for the formation of nanostructures of predesigned shapes and compositions.

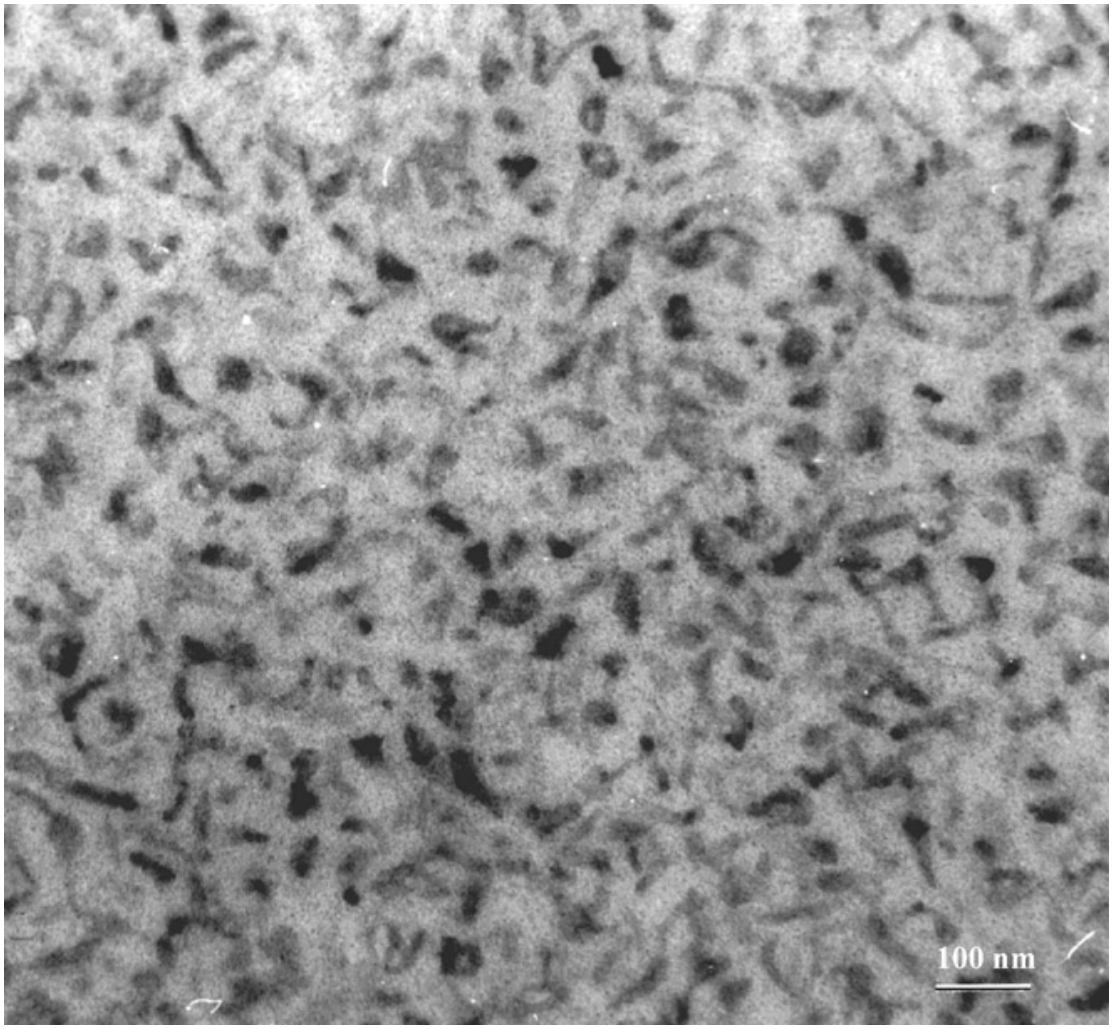

Figure 1.1. Transmission electron microscopy of chitosan-DNA nanoparticles. Homogenous distribution of DNA is seen following staining with uranyl acetate and lead citrate. Bar = 100 nm (Reprinted from reference [8] with permission)

Right now, researchers are devising new methods to mimic, enhance and consolidate the functionalizations of these biological nanostructures.[9-10] To get desired nanostructures, different fabrication methods are used depending on the type of the material. The new methods used to assemble biomolecule-based nanoscale materials

include molecular self-assembly,[11-12] biological aggregation,[13] molecular printing,[14] layer-by-layer electrostatic deposition,[15-16] vapor deposition,[17] and scanning probe manipulation.[18]

The biomolecule-based nanostructure which have been developed for drug delivery systems and tissue engineering scaffolds include nanoparticles,[19-21] lipids,[22-24] nanotubes,[25] emulsions,[26-28] liposomes,[29-32] large molecules,[33-36] peptides,[37-38] viral nanostructures[39] and nucleic acid nanostructures.[40]

2.2. Nanostructured Materials Made by Synthetic Polymers

Synthetic polymers exhibit many desirable properties for drug delivery systems and tissue engineering scaffolds. These properties include biocompatibility, biodegradability, function capability, and low cytotoxicity. Biodegradable polymeric materials are promising for drug delivery and tissue scaffold applications.[41] For example, through functional and structural manipulation of synthetic polymers, drug molecules can be associated with polymer molecules and scaffolds with specific structures.

Some polymeric nanoparticles are consisting of polylactic acid (PLA), polyglycolic acid (PGA), or a copolymer of PLA and PGA. They have been investigated as carriers for anticancer drugs,[42-44] vaccines,[45-46] proteins,[47-48] genes,[49] ocular drugs,[50-51] or cytokines.[52] Other synthetic polymers studied for nanostructured materials include polyalkylcyanoacrylate,[53] poly (3-hydroxybutanoicacid)(PHB),[54] poly(organophosphazene)(POP),[55] poly(ethylene glycol)(PEG),[56-58]

poly(caprolactone)(PCL),[59-60] poly(ethylene oxide)(PEO),[61] and copolymers such as PLA-PEG.[62-63]

Methods employed to make nanoscale materials by synthetic polymers include multisolvent emulsion evaporation,[64] interfacial polymerization,[65] mold replication,[66] colloidal lithography,[67] nanoprecipitation,[68] nanoimprinting,[69] and electrospinning.[70]

2.3. Nanostructured Materials Made by Silicon-based Materials

Silicon-based nanoscale structures could be manufactured by the common techniques that used in producing semiconductors and microelectronic systems, such as photolithography, etching, and deposition. The most widely investigated silicon-based nanomaterials for drug delivery systems and tissue engineering scaffolds are porous silicon materials. The architectures include nanopores, nanoneedles, and nanoparticles.[71] Porous hollow silica nanoparticles can be made in a suspension containing sacrificial nanoscale templates, such as calcium carbonate.[72]

It was reported that the density and diameter of the silica nanopores can be accurately controlled to achieve a constant drug delivery rate through the pores.[73] Through controlling the size of the pores and the diameter of the particles, a near zero-order drug release kinetics can be gotten from the porous hollow silica nanoparticles as depicted in Figure 1.2.[74] Some clinical applications have also been performed. A porous silicon nanostructure embedded with platinum was used as drug loaded scaffold to deliver an antitumor agent.[75] Calcified porous silicon nanoparticles can be designed to deliver an artificial growth factor for bone tissue engineering.[76]

Silicon nanoporous materials also can be used for antibody delivery,[77] and gene therapy.[78-79]

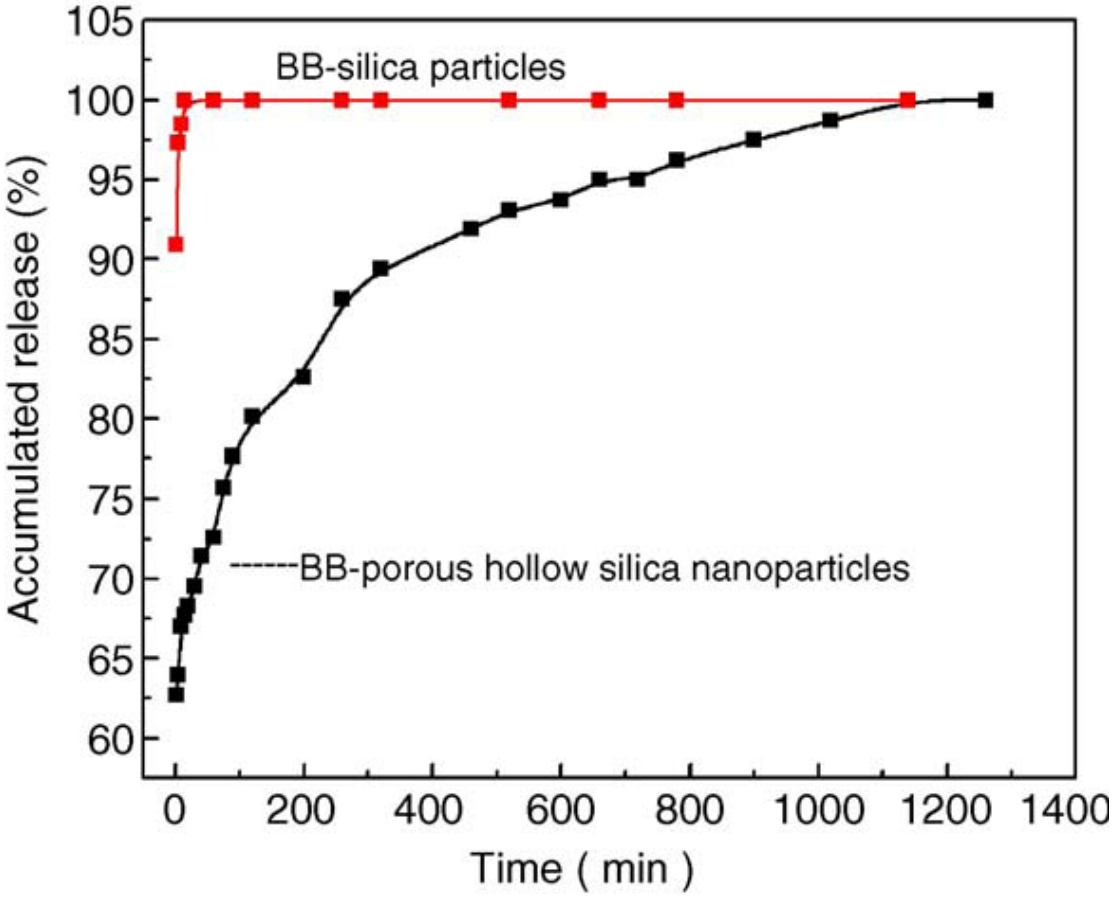

Figure 1.2. Comparison of release profiles of normal silica nanoparticles and porous hollow silica nanoparticles. Released drug was brilliant blue F (BB). (Reprinted from reference [73] with permission)

2.4. Nanostructured Materials Made by Carbon-based Materials

Recently, carbon nanotubes and fullerenes have received intense attention due to their unique hollow, carbon-based, cage-like structures. The size, geometry, and surface properties make these materials appealing for drug delivery systems and tissue engineering scaffolds. Single-wall nanotubes and C60 fullerenes have diameters on the order of 1 nm, about half of the diameter of the average DNA helix. The diameter

of Multi-wall nanotubes is ranging from several nanometers to tens of nanometers depending on the number of walls in the structure. Carbon nanotubes and fullerenes are typically fabricated through electric arc discharge, laser ablation, chemical vapor deposition, and combustion processes.[80-82]

The surface of carbon nanotubes can be functionally modified and be internalized within mammalian cells.[83] If the carbon nanotubes were linked to peptides, the resulted nanostructures can be used as vaccine delivery systems.[84-85] With the help of molecular dynamics simulations, the flow of water molecular through carbon nanotubes can be modeled.[86-88] Carbon nanotubes can also be used as gene delivery tools[89] and composited with other materials to form temperature-stabilized hydrogels for tissue engineering.[90] Fullerenes have also shown tissue-selective targeting[91] and intracellular targeting[92] of mitochondria. Other research revealed that fullerenes can exhibit antioxidant[93-94] and antimicrobial properties.[95]

2.5. Nanostructured Materials Made by Metal

Typical metals employed to manufacture nanoparticles are gold, silver, platinum, and palladium. When linked to or embedded within polymeric matrix, metal nanoparticles can be used as thermal release triggers if irradiated with infrared light or activated by an alternating magnetic field.[96-97] The metal nanoparticles can be conjugated with biomolecular through biofunctional linkages, lipophilic interactions nanobead interactions, silanizations, and electrostatic interactions.[98-99]

It was reported that hollow metal nanoshells have been used as drug delivery carriers.[100] Au nanoparticles modified with shells of bovine serum albumin can penetrate the biological membrane and target the nuclei.[101] Similarly, bovine serum albumin was conjugated to various cellular targeting peptides to provide functional nanoparticles.[102] Also, other metal nanoparticles were applied as targeted biomarkers and drug delivery agents in the analysis and medical treatment of cancers.[103-104] Hybrids of silver nanoparticles with amphiphilic hyperbranched macromolecules exhibited antimicrobial properties and were used as drugs.[105-106]

3. Applications of Nanostructured Materials in Tissue Engineering

Tissue engineering seeks to replace or facilitate the regeneration of damaged or diseased tissue by applying a biomaterial support system or scaffold, and/or a combination of cells, bioactive molecules such as drugs or growth factors.[107] The fundamental principle of tissue engineering is that the materials facilitate regeneration of structures that resemble the original tissue. In order to control and direct cell behavior, a desirable biomimetic environment which surrounds the cells and promotes specific cell interactions is wanted.[108] Three-dimensional artificial scaffolds are designed to provide initial structural integrity for cells, direct cell differentiation and proliferation, and finally lead to assembly of functional tissue.[109] So the chemical, physical and mechanical properties of scaffolds are critical to functional tissue regeneration.[110-113]

In the last couple decades, research in the area of tissue engineering has

witnessed tremendous progress.[110,114-116] Meanwhile, the development of nanotechnology provides opportunities to fabricate, characterize and utilize materials systematically at the nanometer scale. Biodegradable nanomaterials have been widely used as controlled release reservoirs for drug delivery and artificial matrices or scaffolds for tissue engineering.[117-118] Whereas traditional tissue engineering scaffolds were based on hydrolytically degradable macroporous materials, current approaches emphasize the control over cell behaviors and tissue formation by nanoscale topography that closely mimics the natural extracellular matrix.[119-120] The understanding that natural extracellular matrix is a multifunctional nanocomposite motivated scientists to develop nanostructured materials for tissue engineering.[121-122]

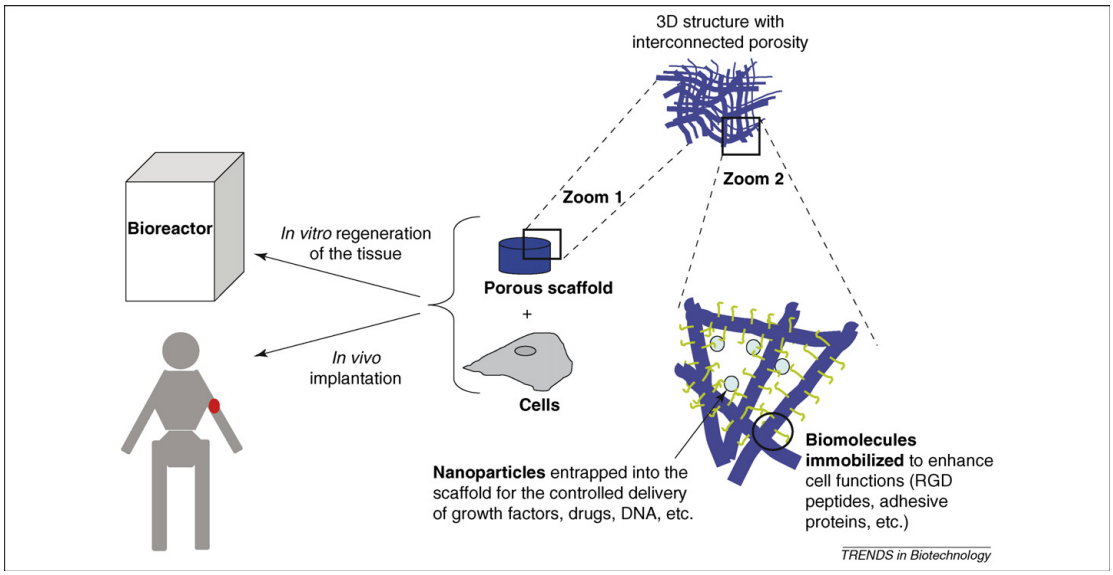

Figure 1.3. Two tissue engineering approaches for tissue regeneration: in vivo or in vitro regeneration of the living tissue. Both methods use a porous scaffold that is loaded with cells. Topographical structure at the nanoscale can be generated onto the scaffold surface. The scaffold can also be functionalized with different molecules to enhance cell function. (Reprinted from reference [122] with permission)

Nanostructured materials for use in tissue engineering share the same advantages

as those used in drug delivery systems. These nanostructured materials can be synthetic with controlled composition, shape, size and morphology. Their surface properties can be manipulated to enhance biocompatibility, immunocompatibility and cell adhesion of produced scaffolds.[119] Cells are seeded on these scaffolds, which can aid and abet tissue regeneration by supporting cell proliferation on their surface. Cell characteristics such as proliferation, adhesion, and morphology are examined with the ultimate aim of implanting the seeded scaffolds in human patients to replace or regenerate cells in the knee, the inter-vertebral discs, the skull, and other areas of the body (Figure 1.3). To buttress cell growth, signaling moieties or growth factors, encapsulated in nanoparticles or microparticles to afford sustained release, can further be integrated in the scaffold material.[123]

Drug delivery and tissue engineering are closely related fields. Actually, tissue engineering can be recognized as a special case of drug delivery since the goal is to accomplish controlled delivery locally to mammalian cells. Controlled release of therapeutic factors has been shown to promote the regeneration of tissue. From a materials point of view, both drug delivery systems and tissue engineering scaffolds should be biocompatible and biodegradable. The medical functions of encapsulated drugs and cells could be dramatically improved by designing materials with controlled structures at the nanometer scale. The aim of this thesis is to create a novel injectable nanostructured material with combined features beneficial for tissue scaffolding and for drug delivery. In this section, some new nanostructured materials utilized in tissue engineering will be reviewed, followed by a brief review of pertinent

drug delivery systems using nanotechnology. Special emphasis is placed on bone tissue engineering and drug delivery.

3.1. Nanoparticles Used in Tissue Engineering

The native extracellular matrix is a dynamic and hierarchically organized nanocomposite that not only provides mechanical support for embedded cells, but interacts with cells to control cellular functions such as adhesion, migration, proliferation, differentiation and morphogenesis.[124] Nanoparticles may be an excellent candidate to improve the properties of tissue engineering scaffolds since they possess a similar length scale of many cellular and molecular components of the extracellular matrix. Nanoparticles can recapitulate the *in vivo* environment of the extracellular matrix, and thus offer an environment conducive to cell adhesion, cell migration, and cell differentiation.[123] Furthermore, since the surface roughness of cells approximates that of materials having nanometer dimensions, and since most proteins, which facilitate cell adhesion also measure in nanometers, better cell adhesion may be achieved when using nanoparticles.[125]

A broad variety of nanoparticles based on biopolymers have been utilized as scaffolds for tissue engineering applications, such as PLLA,[126] PLGA,[127] PCL,[128-129] chitosan,[130] and fibrinogen.[131] For example, a PLGA nanoparticle scaffold was used for bladder replacement.[132] Representative SEM pictures illustrate the three-dimensional, nanostructured PLGA scaffolds (Figure 1.4). Results from this study showed that PLGA nanoparticles firmly attached human bladder smooth muscle

cells and that cell growth, adhesion, and collagen/elastin production was not influenced by 10 cm of water pressure. The performance of bladder cells under conditions simulating the native environment was satisfactory.

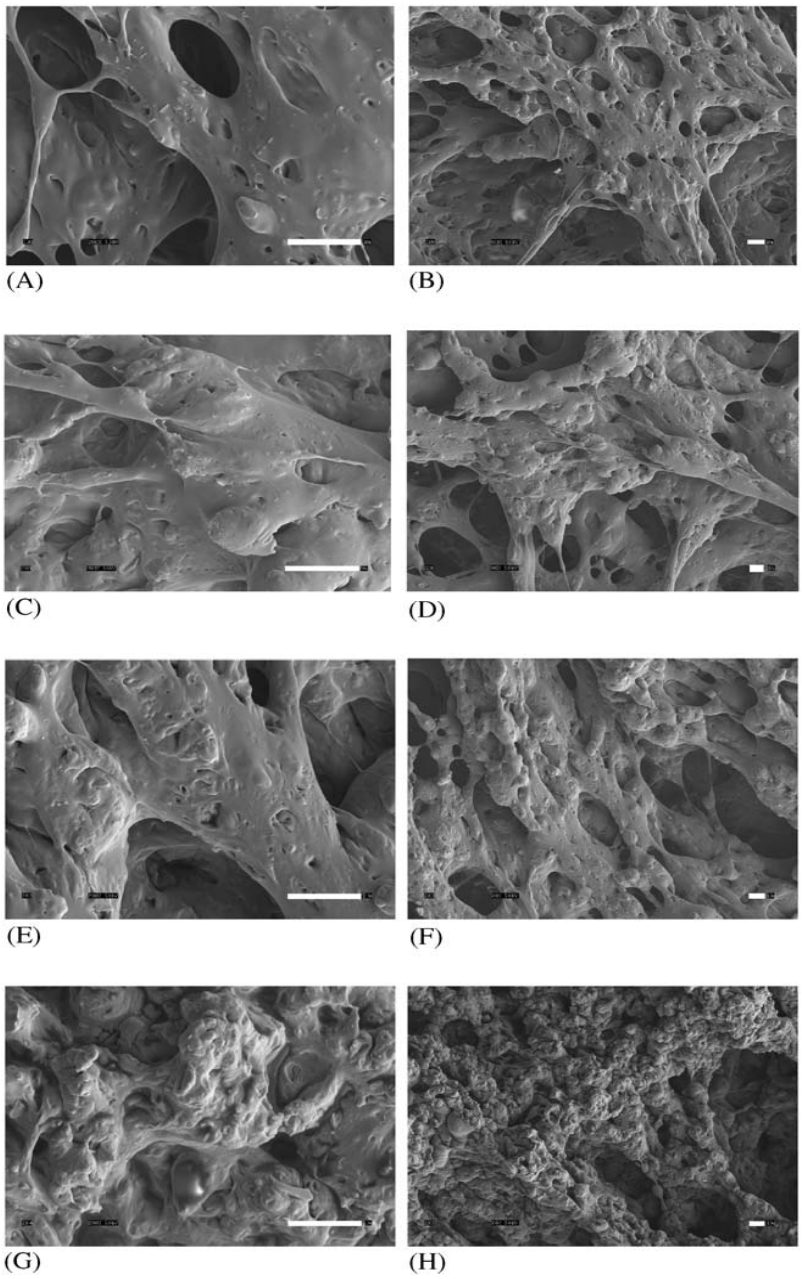

Figure 1.4. Representative SEM of PLGA scaffolds for bladder replacement. Images of conventional PLGA at (A) 500x and (B) 2500x magnification, small-micron PLGA at (C) 500x and (D) 2500x magnification, sub-micron PLGA at (E) 500x and (F) 2500x magnification, and nano-PLGA at (G) 500x and (H) 2500x magnification. Scale bar =10 mm. (Reprinted from reference [131] with permission)

Other materials used to make nanoparticles for tissue engineering include hydroxyapatite,[133-134] silicon,[135-136] carbon,[137-138] and titania.[125] Recently, nanoparticles based composite scaffolds have been widely used in tissue engineering.[139-142] Recently, a three-layered composite made from nano-carbonated hydroxyapatite, mineralized collagen, and PLGA has been evaluated with respect to biocompatibility, biodegradability, and osteconductivity for dental applications.[143] The three-layered membranes exhibited tensile strength approaching that of cancellous bone.

3.2. Nanofibrous Scaffolds Applied in Tissue Engineering

Extracellular molecules exhibit complex supramolecular structures. They are linked together by multiple binding domains which lead to a stable multifunctional matrix. High surface area, high porosity and high spatial interconnectivity are most important factors to maximize the interaction between cells and extracellular matrix, and to further promote tissue regeneration. The porosity may also play a significant role in the transport of nutrients and cell migration during tissue regeneration. In a typical tissue, structural protein fibers, such as collagen fibers and elastin fibers, have dimensions ranging from 10 to hundreds of nanometers. The nanoscale protein fibers entangle with each other to form a non-woven mesh structure that provides tensile strength and elasticity for the tissue. Adhesive proteins, such as fibronectin and laminin which provide specific binding sites for cell adhesion, also function as nanoscale fibers in the extracellular matrix environment. Therefore, artificial nanofibrous materials may have great potential to form tissue scaffolds. And with the

development of nanotechnology, critical insight on fabrication of nanofibrous organization of extracellular matrix components and how they interact with each other to form a functional extracellular matrix are under investigation.[144-145]

In many studies, biodegradable synthetic polymers, such as PCL, PLA, PGA and PLGA, have been used in the fabrication of nanofibers in the engineering of blood vessel,[146] cardiac tissue,[147] bone,[148] cartilage,[120,149-150] peripheral nerves,[151] ligaments,[152] liver[153] and skin.[120] In addition, naturally occurring biomolecules, such as collagen,[154] silk protein,[155-156] fibrinogen,[157] elastin mimetic polypeptides,[158-160] chitosan,[161] dextran[162] and hyaluronic acid,[163] have been fabricated into nanofibers through electrospinning. Effective nanofibers thus produced are expected to possess high axial strength combined with extreme flexibility.[164]

Recently, a novel method called molecular self-assembly has been used to make artificial nanofibrous scaffolding materials for tissue engineering to emulate natural extracellular matrix both structurally and functionally. For instance, Stupp et al. designed small, self-assembling molecules with a hydrophobic alkyl tail and a hydrophilic oliogopeptide head. The amphiphilicity of these molecules creates cylindrical nanofibers with well-defined diameters, which subsequently induce a liquid-to-gel transformation, which can be of benefit in tissue engineering.[165-167] Zhang and co-workers developed another class of nanofibrous hydrogels with very high water content through self-assembly of self-complementary amphiphilic peptides in physiological conditions.[168-169] The developments of self-assembled nanofibrous scaffolds hold great promise in tissue engineering.

3.3. Nanostructured Materials Utilized in Bone Tissue Engineering

The ideal scaffolds for bone tissue engineering should act as temporary frameworks to support bone growth, and release growth factors to induce regeneration of bone-forming cells. However, most tissue scaffolds have limitations in bone regeneration, including insufficient mechanical strength, ineffective cell growth and osteogenic differentiation, as well as insufficient and unstable growth factors release to stimulate bone cell growth.[170] Nanostructured materials have been widely used to enhance bone tissue engineering strategies. The extremely small size, large surface to volume ratio, and surface functionality make nanostructured materials a potential candidate to create novel bone tissue engineering scaffolds.[171-172]

Calcium phosphate nanoparticle cements, such as hydroxyapatite and tricalcium phosphate, are common materials for bone tissue engineering since these 'bioceramics' reflect the chemistry and structure of the native mineral components of bone tissue. Bioceramics can enhance osteodifferentiation and osteoblast proliferation; however, brittleness, processing difficulty and slow degradation rate still limit their applications in tissue engineering. In order to combine the osteoconductivity of hydroxyapatite and biodegradability of polymers, the incorporation of those two has been examined.[139,173] TEM pictures depict a hydroxyapatite nanoparticle composite used to reconstruct the defects of bone (Figure 1.5).[174] Other nanoparticles, such as carbon nanotubes,[175] silicon nanoparticles[176-178] and metal nanoparticles[179-180] have also been utilized in bone tissue engineering.

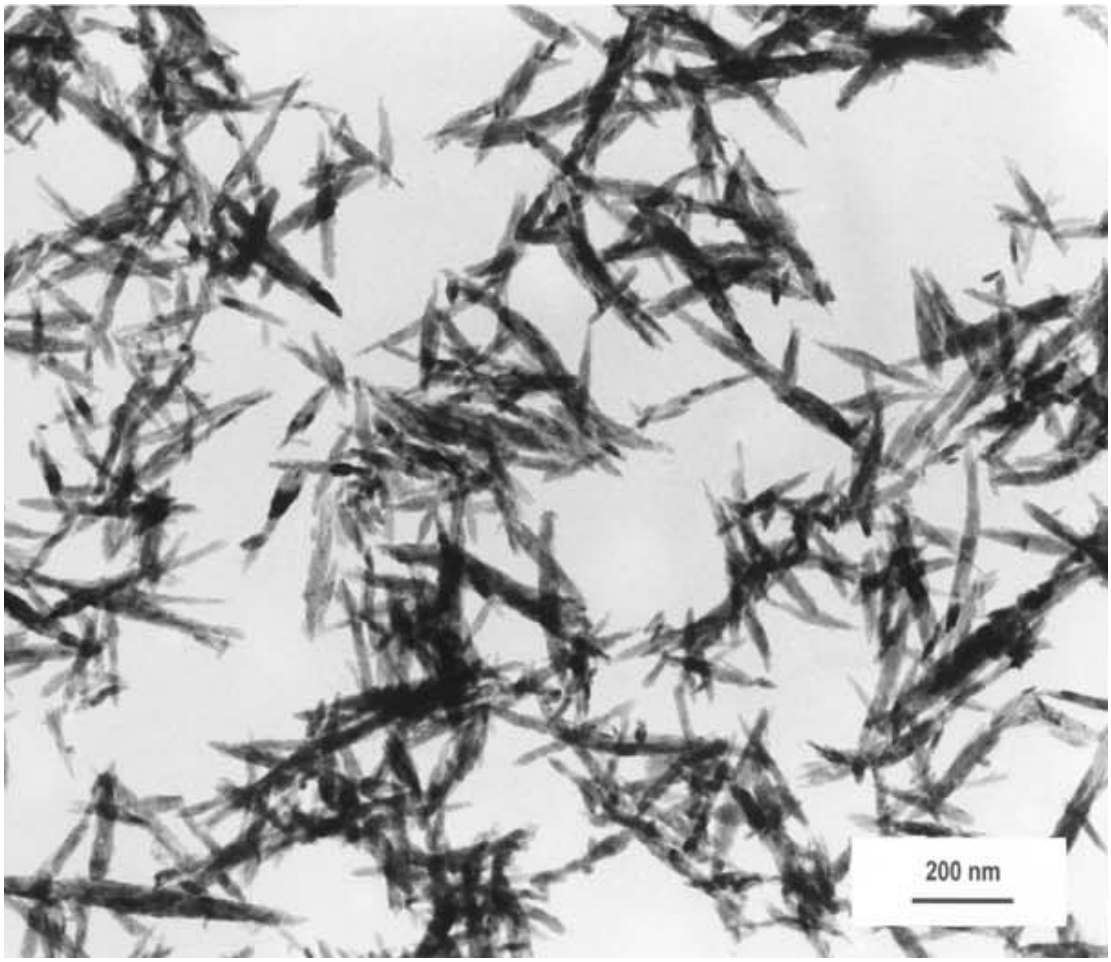

Figure 1.5. Pure nanoparticulate hydroxyapatite which was used for the composite material forms aggregates of needlelike crystals in transmission electron microscopy. (Reprinted from reference [174] with permission)

Nanofibrous scaffolds are another approach to engineering scaffolds designed for bone tissue engineering. Due to the architectural, functional and morphological similarities to extracellular matrix in bone, nanofibrous scaffold may provide a more favorable environment for cellular in-growth and subsequent bone generation.[119] For example, a three-dimensional PLLA nanofibrous scaffold has been shown to improve protein absorption, mediating cellular interactions within the scaffold.[181] Subsequently, it was verified that osteoblasts cultured on PLLA nanofibrous scaffolds exhibited significantly increased proliferation, mineralization throughout osteogenic

differentiation zones, and bone marker protein expression when compared to unmodified PLLA.[182] Furthermore, it was also shown that the presence of the nanofibrous scaffold influenced cell shape and morphology, inducing increased cell spreading.[183] Overall, utilization of various nanostructured materials into scaffold materials has generally enabled improved mechanical properties and facilitated improved regeneration for bone tissue engineering.

3.4. Conclusions

Overall, the use of nanostructured materials in tissue engineering is rapidly expanding. Research emphasis has been placed on a variety of nanomaterials that have found use as tissue scaffolds. These materials include ceramics, biological and synthetic polymers, inorganic materials such as hydroxyapatite that mimic bone, minerals such as carbon and silicon, and composites of nanomaterials. Most of these results have largely been obtained through *in vitro* studies, but pilot in vivo studies have also been encouraging. There is a need for more *in vivo* studies before the use of nanoparticles in tissue engineering becomes more widespread.

4. Applications of Nanostructured Materials in Drug Delivery

Nanotechnology has opened new therapeutic opportunities for drugs which can not be used effectively in traditional drug formulations due to poor bioavailability or less instability. In previous research, microparticles have been used to protect agents susceptible to degradation or denaturation while prolonging the duration of action of a

drug by increasing systemic exposure or retention of the formulation.[184-188] Nanostructured materials have advantages in enhancing dissolution, providing stability during circulation and enhancing transport. Some groups in the field have used nanospheres as carriers to deliver small molecules, proteins and other therapeutic agents.[189-195] The importance of biodegradable nanoparticles is related to the enhanced bioavailability through uptake, followed by degradation and disappearance of the vehicle from the body. Here, focus will be placed on the applications of such nanostructured materials in drug delivery.

4.1. Applications of Nanomedicine in Different Therapeutic Areas

Nanomedicine is one category of nanotechnology bearing fruit from the considerable investments being made in nanotechnology research.[196] One subdivision of nanomedicine is the development of targeted drug delivery and diagnostic approaches based on particulate design. Research into the delivery and functionalization of nanoparticles is a very important area in biomedical applications, especially for targeted entry into cells via nanoparticles.[197] The potential elongation of circulation time of drug loaded nanoparticles improves passive targeting of drugs to areas where inflammation or disease produces greater local vascular permeability together with enhanced local extravasations into the neighboring tissue. Elements of these and other unique features of nanoparticles that offer great therapeutic potential will be briefly addressed.

4.1.1. Applications of Nanoparticles in Immunotherapy

The creation of vaccines is one of the medicine's most important accomplishments. Diseases such as measles, mumps, rubella, diphtheria, tetanus, pertussis, polio and yellow fever are now under control because of the vaccines.[198] More work has focused on using technologies such as recombinant DNA methods to develop DNA and subunit vaccines, as well as conjugates vaccines in which a weak antigen is linked to a stronger immunogen such as a protein or membrane complex.[199-202] With the development of these new vaccines, there is a critical demand for novel delivery vehicles as well as new adjuvants since immunity can be limited by vaccine degradation and low loading efficiency. Therefore, nanoparticle based drug delivery systems offer several advantages for vaccine delivery.[203-204]

There are several types of nanostructured vaccine delivery systems. Among them, polymeric nanoparticles have been extensively studied for use in the formulation of vaccine antigens.[205-206] In these formulations, antigen can be either entrapped or adsorbed to the surface of the particles, and further be tailored to degrade over a range of rates.[189] Additionally, polymeric nanoparticles may offer protection to encapsulated antigens delivered orally and facilitate uptake by M-cells when administrated nasally, thus serving as a vehicle for mucosal immunization.[207-208]

Liposomes are spherical entities composed of a phospholipid bilayer shell with an aqueous core. For vaccine delivery, adjuvants have been either encapsulated in the core of the liposome or adsorbed on the surface for presentation to antigen presenting cells.[209-210] There are a few liposomal vaccines that have been investigated in clinical

trials, such as vaccines against malaria,[211] HIV,[212] hepatitis A,[213] influenza,[214] prostate cancer,[215] and colorectal cancer.[216] These were all found to be safe and highly immunogenic.

Proteosomes are another common form of nanostructured vaccine delivery system composed of the outer membrane proteins.[217] Due to the hydrophobic nature of the proteins, this immunogenic delivery system is appropriate for delivering apolar or amphiphilic antigens.[218] A similar vaccine delivery system is the conjugate vaccine. It consists of a relatively non-immunogenic antigen linked to a more immunogenic carrier.[217] Prevnar, the world's best selling vaccine, is an example of this conjugate vaccine.[219-220]

Inorganic nanoparticles may also be utilized in vaccine drug delivery systems. Calcium phosphate has been used as vaccine delivery vehicle since it is naturally occurring in the body thus reducing the risks of side effects to our body.[221-222] Calcium phosphate has been clinically tested to be a safe and non-toxic vaccine delivery system.[223] Some clinical results indicated that vaccines containing calcium phosphate resulted in immune responses similar to or greater than those adjuvanted with aluminum salts, and the duration of the response was longer.[224] Other inorganic nanomaterials explored for vaccine delivery are gold,[225] latex,[226] silica,[79] and polystyrene.[227]

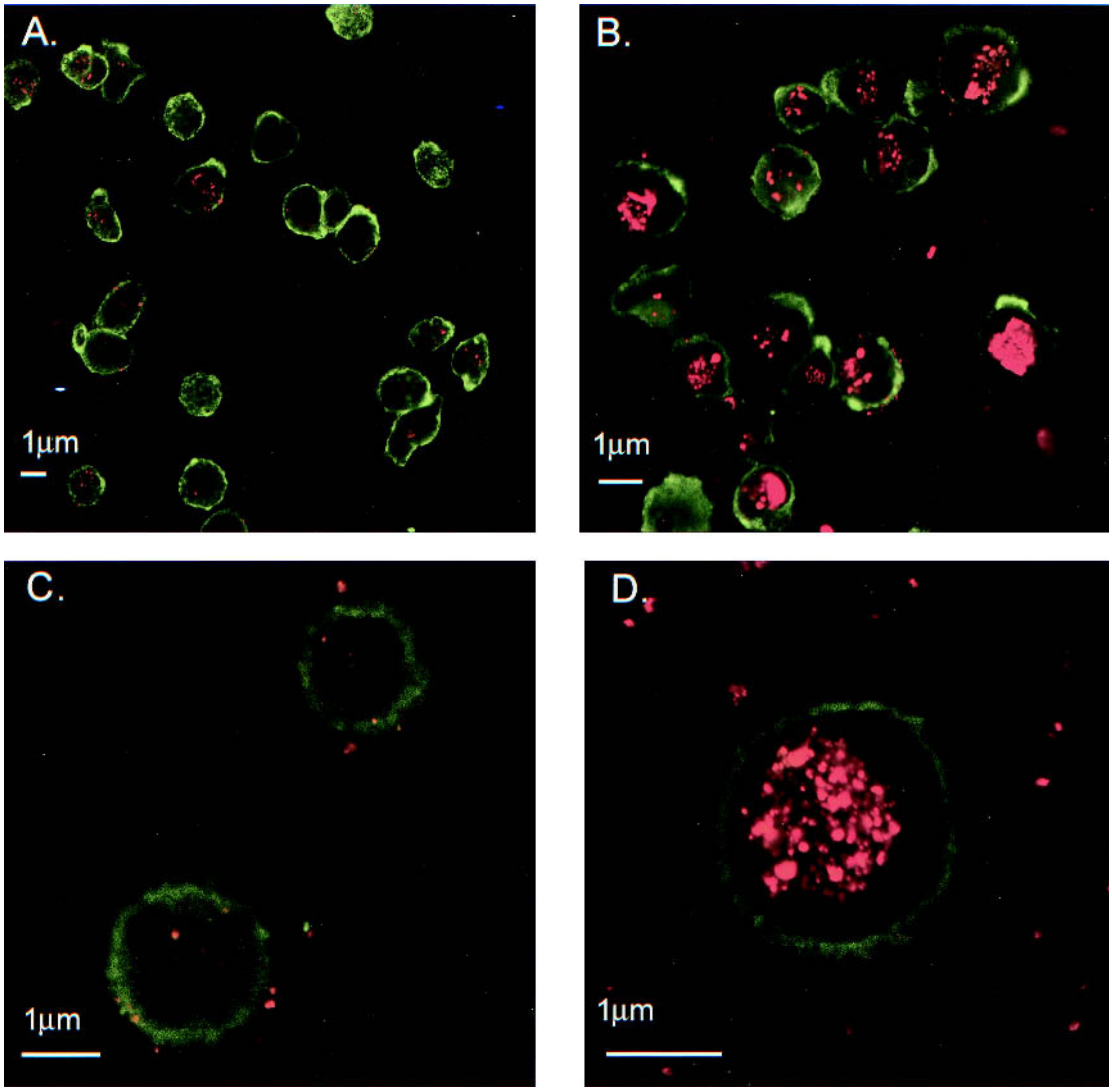

Figure 1.6. Uptake of PLGA nanospheres by human dendritic cells (DCs). On day 4, after 24-h incubation with tetramethylrhodamine-dextran–containing nanospheres, cell membranes of DCs were stained with fluorescein- 5-isothiocyanate-labeled concanvalin A and examined using a confocal microscope. Note the veiling on the cell membranes, which is a morphological trait of DCs. Control cells (A and C) were pretreated with cytochalasin B. Test cells (B and D) were untreated. (Reprinted from reference [228] with permission)

Recently, researchers tried to create a novel nanostructured delivery system to increase the encapsulation.[184,186,228] Lutsiak *et al*.[229] found that human dendritic cells uptake PLGA nanoparticles loaded with tetramethylrhodamine-labeled dextran *in vivo*. (Figure 1.6) This work implicated selective activation of a T-cell mediated immune

response. Nanoparticles have also been used for pulmonary delivery of vaccines to increase encapsulation efficiency. Proteins such as lysozyme, albumin, and insulin can be precipitated as 100-500 nm particles for aerosolized delivery without significant loss of biochemical integrity or marked aggregation.[230-231]

4.1.2. Applications of Nanoparticles in Gene Therapy

With the understanding and development in genetic, molecular, and cellular sciences, more and more new methods have been achieved in prevention, diagnosis, and treatment of diseases. Gene therapy represents a novel approach for treatment that is designed either to alleviate the genetic defect in cells or to provide additional protective effect.[232-233] For example, gene therapy strategies for solid tumors can be divided into methods that induce anti-tumor immunity, confer drug sensitivity, restore cellular growth control, or inhibit neo-angiogenesis.[234-235] For *in vivo* gene delivery, it is necessary to develop new delivery carriers which can transport therapeutic genes to a specific region either locally or systemically, in order to efficiently express encoded proteins at the target site.[236]

Nanoparticles are becoming increasingly critical components of gene therapy. Nanoparticulate systems are attractive methods of gene delivery because of their versatility, ease of preparation, and protection of the encapsulated plasmid DNA.[189] They can efficiently encapsulate various sizes of plasmids and provide protection during transit in systemic circulation.[237] They can also be targeted to specific tissues and cells in the body and avoid uptake by the mononuclear phagocytic system after systemic administration.[56] Nanoparticles can be designed to reach a targeted site by

virtue of their size and charge.[238] Nanoparticles usually have a high surface area to volume ratio and thus are able to efficiently encapsulate DNA even without precondensing the DNA.

One of the most investigated approaches for gene delivery is the liposome. They are particularly useful for gene delivery due to their ability to pass through lipid bilayers and cell membranes. Liposomes consist of a lipid bilayer surrounding a core containing a therapeutic molecule or gene. Recently, composite liposomes containing poly (cationic lipid), cholesterol, and DNA produced higher transfection than naked DNA alone when injected directly into portal circulation following a partial hepatectomy.[239] Targeted therapy has also been achieved via liposomes through blood-brain barrier.[240]

Gene delivery can also use nanoscale polymeric vehicles. For example, 300 nm diameter nanoparticles loaded with platelet-derived growth factor beta-receptor antisense DNA were delivered to treat restenosis.[241] Berton *et al*. have also successfully delivered oligonucleotides.[242] Plasmid DNA can also be delivered by biodegradable nanoparticles.[243-244] Dendritic and hyperbranched polymers also offer potential for gene delivery.[245-247] Such polymers can form extremely small particles on the order of several nanometers as effective DNA-complexes, which are often associated with prolonged *in vivo* retention.[248] It was reported that polyamidoamines complexed to the pCF1CAT plasmid for intravascular and endobronchial delivery of chlroamphenicol acetyl-transferase are being explored as a potential treatment for cystic fibrosis.[244] Moreover, significant improvements in transfection using

polyamidoamines dendritic complexes have also been achieved.[249-251] These complexes give a hint to the future of targeted gene therapy and the importance of nanoscale structures for the achievements of molecular medicine.

4.1.3. Applications of Nanoparticles in Cancer Therapy

One of the most important impacts of nanoparticle-based drug delivery systems appears to be the localized treatment of solid tumors. Novel nanoparticle delivery technologies provide the opportunity for passive accumulation of intravenously injected nanoparticles (20-150 nm) from leaky vasculature. Nanostructured vehicle can penetrate tumors because of the discontinuous and leaky nature of the tumor microvasculature which contains larger pores (100-1000 nm in diameter). For example, the diffusion distances of nanoparticles from the vasculature into adjacent parenchyma are typically very short.[252-253] Meanwhile, tumors may be selectively targeted using nanoparticles greater than the intercellular gap of healthy tissue but smaller than the pores of tumor vasculature. Although the irregular and poorly perfused vasculature of tumors may impede the efficiency of nanoparticles delivery to some degree, formulations with long circulating time and tumor localization are possible.[254-255] These formulations have favorable pharmacokinetics compared to drug alone including an increased area under the curve (300-fold) and reduced clearance and volume of distribution.[256]

Other effective approaches to treat cancer via nanostructured materials include liposomes and polymeric micells. Drug release can be triggered from liposomes that

have accumulated at interstitial sites using enzyme-mediated liposome destabilization.[257-258] The release of the drug can be extended by choosing an appropriate lipid composition.[259] These nanostructures may be delivered to cancer cells and related extracellular elements through passive delivery approaches. One interesting method used *in vivo* detection and imaging of tumor-associated matrix metalloproteinase-7 activity with a dendrimer-based fluorogenic substrate.[260] In other studies, active targeting has proven effective. Folic acid has been attached to the distal end of poly (ethylene glycol) chains on the surface of long circulating, macrophages-evading nanoparticles.[261-267] Folate-based targeting offers distinct advantages over approaches such as monoclonal antibodies.[268-269] Folate is nonimmunogenic and folate nanoparticles are rapidly internalized by activated cells in a manner that bypasses cancer cell multi-drug-efflux pumps.[263-264]

Furthermore, the idea of targeting drugs to blood vessels of tumors is intuitively appealing and nanotechnology can contribute to the early detection and treatment of metastatic cancers or in the diagnosis and therapy of resistant tumors.[270-271] Recent developments in molecular biology are unveiling potential targets within the vasculature of tumors such as integrins with roles in tumor angiogenesis.[272-275] Integrins bind to sequence containing RGD (Arg-Gly-Asp) motifs and have been constructed into a cyclic peptides, such as RGD-4C.[276] Coupling of RGD-4C to doxorubicin increase chemotherapeutic activity with the added benefit of less liver and heart toxicity relative to doxorubicin alone.[277] Other binding sequences, such as the HWGF peptide motif and the hexapeptide NGR can fight against human breast

cancer cells[278] and murine tumor cells,[279] respectively. Cationic nanoparticles complexed with targeted therapeutic gens is another anti-angiogenic pathway to treat solid tumors.[280-281] Similar methods for site-specific magnetic resonance (MR) paramagnetic nanoparticles have been used to detect early tumor angiogenesis.[282-283] Other methods include selectively targeting peptide coated quantum dots to blood and lymphatic vessels in tumor[284] and using nanoparticles motif-decorated liposomes to reduce tumor burden by shutting down their blood supply.[285]

Nanoparticle-based therapy has been widely used to treat different types of cancer in animal models. Chen *et al.* studied the therapeutic efficiency of targeting PLGA nanoparticles loaded with a model toxin against HER-2-positive tumors in breast.[286] The determination of anti-HER2 Fab' on the surface of nanoparticles was depicted in Figure 1.7. Crosslinked human serum albumin [287] and quantum-dot loaded chitosan nanoparticles with absorbed siRNA[288] also showed promising results with HER-2 targeting to breast cancer. Recently, several novel targeting approaches for the therapy of localized and metastatic colorectal cancer have been proposed.[289] The investigators utilized gold nanoparticles, which absorb near infrared light, together with conjugated bacterial heat-stable enterotoxins to thermally ablate targeted cancer cells.[290] Passive targeting to colorectal cancer has been investigated with the use of nanoscale polymeric micelles loaded with chemotherapeutic agents.[291-293]

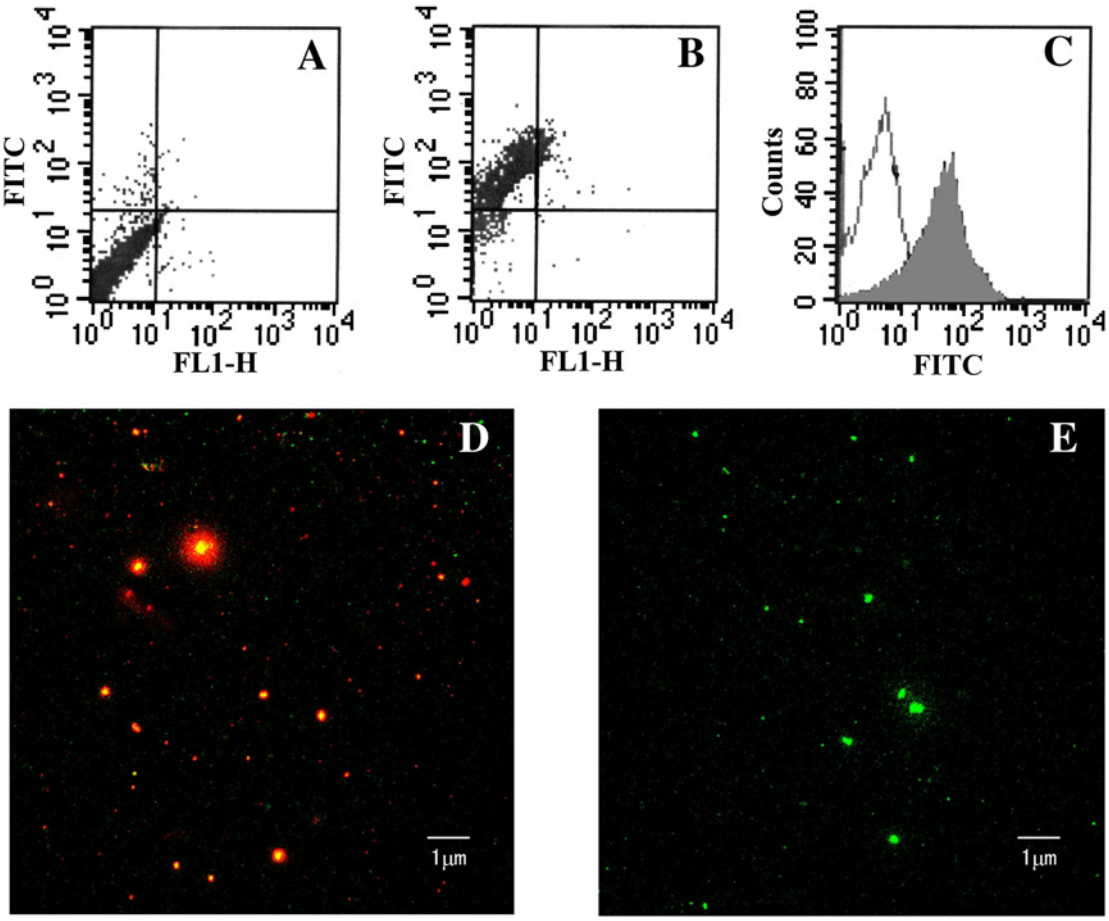

Figure 1.7. Determination of anti-HER2 Fab' on the surface of nanoparticles by flow cytometric analysis and confocal microscopy. Density plots of antibody modified nanoparticles (A) and antibody modified nanoparticles (B) showed that antibody modified NPs were conjugated with FITC-labeled Fab' and could be distinguished from non-antibody modified NPs by their fluorescence. A shift of FITC fluorescence intensity (C) could be seen for antibody modified NPs (grey histograms) in comparison to non-antibody modified NPs (white histograms), indicating the presence of Fab' on the surface of NPs. Furthermore, confocal microscopic images of FITC FITC-labeled nanoparticles (green) with conjugated phycoerythrin labeled anti-HER2 Fab' (red) showed merged red/green fluorescence (D). The control sample of nanoparticles showed only green fluorescence (E). (Reprinted from reference [285] with permission)

Moreover, nanostructured polymeric conjugates have also been used for improved treatment of colorectal cancer.[294] For lung cancer, nanoparticles using targeting aptamers have been developed to target small-cell lung cancer cells, recently.[295] The use of the EGF ligand has been performed for targeting nanoparticles

to lung cancer.[296] Delivery of antisense oligodeoxynucleotides or siRNA for the treatment of lung cancer has also been attempted with the use of targeted nanoscale carriers.[297-298] Prostate specific membrane antigen (PSMA) is one of the most investigated targets for prostate cancer therapy. Robert Langer's lab of MIT has studied the target potential of drug/aptamer conjugates and polymeric stealth nanoparticles with surface conjugated aptamer for targeting chemotherapeutic agents to prostate cancer.[299-302] The *in vivo* studies with the aptamer-conjugated nanoparticles have shown promising results for treating prostate cancer.[303] Another approach has been utilized to targeting prostate tumor through PSMA is folate ligands.[304-305]

More effective delivery of nanoparticles to cancer has resulted in the development of more new approaches to treat this desease. Nanoparticle based therapeutic systems are able to target various portions of the tumor using specific targeting moieties and avoid some of the problems associated with drug resistance.

4.2. Nanostructured Materials Used in Different Drug Delivery Routes

The traditional methods to deliver drugs are oral and injection pathways, which have limited the development of new drugs. Most drugs have been produced and formulated to accommodate the oral or injection delivery routes, which are not always the most efficient delivery method for a particular drug. In addition to the commonly used oral and injection pathways, drugs can also be delivered via other routes, including transdermal, pulmonary, ocular, and implantation. Furthermore, the efficiency of drug delivery to different parts of body is often determined by the size

and charge of the vehicles. Nanoscale drug delivery systems have the potential to enhance drug bioavailability, improve the release profiles of drug molecules, enable precise drug targeting,[306] reduce drug toxicity and distribute the drug more efficiently.[307] Nanoparticle drug delivery systems can also be implemented with pulmonary therapies,[308] as gene delivery vehicles,[189,309] and for the stabilization of drug which maybe degrade very fast.[310-311]

Normally, many desired physiological targets are difficult to treat because of anatomical features, such as the tight epithelial junction of skin, the branching pathways of the pulmonary system, and the blood brain barrier. Nanostructure drug carriers may penetrate or overcome some of these barriers for drug delivery. For instance, it has been reported that the most efficient carrier for delivery into the pulmonary system is a particle size below 100 nm.[308] If the particle size is between 50 nm and 100 nm, improved uptake has been shown for gastrointestinal absorption[186,312] and transcutaneous permeation.[313] As the drug carrier penetrates further into the lung, additional shedding will allow the encapsulated drug to be released.[314] Another advantage of nanoparticle drug delivery systems is the ability to deliver drug molecules directly into cells.[315] Moreover, DNA and RNA maybe packaged within a nanoscale delivery system and transported into the cell to fix genetic mutations or alter gene expression.[316]

4.2.1. Pulmonary Drug Delivery via Nanoparticles

The primary function of lung is to enable air exchange between blood and

external environment, further to maintain homeostatic systemic pH. The large surface area, over 100 m^2, and the thin barrier between pulmonary lumen and capillaries create suitable conditions for efficient mass transfer.[317] So lungs are suitable for both local and systemic drug delivery. Meanwhile, nanostructures drug formulations offer many advantages over traditional aerosol powders and liquid pulmonary dose formulations. The nanoparticle drug formulation not only can greatly enhance the bioavailability of poorly water-soluble drugs by its large surface area, but can be particularly formulated to offer improved control over the morphology of dry powder drug formulations.

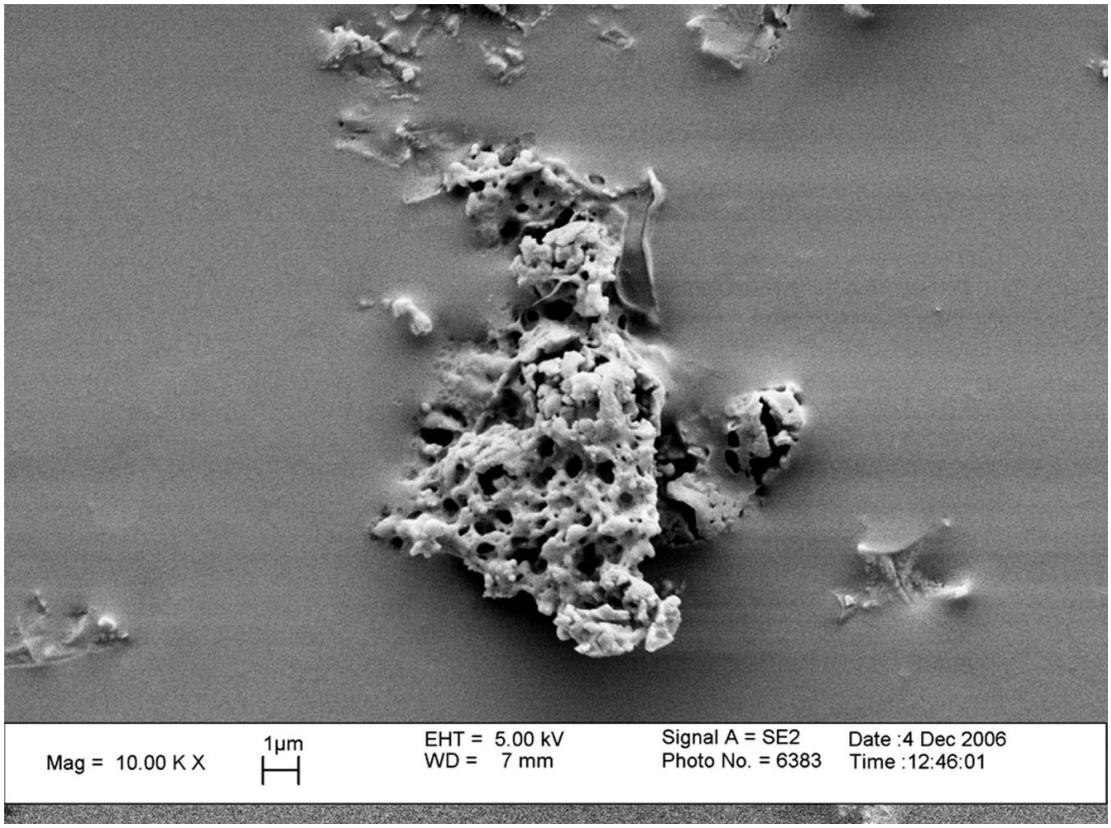

Figure 1.8. Scanning electron micrograph depicting the porosity of a nanocluster imaged at 10 000× magnification. The nanocluster contains nanoparticles cross-linked with protein, which results in a webbed appearance. (Reprinted from reference [328] with permission)

Nanotechnologies have been widely used to produce nanostructured materials suitable for pulmonary drug delivery. The normal processes that are currently under investigation are wet milling,[318-319] spay drying,[320-321] electro spray,[322-323] supercritical fluid extraction,[324] and high pressure homogenization.[325] Recently, some new polymeric nanoparticle fabrication technologies have also been studied as pulmonary drug formulations. These technologies generally involve polyelectrolyte complex formation,[326-327] double emulsion and solvent evaporation techniques,[328-329] or emulsion polymerization techniques.[330-331] A nanoparticle agglomerate as protein carrier for pulmonary drug delivery is shown in Figure 1.8. Liposomal formulation is another typical nanotechnology employed in pulmonary drug delivery which is normally produced by extruding or homogenizing a suspension of dissolved, hydrated lipids.[332] Liposomes prepared via this method have been dispersed into lactose and spray dried to produce nanoparticle-containing dry powders for pulmonary drug delivery.[333] Aerosolized liposomes have been used for a variety of pulmonary drug delivery applications, such as local drug delivery of chemotherapeutics to the lung[334] and systemic drug delivery of peptides.[335] With the development and unique advantages of pulmonary nanoparticle drug formulations, the lung may become a preferred route of drug delivery for more local and systemic therapeutic interventions.

4.2.2. Nanoparticle-based Drug Delivery via GI Tract

Oral delivery is one of the most desirable routes of drug delivery because of improved patient compliance and ease of administration.[336-337] These features make

oral delivery especially attractive for mass immunization and self-administration of medications. Oral drug delivery formulation often provides a longer shelf-life due to drug stabilization in polymeric matrices. Oral drug delivery systems include monolithic matrix tablets,[338] osmotic pumps,[339] biodegradable microparticles and nanoparticles with encapsulated drug,[340] microcapsules,[341] and so on. These oral delivery systems are selected for the clinic based on release properties, targeting, biocompatibility, expense, and the timeframe of the effective therapeutic levels.

Nanotechnology has been adopted in the manufacturing process to improve oral drug delivery systems. Nanoparticles are promising oral drug delivery vehicles since they have potential to increase the efficiency in each section of the GI tract.[342] Nanostructured materials can protect the encapsulated biomolecules from degradation in the stomach and intestinal lumen,[343] prolong the GI tract residence time via mucoadhesion,[344] and promote epithelial cell targeting and endocytosis.[345-346] The small size of the nanoparticles allows them to diffuse through the mucosal layers and sometimes, to be transferred across the epithelium.[347] They still have the potential to protect their payload and increase the efficiency of the delivery even after the transportation across the epithelium by mediating the post GI tract delivery actions, such as endosomal escape[348] and nuclear targeting.[349]

Biopolymers are often used to make nanostructured drug carriers for delivery of agents targeted at acute or chronic indications. Encapsulation within biopolymers may circumvent the harsh environment of the stomach and work as a coating material to modulate pharmacokinetics and optimize the drug targeting. Additionally,

biodegradable polymers have the ability to modulate drug release amount, which permits control of drug levels within a therapeutic range.[350] So biodegradable drug release systems have become popular in nanomedicine due to the ability to tailor the delivery platform in size, surface charge, chemical properties and time range to achieve targeted GI tract delivery.[351-352] Some biopolymers can promote mucoadhesive interactions with the tissue of the GI tract, leading to increased exposure to the epithelium and greater potential for uptake into the tissue.[353] This mucoadhesive interaction involves noncovalent bonding with chemical moieties on the surface of the polymer with the mucopolysaccharide coating of the GI tract.[354] Through a variety of novel techniques, including nanotechnology, bioadhesion has been improved and quantified for a number of biomaterials.[355-356]

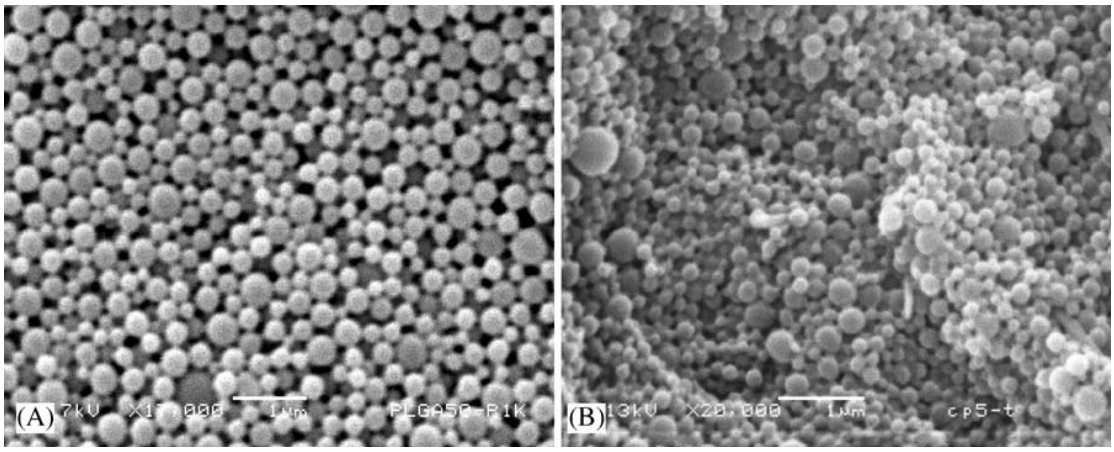

Figure 1.9. SEM images of coumarin 6-loaded PLGA particles coated with PVA (A) and vitamin E TPGS (B) (bar=1 mm). (Reprinted from reference [361] with permission)

It has been reported that particles with varying size cross the mucosal lining of the small intestine and enter systemic and lymphatic circulation within less than one hour.[357-358] It has been speculated that uptake can occur via the gut-associated lymphoid tissue and across the apical membrane of absorptive epithelium through the

paracellular pathway. Normally, the degree of uptake and translocation is inversely proportional to the size of particles. Epithelial uptake in regions free of Peyer's Patches occurs with nanoparticles with size ranging from 10 nm to 2000 nm.[359] Some other polymeric nanoparticles also showed non-lymphatic update.[351-352,360-361] PLGA nanoparticles around 250 nm were fabricated with coating materials of either PVA or vitamin E derivative. These nanoparticles were assessed in an *in vitro* model consisting of a monolayer of Caco-2 cells and showed to be an absorption enhancer (Figure 1.9).[362] Similar studies showed that PLGA nanoparticles were taken up in Peyer's Patches after 1 h while following absorption into epithelium over half to 1 hour.[363] Other nanoparticle coatings have also been reported to enhance absorption in the GI tract.[364-365] Molecular weight also showed to have effects on Peyer's Patch uptake of PLGA Nanoparticles.[366]

4.2.3. Drug Delivery by Nanoparticles to Central Nervous System

Central nervous system disorders are a leading cause of disability and are difficult to treat because of ineffective drug delivery to the brain.[367] A significant treatment obstacle is not drug potency but the physical barriers that render the circulatory routes of delivery ineffective.[368-369] These barriers are presented at distinct interfaces including the blood vessels of the brain, the choroid plexus, the arachnoid layer of the meninges, and within brain tumors. These barriers present greater problems for peptides, proteins, and oligonucleotides that tend to be larger hydrophilic molecules. But nanoparticles can potentially overcome these difficulties

and are being increasingly applied as drug carriers to deliver drugs to the central nervous system. The mechanism of nanoparticle-mediated drug delivery across the blood brain barrier (BBB) is determined by the chemistry and architecture of the nanoparticles.[370]

Although there are no FDA-approved used nanoparticle drug delivery systems for treating central nervous system, in vivo evaluation of some approaches has begun. For example, surfactant coated poly(butyl)cyanoacrylate (PBCA) nanoparticles have been extensively studied and met with some significant success. The leuenkephalin analog dalargin can be rendered efficacious when injected directly into the brain or bound to PBCA Nanoparticles.[371-372] The efficiency of this method is highly dependant on the type of coating materials.[373-374] Polysorbate-80 coated PBCA nanoparticles also enhanced delivery of tubocurarine,[375] loperamide,[376] dipeptide kytorphin,[377] and the anti-tumor antibiotic doxorubicin[378] into the central nervous system.

Nanoparticles formulated with chemotherapeutic agents are other promising areas under investigation. It was reported that a PBCA nanoparticulate formula of doxorubicin improved drug delivery across the BBB to reduce tumor burden and extend survival in a rat glioblastoma model.[379] Doxorubicin-loaded PEGylated solid lipid nanoparticles enhanced delivery across the BBB after intravenous administration.[380] Similarly, when idarubicin-loaded[381] and tobramycin-loaded[382] nanoparticles were systemically administrated, the drug was only detected in the brain. Moreover, the improved brain uptake of paclitaxel across BBB has been achieved via

using emulsifying wax nanoparticles.[383-384] Overall, these data demonstrated that nanoparticles containing chemotherapeutics can improve the delivery of drugs across BBB with certain specificity while retaining/enhancing their tumor killing effects.

4.2.4. Drug Delivery to Bone via Nanoparticles

Bone is a highly specified form of connective tissue which provides an internal support system and sites of muscle attachment for locomotion. It is also the major source of inorganic ions in the body and actively participates in calcium and phosphorus homeostasis in the body.[385] Since the structural features and properties of bone provide a unique opportunity to target drugs to bone tissue engineering, many novel therapeutic targets have been identified in recent years to improve the treatment of bone diseases.[386-389]

The major component of bone is hydroxyapatite and this mineral imparts specific affinity to certain molecules.[390] Molecules with diverse structural features have been reported with bone affinity.[391-392] Growth factors are one of the essential factors that induce or stimulate tissue growth, such as cell recruitment to the healing site, mitogenesis, differentiation into the osteogenic linage, and bone formation.[170,393] Delivering growth factors to targeted sites to induce bone formation is a very important bone regenerative strategy.[394-396] Bone growth in defected sites is normally limited by insufficient cell growth for bone fracture-healing and insufficient growth factor expression to stimulate osteogenic differentiation and proliferation. In order to overcome these drawbacks, nanotechnology has been combined with bone tissue

engineering methods.

PLGA nanoparticles were reported as a bone-seeking material by using a PLGA polymer grafted with alendronate. The nanoparticle diameter was around 50 nm and they were able to encapsulate and release hydrophobic drugs from their PLGA cores.[397] Bisphosphates has been incorporated into liposomes to make nanostructures by using a lipophilic bisphosphate for bone targeting.[398] These nanoscale liposomes were about 100 nm in size and displayed hydroxyapatite affinity in vitro that was dependent on the extent of bisphosphate incorporated in the liposomes.

4.3. Conclusions

The development of biotechnology brings human beings to a new era. New biological drugs such as proteins and nucleic acids require novel drug delivery technologies that will minimize the side effects and lead to better patient compliance.[399-400] The market also needs new and efficient drug delivery methods for the new drugs.[401] It has been estimated that the market of drug delivery accounts for around 40% of all pharmaceutical sales in 2007.[402] At the same time, upcoming patent expirations are driving pharmaceutical companies to develop new formulations for their products. Novel drug delivery systems make this feasible. Pharmaceutical companies can develop new formulations of off-patent and soon-to-be off-patent drugs through new delivery methods, such as nanotechnology.[306] Reformulations of old drugs aim to reduce side effects and increase patient compliance. Drug candidates reformulated with new drug delivery systems do not need to pass through extensive

trials, thus saving money on health care expense. Innovative drug delivery systems also may enable some chemicals or biologics that were previously impractical because of toxicity and rapid clearence. For example, drug targeting via drug loaded nanoparticles is enabling the delivery of chemotherapy agents directly tumors, reducing systemic side effects.[403] Scientists will continue their research to create novel methods to deliver macromolecules that will facilitate the development of new biologic products such as proteins and vaccines.[311,404] Meanwhile, the success of gene therapies will depend on innovative drug delivery techniques.[13,405] Since the success of a drug is highly dependent on the drug delivery method, more than 300 companies based in the United States are involved in developing new drug delivery systems.[406]

5. Approved Therapeutic Nanoparticles Products in the Market

In the past twenty years, the number of commercial nanoparticle-based therapeutic products has undergone a substantial increase. Nowadays, there are more than 150 companies developing nanoscale therapeutics in the world.[407] So far, more than 20 nanoparticle-based therapeutic products have been approved for clinical use, with total sales exceeding $5.4 billion.[407] Among these commercial products, liposomal drugs and polymer-drug conjugates are two major categories, which account for more than 80% of the total. A summary of typical nanoparticle systems in preclinical development are depicted in Figure 1.10.[408]

Liposomes are spherical lipid structures with a bilayered membrane composed of natural or synthetic amphiphilic lipid molecules.[409-410] Doxil was the first

nanoparticle-based liposomal drug formulation approved by the Food and Drug Administration (FDA), USA for the treatment of AIDS associated with Kaposi's sarcoma in 1995.[411] Another widely studied nanoparticle drug delivery platform currently available in the commercial market is polymer-drug conjugates.[412] Normally, small molecule therapeutic agents, particularly anticancer chemotherapeutic agents, have a short circulation time and non-specific targeting. On the contrary, the conjugation of small molecule drugs to polymeric nanostructures can reduce the undesirable adverse effects and extend circulation times. This strategy enhances the passive delivery of drugs to tissue with leaky blood vessels, such as tumors and atherosclerotic plaques.[413-414]

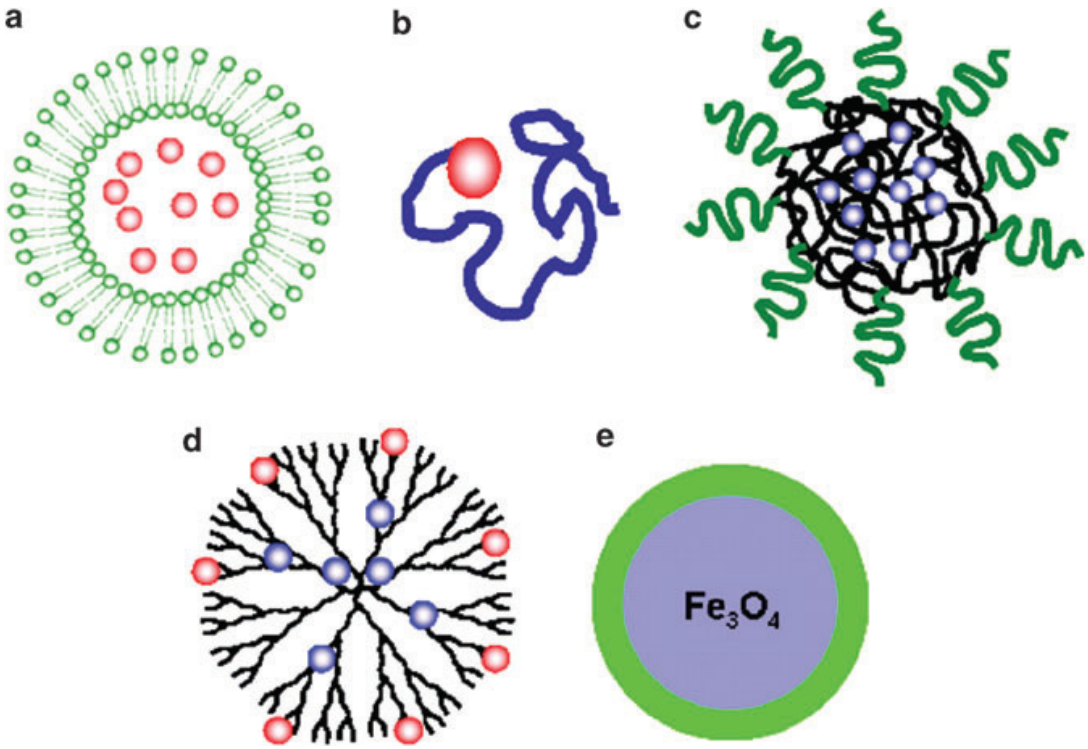

Figure 1.10. Representation of therapeutic nanoparticle systems in preclinical development: (a) liposome, (b) polymer–drug conjugate, (c) polymeric nanoparticle, (d) dendrimer, and (e) iron oxide nanoparticle. The red dots are hydrophilic drugs and the blue dots are hydrophobic drugs. (Reprinted from reference [407] with permission)

Many polymeric nanoparticles have been proposed as drug carriers, but only a few of them with linear architecture have been accepted into clinical practice. PEG-conjugated nanoparticles were first introduced into clinical application in 1990s.[415] This strategy can enhance the plasma stability and solubility of the drug while reducing its immunogenicity. Other polymeric drug conjugates have a hydrodynamic size of 5-200 nm and have also been developed as drug carriers. Abraxane is a 130 nm albumin-bound paclitaxel drug which was approved by the FDA in 2005 to treat breast cancer. It almost doubles the therapeutic response rate, increases time to disease progression, and enhances the overall survival in patients with breast cancer.[416]

In addition to the already approved nanoparticle-based therapies in clinical use to improve the therapeutic index of drugs, numerous other nanoparticle platforms are currently under various stages of preclinical and clinical development, such as various liposomes,[417-418] polymeric conjugates,[419-420] dendrimers,[421] quantum dots,[422] gold nanoparticles,[423] and calcium nanoparticles.[424-425] For example, Ostim® is a commercially available nanostructured hydroxyapatite paste used in bone tissue engineering.[174,426] With continued research and development efforts, more and more nanoparticle-based therapeutic products will become commercially available in the market.

6. Conclusions

Nanotechnology holds great promise for delivering technological breakthroughs

to the medical community. Here, several developments in the application of nanoparticles for drug delivery and tissue engineering were reviewed. To some extent, the currently approved nanoparticle-based therapeutic platforms have improved the therapeutic index of drugs by reducing drug toxicity, mitigating side effects, or enhancing drug efficacy. The next generation of nanoparticle systems will have targeting ligands such as antibodies, peptides, or aptamers, which may further improve efficacy or reduce toxicities. In order to achieve this goal, more complex systems such as multifunctional nanoparticles that are concurrently capable of targeting, imaging, and therapy are the direction of future investigational research. The introduction of safer nanomaterials together with novel engineering approaches that result in optimally designed nanoparticles is anticipated to lead to an increasing number of multifunctional nanoparticles in the clinic in the future.

Among all these nanotechnologies, colloidal nanotechnology has been widely used in many different areas. A colloid system is a suspension in which the dispersed phase is so small (1-1000nm) that gravitational force are negligible and interactions are dominated by short-range and temporary forces, such as van der Waals force, electrostatic force (charge), and steric force. The colloidal gels researched here exhibited shear-thinning behavior due to the disruption of interparticle interactions as the applied shear force was increased. Once the external force was removed, the cohesive property of the colloidal gel was recovered. This unique shear-thinning behavior makes colloidal gels an ideal material both for tissue engineering and drug delivery, such as moldable tissue scaffolds to treat bone defects

and as injectable drug carriers for local drug delivery. In this thesis, emphasis was on creating novel injectable and biodegradable gels using colloidal nanotechnology as a material suitable for biomedical applications.

CHAPTER II

Biodegradable Colloidal Gels as Moldable Tissue Engineering Scaffolds

1. Introduction

Colloidal gels composed of oppositely-charged nanoparticles at high concentration exhibit pseudoplastic behavior facilitating the fabrication of shape-specific microscale materials. Emerging applications in materials science have aimed to leverage the unique properties of colloidal gels.[427-431] For example, Lewis and others have reported that freeform printing of colloidal gels may be utilized to produce 3-D, microperiodic networks exhibiting precise structure.[432-437] The cohesive strength of these materials depends upon interparticle interactions such as; electrostatic forces, van der Waals attraction, steric hindrance, *etc*.,[438] which may be leveraged to facilitate the synthesis of ceramic devices, sensors, or drug delivery systems.[6,15,439-447] The application of moldable colloidal gels towards generating tissues has also been proposed,[448-449] but has yet to be applied with the integration of biodegradable nanoparticles composed of materials commonly used as tissue engineering scaffolds.

Poly(D,L-lactic-co-glycolic acid) (PLGA) is an FDA approved, biocompatible and biodegradable polymer, which has been widely used in pharmaceutical products and as tissue engineering scaffolds.[450-451] Here, oppositely charged PLGA nanoparticles were combined to create a cohesive colloidal gel. The colloid self-assembled through electrostatic force resulting in a stable 3-D network that was easily molded to the desired shape. The colloidal gel demonstrated shear-thinning behavior due to the disruption of interparticle interactions as the applied shear force was increased. Once the external force was removed, the strong cohesive property of

the colloidal gel was recovered. This reversibility makes the gel an excellent material for molding, extrusion, or injection of tissue scaffolds (Figure 2.1).

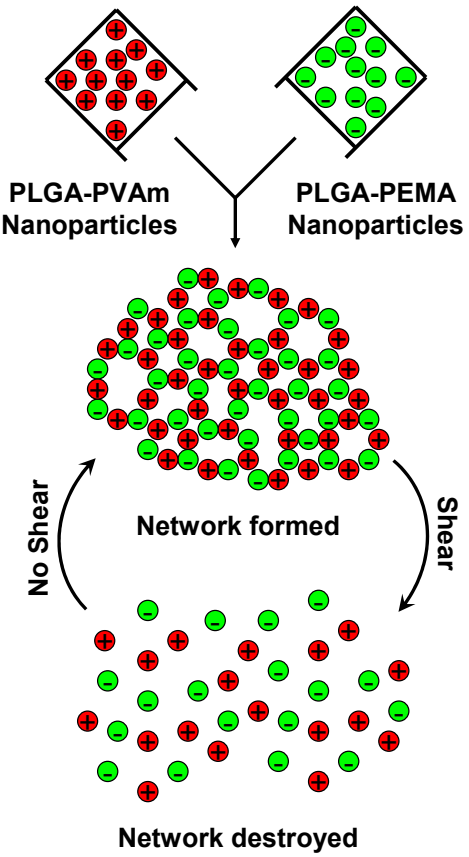

Figure 2.1. Schematic representation of colloidal gel formation and properties.

2. Materials and Methods

2.1. Materials

All materials were purchased from Aldrich unless otherwise stated. PLGA (75:25) (inherent viscosity: 0.47 dL/g in Chloroform at 30°C) was purchased from Absorbable Polymers. PEMA was purchased from Polysciences, Inc. Fluorescein was purchased from Acros Organics. Rhodamine B was purchased from MP Biomedicals, Inc. All cell culture media were supplied by Invitrogen.

2.2. Preparation of Charged PLGA Nanoparticles

100 mg of PLGA was dissolved in 10.0 mL acetone and then the solution was added into 0.05% PVAm or PEMA (150 mL) through a syringe pump (20 mL/h) under stirring at 200 rpm overnight to evaporate acetone. Nanoparticles were collected by centrifugation (16,000 rpm, 20 min). The nanoparticles were washed using deionized water three times to remove excess surfactant. A fine powder of charged nanoparticles was obtained by lyophilization for ~2 days.

2.3. Preparation of Colloidal Gels

Lyophilized nanoparticles (PLGA-PVAm or PLGA-PEMA) were dispersed in deionized water at 20% wt/vol. These dispersions were mixed in different proportions to obtain the different weight ratios studied. Homogeneous colloid mixtures were prepared in a bath sonicator for 3 minutes and stored at $4^{\circ}C$ for 2h to allow stabilization before use.

2.4. Characterization of Nanoparticles and Colloidal Gels

The sizes and zeta potentials of the different PLGA nanoparticles were determined using a ZetaPALS dynamic light scattering system (Brookhaven, ZetaPALS). SEM was performed using a LEO 1550 field emission scanning electron microscope at an accelerating voltage of 5 kV. Laser scanning confocal microscopy was performed on an Olympus/Intelligent Innovations Spinning Disk Confocal Microscope with epifluorescence attachment.

2.5. Rheological Experiments

Rheological experiments were performed by a controlled stress rheometer (AR2000, TA Instrument Ltd.). Cone steel plates (2°, 20 mm diameter) were used and the 500 μm gap was filled with colloidal gel. A solvent trap was used to prevent evaporation of water. The viscoelastic properties of the sample were determined at 20 °C by forward-and-backward stress sweep experiments. The viscosity (η) was monitored while the stress was increased and then decreased (frequency = 1Hz) in triplicate with 10 minutes between cycles. The gel recoverability was assessed using no time break between cycles.

2.6. Cell Culture

Human umbilical cord matrix stem cells (HUCMSCs) were harvested and cultured until passage 1 as previously described[452] and then frozen in media consisting of 80% fetal bovine serum (FBS) and 20% dimethyl sulfoxide until use. Cells were thawed and expended to passage 4 for cell seeding at culture medium including low glucose Dulbecco's Modified Eagle's Medium**,** 20% FBS, and penicillin streptomycin (PS).

2.7. Cell Seeding and Viability

HUCMSCs were seeded onto colloidal gels at a density of 1×10^6 cells/mL. The colloidal gel was sterilized under UV light for 10 min. Cells were deposited on colloidal gels in the individual wells of a 24-well untreated plate, then 1 mL of defined medium

was added into wells.[452] Cells were cultured in monolayer on the gel surface for 2 wks, with half of the media changed every other day. Subsequently, the scaffolds were stained with LIVE/DEAD reagent (dye concentration 2 mM calcein AM, 4 mM ethidium homodimer-1; Molecular Probes) and incubated for 45 min, before being subjected to fluorescence microscopy (Olympus/Intelligent Innovations Spinning Disk Confocal Microscope).

3. Results and Discussion

3.1. Characterization of PLGA Nanoparticles and Colloidal Gels

Oppositely charged PLGA nanoparticles were prepared by a solvent diffusion method. PLGA dissolved in acetone was titrated into a water phase containing polyvinylamine (PVAm)[453] or poly(ethylene-co-maleic acid) (PEMA)[454] resulting in the precipitation of PLGA nanoparticles coated with the respective polyelectrolyte. The particle size of PLGA-PVAm nanoparticles was slightly smaller than that of PLGA-PEMA nanoparticles and the absolute value of the particle zeta potential of PLGA-PVAm nanoparticles was significantly larger than that of PLGA-PEMA nanoparticles (Table 2.1). These differences influenced gel properties since zeta potential and particle size are two critical factors influencing the properties of colloidal gel systems.[436]

Colloidal gels exhibiting different degrees of cohesiveness were formed by mixing different ratios of positively and negatively charged PLGA particles and by controlling the total concentration of particles in suspension. For initial studies,

cationic or anionic nanoparticles were suspended in deionized water at 20% (w/w). Scanning electron micrographs of dried colloidal networks revealed little difference in the structure of dried gels containing different mass ratios of nanoparticles (Figure 2.2). When dried, each mass ratio (3:7, 1:1, and 7:3; PLGA-PEMA: PLGA-PVAm) exhibited a loosely organized, porous structure. Nanoparticles were linked together into micrometer-scale, ring-like structures, which interconnected to form the bulk porous structure observed. Domains of more tightly packed nanoparticle agglomerates were also evident suggesting that the cohesive nature of these colloidal gels results from an equilibrium of nanoparticle attraction (tight agglomerates) and repulsion (pores).

Table 1. PLGA nanoparticle properties

	PLGA-PEMA	PLGA-PVAm
Size (nm)	181±15	144±12
Polydispersity	0.116	0.095
Zeta potential (mV)	-20.1±1.0	+32.2±1.3

Table 2.1. Sizes and zeta potentials of PLGA Nanoparticles

Laser scanning confocal microscopy (LSCM) was used to probe the structure of colloidal gels in solution. For this study, PLGA-PEMA nanoparticles were dyed with fluorescein (green) and PLGA-PVAm nanoparticles were dyed using rhodamine B (red). Colloidal gels were diluted by deionized water to 5% (w/w) for LSCM studies since high concentrations encumbered image acquisition. 3-D projections of colloidal

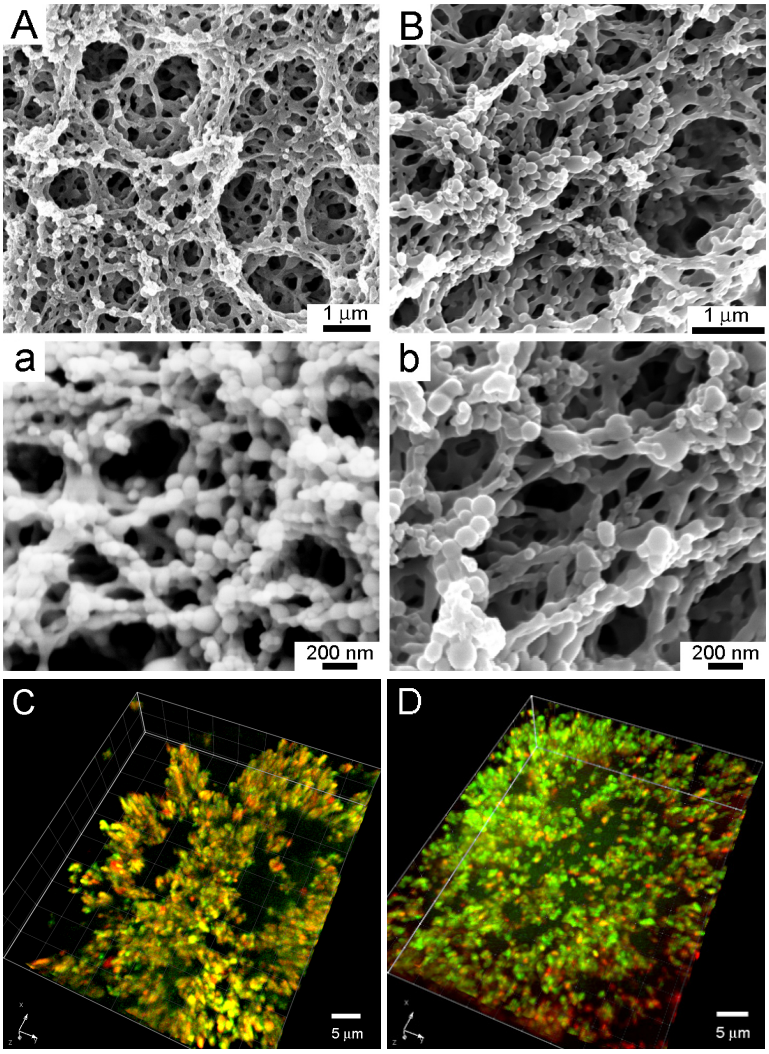

Figure 2.2. SEM observation of colloidal gels revealed similar porous microstructure and nanostructure for (A and a) 1:1 and (B and b) 7:3 (PLGA-PEMA:PLGA-PVAm) weight ratios in the dry state. Laser scanning confocal micrographs (LSCM) of more dilute colloidal gels (5% wt/vol) revealed that (C) 1:1 weight ratio contained nanoparticles organized into networks, but (D) the 7:3 ratio did not exhibit similar long-range structure [PLGA-PEMA nanoparticles (green): PLGA-PVAm nanoparticles (red)].

gels formed from mass ratios of 1:1 revealed long-range structure in the form of rings or bridges that were interconnected by more tightly agglomerated particles (Figure 2.2.C). 7:3 mass ratios appeared more homogeneous with discrete agglomerates of nanoparticles evident, but a lesser degree of long-range structure (Figure 2.2.D). Notice of these structures *in situ* supported the evidence of micro- and nanostructure

of dried colloidal gels observed by SEM. 3-D LSCM composite images for 3:7 mass ratios were not attainable because of high particle mobility, which lead to image smearing during acquisition.

One may suspect that 3:7 and 7:3 mass ratios of nanoparticles may behave similarly; however, colloidal gels composed of excess positively charged particles (3:7 mass ratio) exhibited more fluidity. LSCM video clips demonstrated the confined mobility of nanoparticles and fewer agglomerates compared to the 1:1 and 7:3 mass ratios (see supplementary video of reference [455]). In contrast, nanoparticles in colloidal gels comprising 1:1 and 7:3 mass ratios were essentially motionless. The larger zeta potential of positively charged nanoparticles resulted in a more equal overall charge balance when negatively charged particles were in excess, thus, providing a probable explaination for the stronger cohesion observed in the 7:3 mass ratio compared to the 3:7 mass ratio.

3.2. Rheological Properties of Colloidal Gels

Rheological studies were employed to further probe the differences in plasticity of colloidal gels (Figure 2.3). Equal mass ratios of nanoparticles yielded the highest viscosity gel. As expected, mass ratios containing more negatively charged particles (7:3) exhibited higher viscosity than the inverse mass ratio. Pure nanoparticle suspensions exhibited minimal shear-thinning behavior. Viscosity was enhanced and shear-thinning more pronounced as the concentration of nanoparticles increased (Figure 2.3.B). Consecutive acceleration/deceleration cycles of the shear force

revealed that these colloidal gels do not rapidly recover. Delaying shear cycles for more than one hour, however, enhanced the recovery of gel viscosity (Figure 2.3.C).

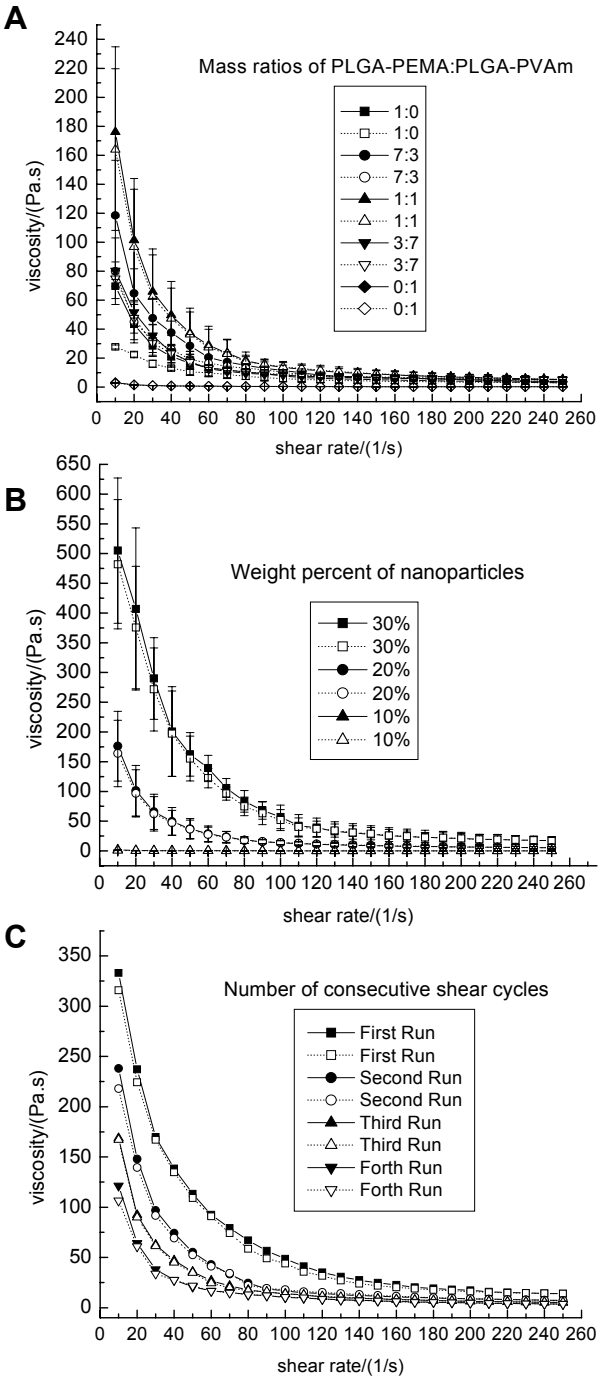

Figure 2.3. (A) High viscosity and shear-thinning behavior were observed in colloidal gels mixed at different ratios compared to pure nanoparticles for accelerating (solid symbols) and decelerating (open symbols) shear force. (B) Increasing nanoparticle mass per volume of water systematically increased viscosity trends. (C) Colloidal gels with a 1:1 mass ratio showed a steady decrease in viscosity for each cycle when no recovery time was allowed between shear cycles.

3.3. Cytotoxicity of Colloidal Gels to hUCMSCs

The pseudoplastic behavior of colloidal gels was leveraged to construct

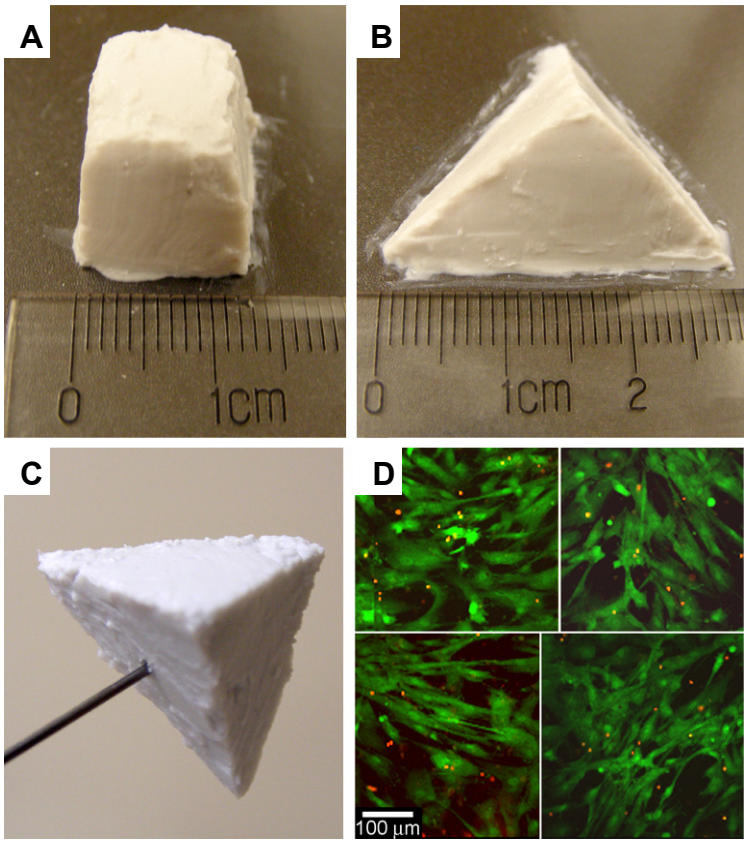

Figure 2.4. (A and B) Different shapes of tissue scaffolds made from 20% wt/vol colloidal gels (1:1 mass ratio). (C) Gels possessed sufficient cohesiveness to be handled by a 20 gauge needle. (D) Human umbilical cord matrix stem cells cultured on colloidal gels demonstrated high viability (green) and minimal cell death (red).

differently shaped tissue scaffolds (Figure 2.4). Molded scaffolds exhibited stable structure and shape retention when handled (Figure 2.4.C). The compatibility of colloidal gels with human umbilical cord matrix stem cells (HUCMSCs) was also assessed. For this study, colloidal gels were deposited and shaped in well plates. The scaffolds maintained integrity when culture media was introduced. HUCMSCs seeded onto the surface of the scaffolds were highly viable (green fluorescence), exhibiting

minimal cell death (red fluorescence), which suggested that these colloidal gels were non-toxic to HUCMSCs (Figure 2.4.D). In addition, cell morphology was indicative of substantial cell adhesion to the scaffold.

3.4. Discussion

Colloidal gels were fabricated using oppositely-charged, biodegradable PLGA nanoparticles that interact to form stable 3-D structures. Malleability under shear and strong static cohesion facilitated fabrication of shape-specific tissue scaffolds. The overall theme of this work is depicted in Figure 2.5.

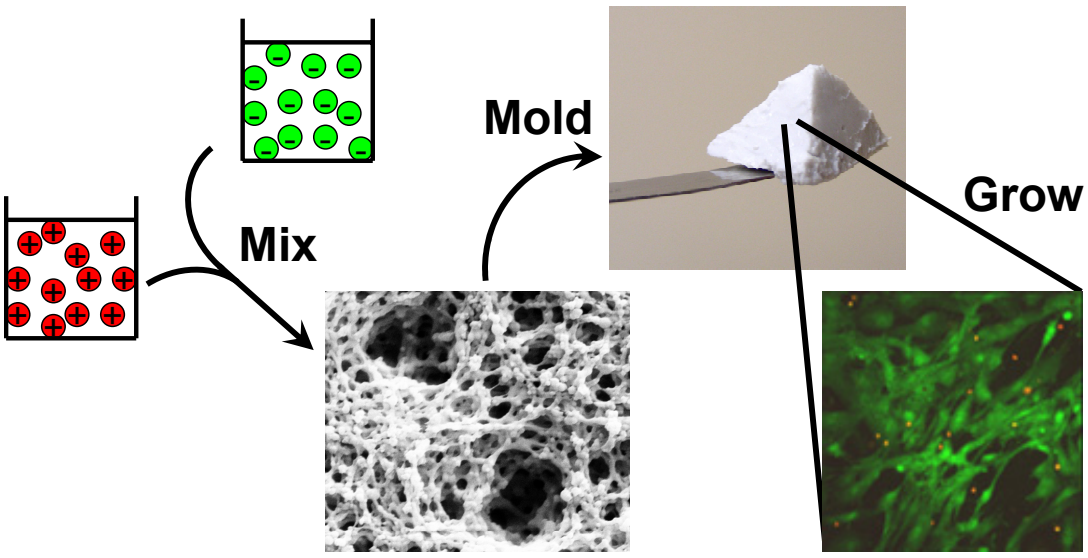

Figure 2.5. Colloidal gels were fabricated using oppositely-charged, biodegradable PLGA nanoparticles that interact to form stable 3-D structures; malleability under shear and strong static cohesion facilitated fabrication of shape-specific tissue scaffolds.

In colloidal gel systems, the volume fraction (ϕ) and movement frequency (ω) of solid particles determines the viscosity of the system[455] as described by:

$$\eta(\phi,\omega) = \eta_1(\phi) + \eta_2(\omega) \qquad (1)$$

The variable η is the viscosity of the colloidal system and is ascribed two parts: η_1 designated as the contribution of volume fraction of solid nanoparticles (increasing viscosity with higher fraction of solids, see Figure 2.3.B) and η_2 designated as the contribution of particle movement frequency as determined by interparticle interactions (*e.g.* electrostatic force, van der Waals attraction, steric repulsion). In cohesive colloidal gels, the movement frequency describes how easily a particle can escape from energy barriers associated with neighbor particles. Under static conditions, ϕ may strongly dictate the viscosity and structure of colloidal assemblies leading to a stable structure exhibiting high viscosity at equilibrium. If the particle-particle equilibrium is disrupted by an external force, the requisite activation energy for nanoparticle escape from the colloidal structure decreases simultaneously, thus, propagating a tendency towards viscosity reduction (shear-thinning) as the external force is increased. The composite balance of these attractive and repulsive forces under static conditions also directs the formation of the porous structures observed (Figure 2.2).

4. Conclusions

Advancing the applications of moldable or injectable tissue scaffolds will require continued efforts to add control of material plasticity and recoverability to proven biodegradable materials. PLGA-based colloidal gels reported here offered desirable properties for molding tissue scaffolds and demonstrated negligible toxicity to HUCMSCs. Multiple avenues exist for expanding the applications of these materials.

For example, scaffolds may be molded and freeze dried to create more rigid structures or directly injected as *in situ* forming scaffolds. Application of porogens to these materials may ultimately be leveraged to promote in-growth of cells and enhance interconnected pore 3-D structure. In addition, integration of controlled release strategies (*e.g.* growth factors) would be straightforward and would allow advanced combination strategies for tissue engineering coupled with growth factor delivery.

CHAPTER III

Injectable PLGA Based Colloidal Gels for Zero-order Dexamethasone Release in Cranial Defects

1. Introduction

Injectable biomaterials that form three-dimensional (3-D) structures *in situ* are being pursued as tissue engineering scaffolds,[442], biosensors[443] and drug delivery systems.[66] Colloidal gels composed of oppositely-charged nanoparticles at high concentration can exhibit pseudoplastic behavior facilitating the fabrication of shape-specific macroscale materials with 3-D architectures.[428,441] Emerging applications in materials science have also aimed to leverage the unique properties of colloidal gels.[427,430,432,456-459] For example, freeform printing of colloidal gels may be utilized to produce 3-D, microperiodic networks exhibiting precise structure.[433-435,437] The cohesive strength of these materials depends upon interparticle interactions such as electrostatic forces, van der Waals attraction, steric hindrance, *etc*. [438] which may be leveraged to achieve unique bulk material properties for many different applications.[439-440,444,446-447,460] The application of moldable colloidal gels towards generating tissues has also been proposed,[448-449] but has not yet to be applied with the integration of biodegradable and non-cytotoxic nanoparticles composed of materials commonly used as tissue scaffolds.

The injectability of 3-D scaffolds is desired as a means to fill tissue defects of irregular size and shape. Injectable scaffolds are particularly desired in order to avoid the invasive surgery typically required for tissue implantation.[461] From a clinical perspective, the use of injectable scaffolds is an attractive alternative to surgery as it reduces the risk of infection, scar formation, patient discomfort and the cost of treatment.[462] Recently, many scaffolds that stiffen or solidify *in vivo* have been

applied as injectable scaffolds.[463] Often injectable scaffolds are polymerized or chemically crosslinked to stiffen the material. Chemically crosslinked scaffolds normally form via an *in situ* reaction induced by the presence of water, heat, light or other stimuli. During solidification, However, toxic chemical agents are sometimes employed which may adversely affect the scaffolds, destabilize encapsulated biomolecules, or pose toxicity concerns. On the contrary, colloidal gels that stiffen through interactions such as electrostatic forces, van der Waals attraction and steric hindrance may overcome some of these limitations.

Poly (D,L-lactic-co-glycolic acid) (PLGA) is a biodegradable polymer, which has been widely used in pharmaceutical products and in tissue engineering scaffolds.[450-451,464-465] Previously, oppositely-charged PLGA nanoparticles were used to create a cohesive colloidal gel. The colloid self-assembled through electrostatic forces resulting in a stable, porous 3-D network that was easily molded to the desired shape.[466] The colloidal gel demonstrated shear-thinning behavior due to the disruption of interparticle interactions as the applied shear force was increased. Once the external force was removed, the strong cohesive property of the colloidal gel was recovered. This material also demonstrated negligible toxicity to human umbilical cord matrix stem cells (hUCMSCs). The biodegradability, biocompatibility and reversibility of this unique material make it a potential injectable scaffold for tissue engineering.

In this paper, PLGA colloidal gels were studied as an injectable controlled release system to deliver dexamethasone (DEX). DEX is a glucocorticoid which is usually

used as an anti-inflammatory and immunosuppressive agent in bone tissue engineering.[467-469] In addition, previous reports suggest that glucocorticoids such as DEX may facilitate osteogenesis.[470-471] Here, DEX was used to investigate the drug release properties of PLGA colloidal gels. The rheological properties of the drug-loaded colloidal gel were also evaluated and its application in rat cranial bone defects was assessed.

2. Materials and Methods

2.1. Materials

All materials were purchased from Sigma-Aldrich unless otherwise stated. PLGA (75:25) (inherent viscosity: 0.47 dL/g in chloroform at 30 °C) was purchased from Absorbable Polymers. DEX was obtained from Alfa Aesar Co. Poly (ethylene-co-maleic acid) (PEMA) was purchased from Polysciences Inc. Surfactant PEMA[453] and polyvinylamine (PVAm)[454] were synthetic through reported protocols.

2.2. Preparation of Blank PLGA Nanoparticles

The oppositely-charged blank PLGA nanoparticles were prepared by a solvent diffusion method. 100 mg of PLGA was dissolved in 10 mL acetone and then the solution was added into 0.2 % PVAm or PEMA (150 mL) surfactant solution through a syringe pump (20 mL/h) under stirring at 200 rpm overnight to evaporate acetone. Nanoparticles were collected by centrifugation (Beckman Co., Avanti 30) (16,000 rpm, 20 min). The nanoparticles were centrifuged and resuspended using deionized

water three times to remove excess surfactant. A fine powder of nanoparticles was obtained by lyophilization for ~2 days.

2.3. Preparation of Drug Loaded PLGA Nanoparticles

PLGA naoparticles loaded with DEX were prepared by a single oil-in-water (O/W) emulsion/solvent evaporation method.[472] In order to maximize the encapsulation efficiency (EE) of the DEX in the nanoparticles, different conditions were tested. 90 mg of PLGA was dissolved in 9 ml dichloromethane and then 10 mg DEX was dissolved in 1 ml acetone. The DEX in acetone was added to the PLGA in dichloromethane to form the oil phase. Then 10 ml of the oil phase was added dropwise to a 0.2 % PVAm or PEMA (30 mL) surfactant solution through a syringe pump (1 mL/min). The oil-in-water (O/W) emulsion was formed using a high speed homogenizer (Biosepc Products, Inc.) at 16,000 rpm in an ice bath to prevent overheating. Then the emulsion was added to a 160 ml 0.2 % PVAm or PEMA surfactant solution under stirring at 200 rpm overnight to evaporate the organic phase. DEX-loaded nanoparticles were collected by centrifugation (16,000 rpm, 20 min). The nanoparticles were centrifuged and resuspended using deionized water three times to remove excess surfactant and free drug. A fine powder of drug loaded nanoparticles was obtained by lyophilization for ~2 days.

2.4. Preparation of Colloidal Gels

Lyophilized nanoparticles (PLGA-PVAm or PLGA-PEMA, blank or drug loaded)

were dispersed in deionized water at 20 % wt/vol. These dispersions were mixed in different ratios to obtain the different PLGA-PEMA: PLGA-PVAm ratios studied. Homogeneous colloid mixtures were prepared in a bath sonicator for 3 minutes and stored at 4 $^{\circ}$C for 2 h before use. Several gels with different mass ratios of PLGA-PEMA nanoparticles to PLGA-PVAm nanoparticles were designated as AB73, AB55 and AB37 (A: PLGA-PEMA nanoparticles; B: PLGA-PVAm nanoparticles; the mass ratio of PLGA-PEMA nanoparticles to PLGA-PVAm nanoparticles is 70:30, 50:50 and 30:70, respectively).

2.5. Characterization of Nanoparticles and Colloidal Gels

The sizes and zeta potentials of the different PLGA nanoparticles were determined using a ZetaPALS dynamic light scattering system (Brookhaven, ZetaPALS). All samples were analyzed in triplicate. Scanning electron microscopy (SEM) was performed using a Jeol JSM-6380 field emission scanning electron microscope at an accelerating voltage of 10 kV.

2.6. Rheological Experiments

Rheological experiments were performed by a controlled stress rheometer (AR2000, TA Instrument Ltd.). 2° cone steel plates (20 mm diameter) were used and the 500 μm gap was filled with colloidal gel. A solvent trap was used to prevent evaporation of water. The viscoelastic properties of the sample were determined at 20°C by forward-and-backward stress sweep experiments. The viscosity (η) was

monitored while the stress was increased and then decreased (frequency = 1 Hz) in triplicate with 10 minutes between cycles. The gel recoverability was assessed using the defined time break between cycles. All samples were analyzed in triplicate.

2.7. In vitro Drug Release Tests

The encapsulation efficiency of DEX in the drug loaded nanoparticles was determined by dissolving 10 mg of drug-loaded nanoparticles powder in 2 ml trifluoroethanol (TFE). Samples were rotated for at least 24 h at 10 rpm to ensure complete dissolution in TFE. Blank nanoparticles were treated identically. The concentration of DEX in the resulting solution was determined by measuring the absorbance at 242 nm in a spectrophotometer (Agilent Technologies, 89090A) and then subtracting the absorbance values for the blank nanoparticles. All samples were analyzed in triplicate.

DEX release profiles were determined by suspending 800 mg of drug-loaded nanoparticles in 50 mL of PBS (pH 7.4). The samples were incubated at 37 °C while shaking at 50 rpm in an incubator/shaker (New Brunswick Scientific C24). At selected time points, the supernatants were removed and replaced with fresh buffer. The concentration of DEX in the supernatant was determined using the UV detection method described above. The absorption of supernatant collected from blank PLGA nanoparticles was negligible at 242 nm throughout the release study. The amount of drug in each sample was summed with the amount at each previous time point to obtain the cumulative drug release amount and the total was divided by the amount of drug in

the nanoparticles (encapsulation efficiency times mass of drug-loaded nanoparticles) to calculate the cumulative drug release percentage. Each release experiment was performed in triplicate.

2.8. Animals and Surgical Implantation of Colloidal Gels

The use of animals and the surgical procedures used in this study were approved by the Animal Care and Use Committee at the University of Kansas Medical Center, Kansas City, KS. Male Sprague-Dawley rats, 7 to 8 weeks old, were used in this study. All surgical procedures were performed under general anesthesia and sterile conditions. A longitudinal skin incision was made centered over the mid-sagittal suture of the skull. Following careful dissection and removal of the periosteum, an 8-mm diameter full thickness defect was produced in the parietal bone using a dental burr. The wound was carefully rinsed with normal saline to remove any bone debris in the defect, which was an important step in this procedure for evaluating the formation of new bone within the cranial bone defect as previously described.[473-474] The cranial defects were filled with injectable PLGA colloidal gel, PLGA colloidal gel with DEX, or left untreated. The wound was closed with a 5-0 nylon suture. The day of surgery was designated as day "0". Animals were sacrificed at 4 weeks after surgery and operated calvaria were harvested for histological and histochemical analyses.

2.9. Histology and Histochemistry

To evaluate the cellular and matrix responses to the implants, implanted materials

were retrieved with the surrounding host bone and residual periosteum. Tissue samples were fixed in 2% paraformaldehyde (Boston BioProducts), pH 7.4, decalcified in 25% formic acid (Sigma), embedded in paraffin, sectioned at 5 μm, and stained with either hematoxylin and eosin (H&E) or safranin-O that identifies cartilage cells and cartilage matrix. At least three animals per treatment group were examined histologically and histochemically.

3. Results and Discussion

3.1. Characterization of PLGA Nanoparticles and Colloidal Gels

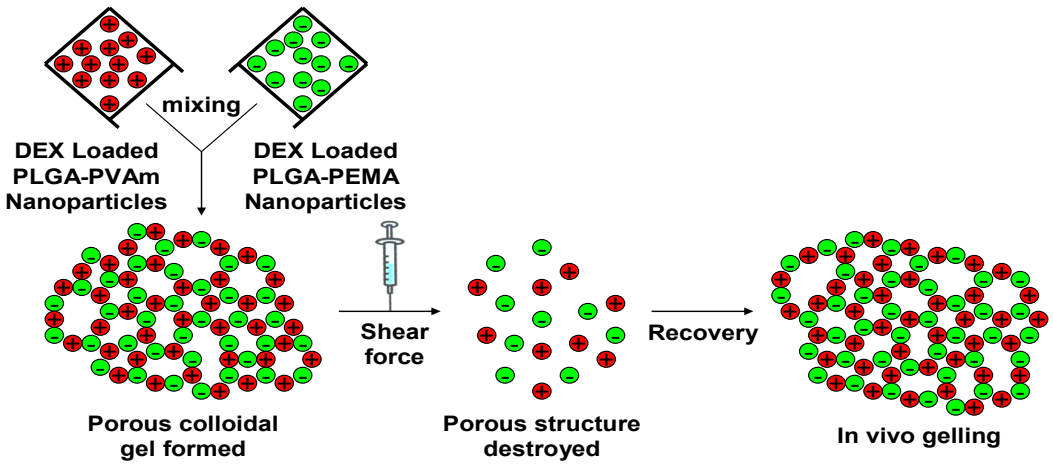

Figure 3.1. Schematic representation of the properties of DEX-loaded colloidal gels.

A schematic representation of the DEX-loaded colloidal gels provides an overview of the concept (Figure 3.1). Drug-free PLGA nanoparticles were prepared by a solvent diffusion method. PLGA dissolved in acetone was titrated into a water

phase containing PVAm or PEMA. The surface charge of particles resulted from the precipitation of PLGA nanoparticles coated with the respective polyelectrolyte. The particle sizes of drug-free nanoparticles were 181 ± 15 nm (PLGA-PEMA) and 144 ± 12 nm (PLGA-PVAm). The zeta potentials of drug-free nanoparticles were -20.1 ± 1.0 mV (PLGA-PEMA) and +32.2 ± 1.3 mV (PLGA-PVAm).[466] The DEX-loaded PLGA nanoparticles were prepared by a single oil-in-water (O/W) emulsion/solvent evaporation method. PLGA and DEX dissolved in a mixture of dichloromethane and acetone was emulsified into a water phase containing PVAm or PEMA. The sizes of drug-loaded nanoparticles were 241 ± 26 nm (PLGA-PEMA) and 182 ± 21 nm (PLGA-PVAm). The zeta potentials of drug-loaded nanoparticles were -28.5 ± 2.1 mV (PLGA-PEMA) and +34.6 ± 2.5 mV (PLGA-PVAm).

Zeta potential and particle size are two critical factors influencing the cohesive properties of colloidal gel systems.[466] Small nanoparticles (~100-200 nm) were selected to provide the desired cohesive strength of colloidal gels. Larger particles yield fewer particle-particle contacts, thus reducing the gel strength. The large zeta potential of the DEX-loaded nanoparticles also facilitated tight particle packing as a result of strong electrostatistic interparticle attraction. These features led to the formation of stable, drug-loaded colloidal gels. DEX-loaded nanoparticles were linked together into micrometer-scale, ring-like structures resulting in a bulk porous structure with microchannels. The formation of these structures is hypothesized to result from an equilibrium of nanoparticle attraction (tight agglomerates) and repulsion (pores) (Figure 3.2). Microchannels were ~1 μm in the dry state; however,

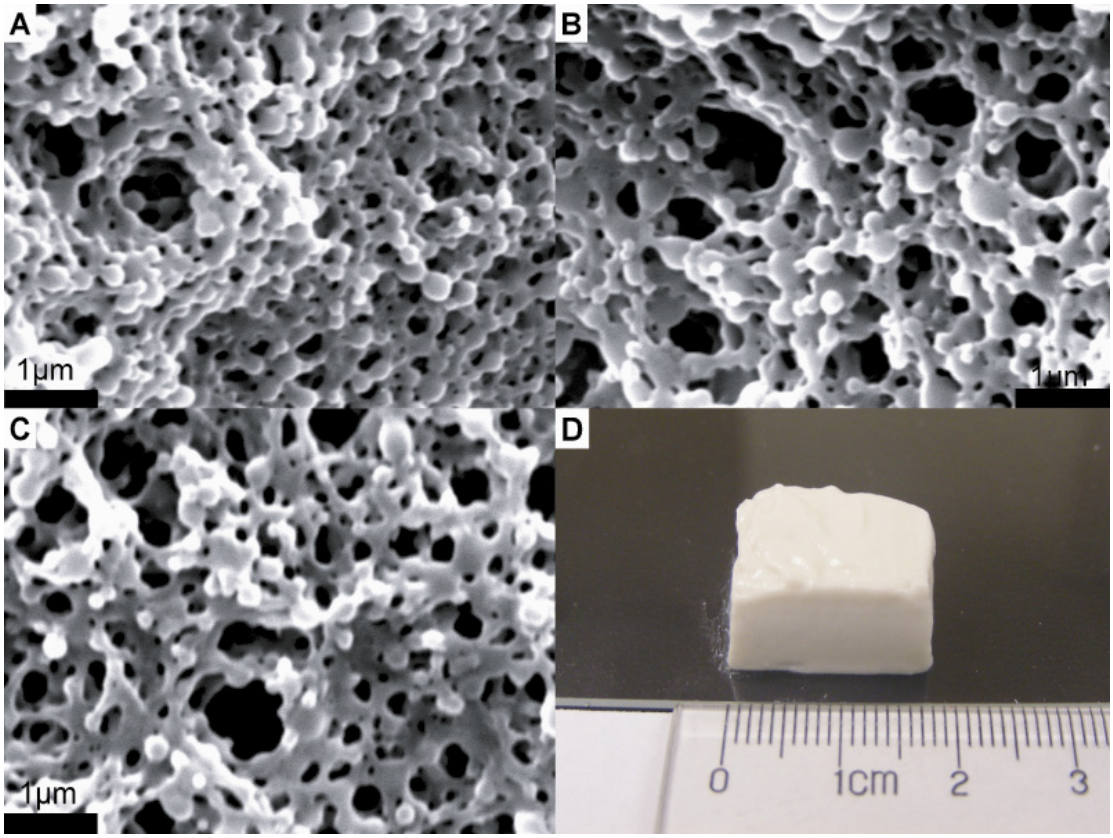

Figure 3.2. Scanning electron micrographs of AB55 colloidal gel with 10% DEX loading (A), AB73 colloidal gel with 10% DEX loading (B), AB55 colloidal gel with 20% DEX loading (C) and shaped tissue scaffold made from AB55 colloidal gel with 10% DEX loading (D) (scale bar = 1µm).

these are large in the hydrated state.[466] The entire drug-loaded colloidal gel exhibited a loosely organized, micro-porous structure which was consistent for different gel compositions and drug loading percentages. Scanning electron micrographs (SEM) of dried colloidal gels showed little difference in the structure of the gels containing different mass ratios of drug-loaded nanoparticles (Figure 3.2.A and 3.2.B), despite the different zeta potential and particle size of the DEX-loaded PLGA-PEMA and PLGA-PVAm nanoparticles. DEX loading percentage also did not affect the dry structure of the drug-loaded colloidal gels (Figure 3.2.C). The appearance of drug-loaded colloidal gels was similar to the unloaded colloidal gels;[466] molded

scaffolds still exhibited stable structure and shape retention when handled (Figure 3.2.D).

3.2. Rheological Properties of PLGA Colloidal Gels

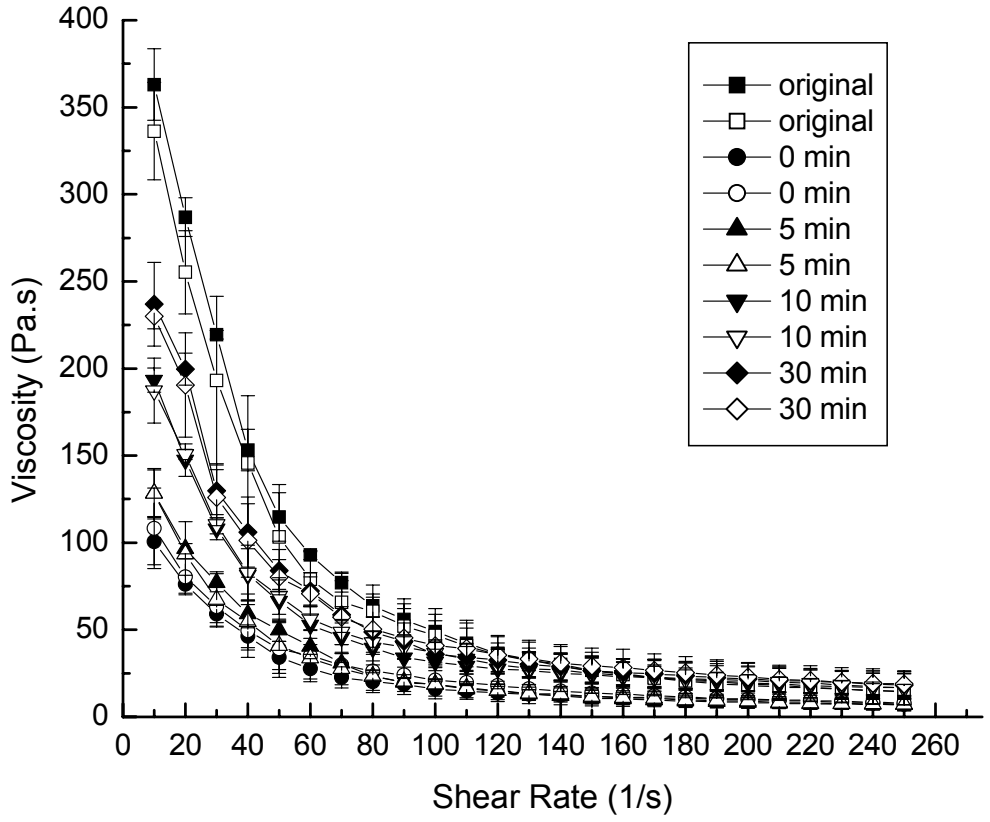

Figure 3.3. Viscosity profiles demonstrate the shear-thinning behavior of AB55 colloidal gel with 10% DEX loading for accelerating (solid symbols) and decelerating (open symbols) shear force using different lag times between cycles.

The strength of colloidal gels depends upon interparticle interactions such as electrostatic forces and van der Waals attraction.[438] Here, the nanoparticle colloid presumably self-assembled through long-range electrostatic interactions resulting in a stable 3-D network as was reported previously.[466] When an external force was applied to disrupt the interparticle interactions, the colloidal gel demonstrated shear-thinning

behavior. Once the external force was removed, the strong cohesive property of the colloidal gel was recovered. This reversibility makes the gel an excellent material for molding, extrusion, or injection of tissue scaffolds. The cohesive strength and the reversibility of colloidal gels depended on the mass ratios of oppositely-charged nanoparticles and the concentration of the nanoparticles in the gels.[466]

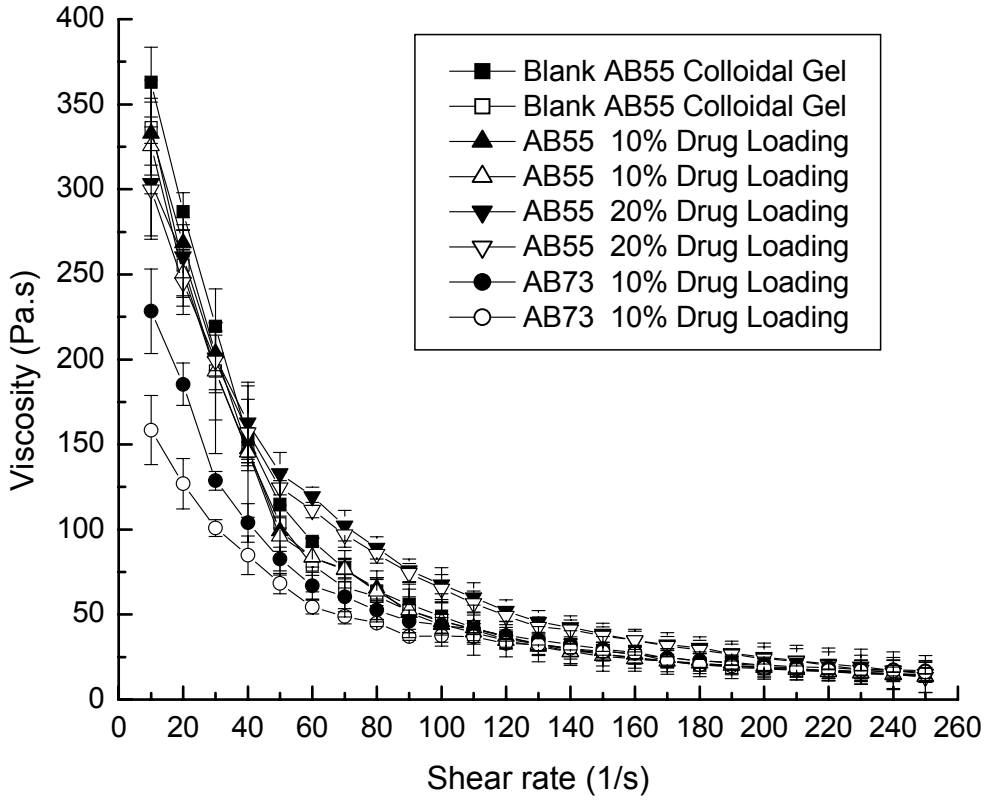

Figure 3.4. Viscosity profiles demonstrate the shear-thinning behavior of AB55 and AB73 colloidal gel with different DEX loading for accelerating (solid symbols) and decelerating (open symbols) shear force.

The dependence of lag time on the recovery of colloidal gel viscosity after accelerating/decelerating shear force cycles was determined for drug-loaded colloidal gels (Figure 3.3). The colloidal gels did not rapidly recover during consecutive acceleration/deceleration cycles of shear force. When no recovery time was allowed,

the viscosity of the colloidal gel only recovered about 30% of the original value. On the contrary, if more time (30 minutes) was provided for the recovery process, the cohesive property of the colloidal gel was restored to around 65% of the original value. The results suggested good recoverability for sheared colloidal gels.

The percentage of drug-loading did not appreciably affect the rheological behavior of colloidal gels (Figure 3.4). Meanwhile, colloidal gels with different compositions showed different viscosity profiles as expected. Equal mass ratios (50:50) of nanoparticles yielded a higher viscosity gel (AB55) than an unequal ratio (70:30) of nanoparticles (AB73). The result confirmed the importance of equilibrating charge as a means to increase cohesion.

3.3 Drug Release Tests

The encapsulation efficiency of DEX in the nanoparticles depended upon the drug loading (Figure 3.5). Because of the slight solubility of DEX in water, some was lost during the fabrication process. The PLGA-PEMA nanoparticles seemed to encapsulate DEX slightly more efficiently than PLGA-PVAm nanoparticles; however, the differences were not statistically significant.

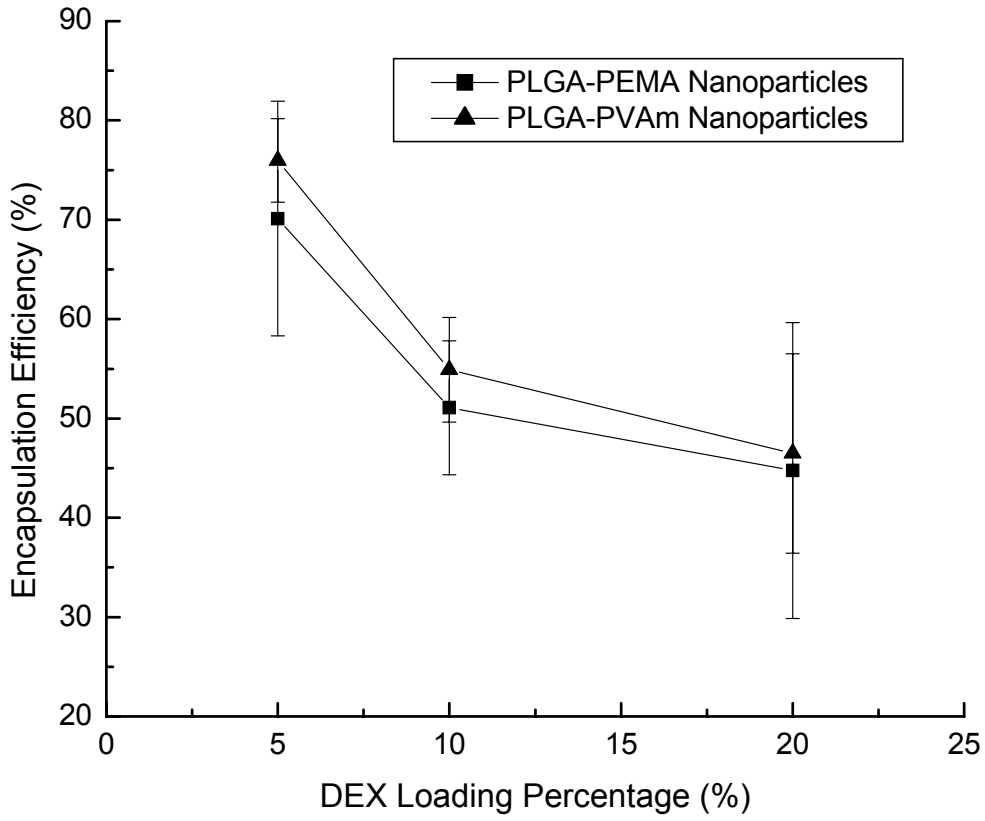

Figure 3.5. Encapsulation efficiency of DEX in PLGA-PEMA and PLGA-PVAm nanoparticles with different loading percentage.

DEX released from the colloidal gels with different drug loading for more than 60 days when the drug was encapsulated in the PLGA nanoparticles (Figure 3.6). More drug was released from the colloidal gel with the highest drug loading (20%) and followed a near zero order release profile. However, only about half of the drug was released over 60 days due to the larger amount of drug present at this high loading. On the other hand, almost all drug was released from 5% drug-loading colloidal gels within 60 days.

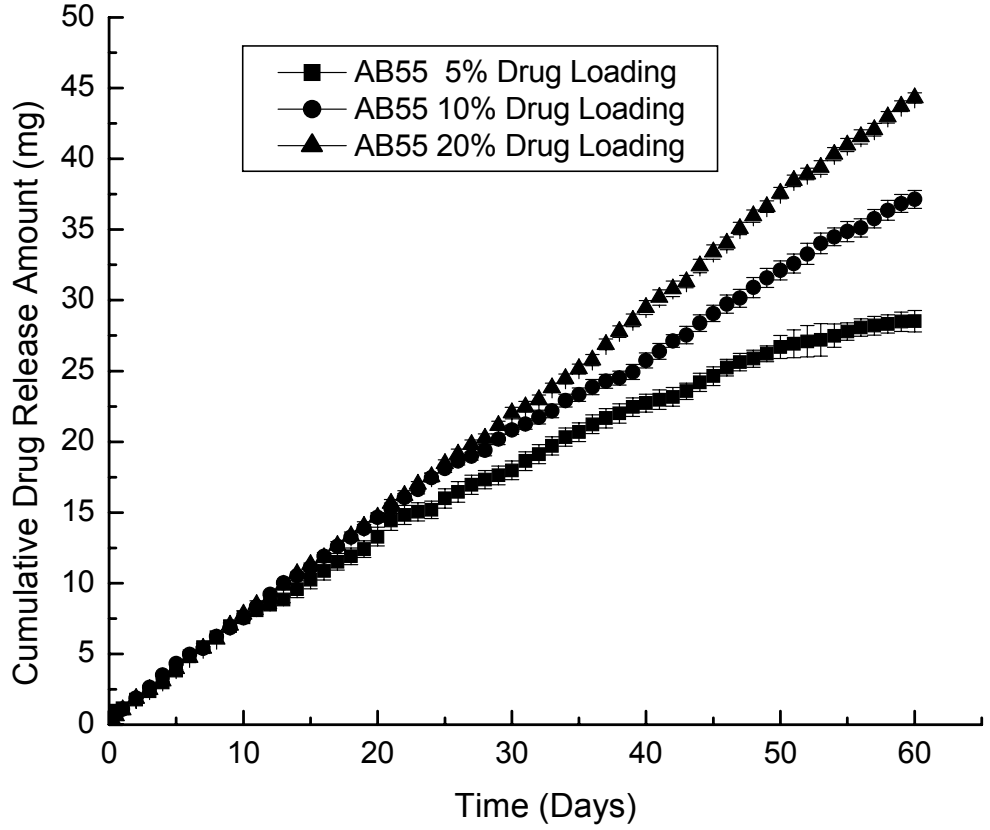

Figure 3.6. Drug release profiles of AB55 colloidal gels with different DEX loading. Drug was loaded into both positively charged and negatively charged PLGA nanoparticles.

Suspensions of purely cationic or anionic drug-loaded nanoparticles released DEX very quickly (Figure 3.7). The entire release process only lasted about 15-25 days. Colloidal gels composed of mixtures of the exact same drug-loaded nanoparticles released the drug for more than 2 months. Again, the kinetics were near linear regardless of the mass ratio of charged, drug-loaded nanopaticles in the colloidal gels. For comparison, when the drug was directly mixed with the colloidal gels, the material still performed as a controlled release drug delivery system for about 35 days (Figure 3.8). Slow diffusion of the drug through the porous

microstructure of colloidal gels and, perhaps, adsorption of the drug onto the nanoparticles, led to the sustained release profile of DEX directly mixed with the colloidal gel. Furthermore, the time required for drug release from suspensions of purely charged drug-loaded nanoparticles plus the time required for drug release from colloidal gels mixed with drug was nearly equal to the duration of drug release from colloidal gels composed of DEX-loaded nanoparticles. This result was supportive of a two-stage drug release process from this material.

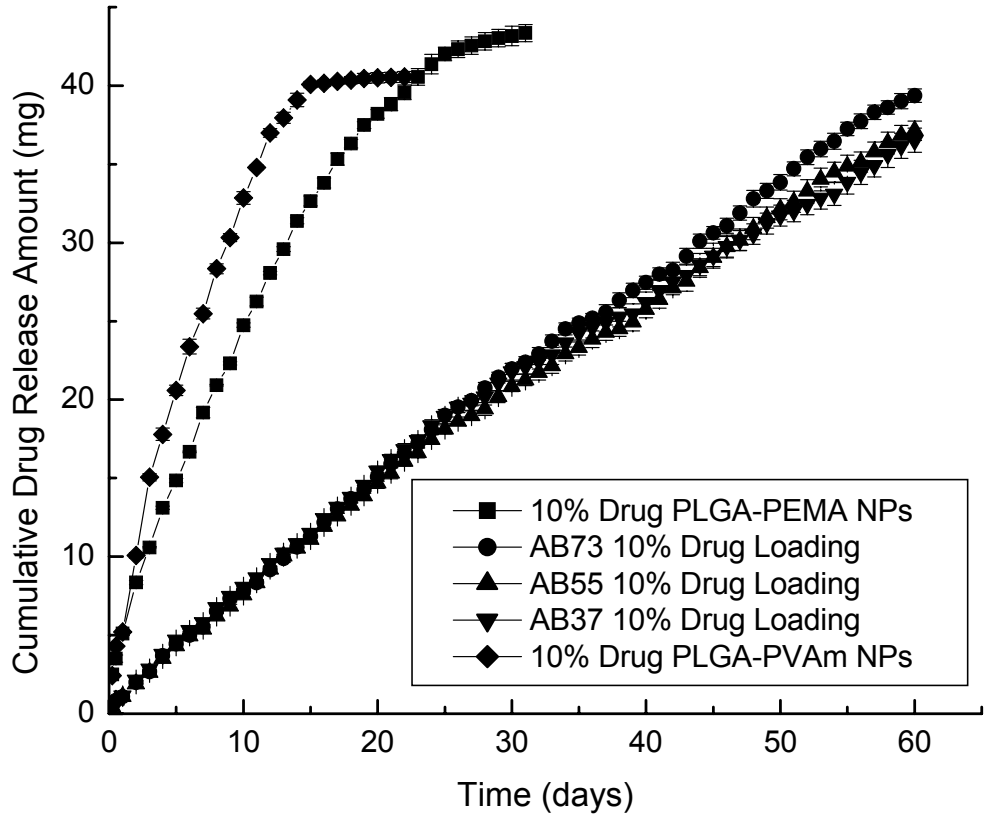

Figure 3.7. Drug release profiles of colloidal gels mixed at different ratios with 10% DEX loading. Drug was loaded into both positively charged and negatively charged PLGA nanoparticles (NPs).

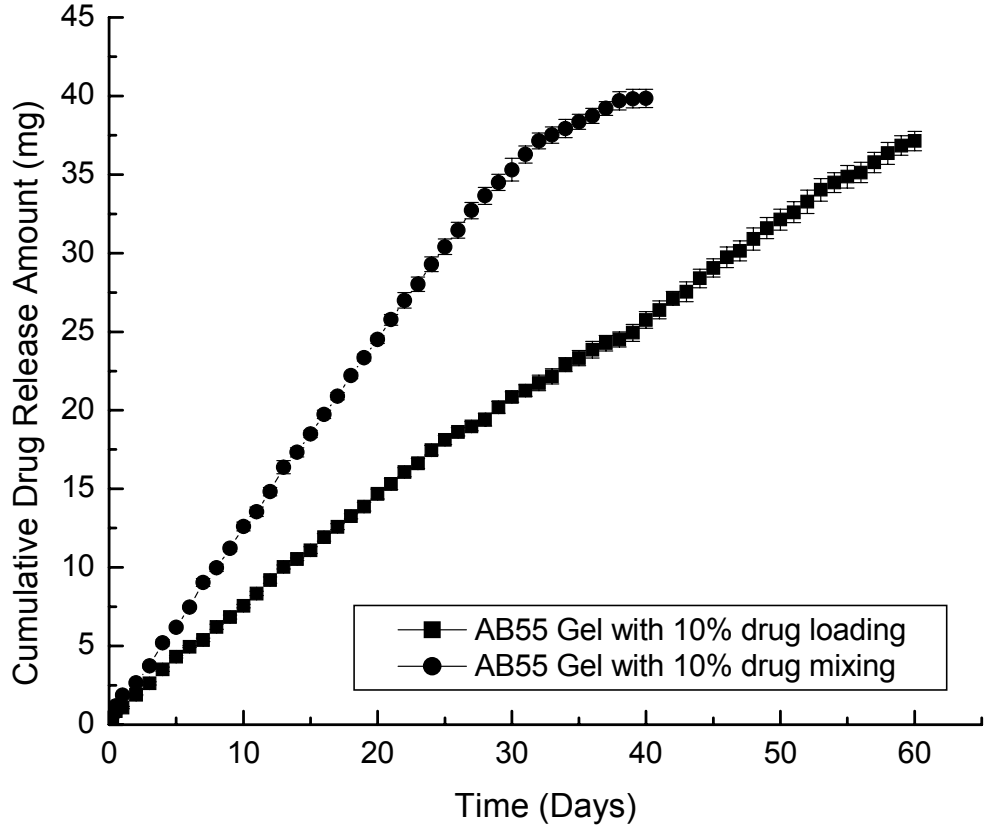

Figure 3.8. Drug release profiles of AB55 colloidal gels with 10% DEX directly mixed into the bulk material (round) compared to the drug encapsulated in both nanoparticle types (square).

In previous reports, porous drug delivery systems composed of nanoparticles with microchannels showed drug release with near zero-order kinetics.[63,475] Presumably, the drug released from the colloidal gels via a two-stage process. Here, DEX was first released from the nanoparticles into the micropores of the colloidal gel. In this process, the drug release rate was faster for colloidal gels containing nanoparticles with higher drug loading, presumably due to the larger concentration of DEX within nanoparticles driving diffusion. Then, the released drug diffused through

the microchannels and was ultimately transferred to the media. It appears that, the second process may have been the rate limiting step of this two step release mechanism. The entire release process exhibited near zero-order release and colloidal gels with higher drug loading released more drug for a longer duration, but with similar kinetics.

3.4. Histological and Histochemical Analyses

Rat cranial defects were used as an *in vivo* model to test the efficacy of PLGA colloidal gels. Previous reports suggested that sustained delivery of low doses of DEX may enhance osteogenesis; therefore, PLGA colloidal gels containing 5% DEX were selected for *in vivo* studies.[470-471] Cranial defects were chosen as a model since the current colloidal gel materials would not be amenable to load-bearing bone regeneration. These materials are desired for cranial defects, however, since they may conform to irregular defect shapes and the viscosity is appropriate for placement and subsequent stiffening *in situ*. Colloidal gels are also malleable, which may facilitate the ingrowth of new tissue.

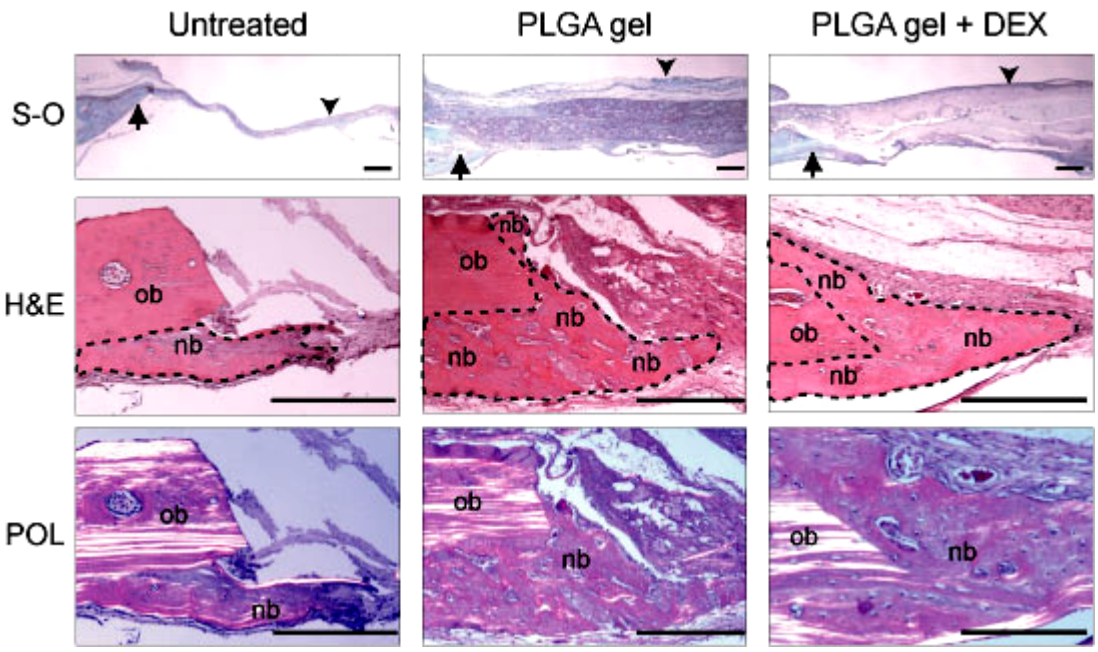

Figure 3.9. Photomicrographs of tissue sections prepared from untreated, PLGA colloidal gel (PLGA gel)-treated, or PLGA colloidal gel with encapsulated DEX (PLGA gel + DEX)-treated rat cranial bone defects. Top row: Low magnification of tissue sections including the bone defect margins (arrows) and the mid-portion of the defects (arrow heads). S-O, safranin-O and fast-green staining. Middle row: Higher magnification of tissue sections showing both the original host bone (ob) and the new bone (nb) formed in the areas adjacent to the host bone. New bone is outlined by a dotted line. H&E, hematoxylin and eosin staining. Bottom row: Polarizing photomicrographs (POL) of the cranial defect margins demonstrate that collagen fibers of the new bone (nb) display non- or low-polarizing orientation compared to that of the highly polarized parallel lamellae of the original host bone (ob) in each treatment group. H&E staining. Scale bar = 100 μm for all photos in this figure.

Untreated rat cranial defects were filled with a thin layer of fibrous tissue resulting in a collapse of the defect area. In contrast, the defects treated with either PLGA colloidal gel or colloidal gel with DEX were filled with residual implant materials surrounded by either new bone or fibrous tissue, which effectively prevented the defects from collapsing (Figure 3.9, Top row). The formation of new bone, which was observed in the areas adjacent to the host bone, was substantially more abundant in the defects treated with PLGA colloidal gel (with or without DEX) compared to the

untreated control defects, indicating that PLGA colloidal gel can stimulate osteoconductive bone formation (Figure 3.9, Middle and Bottom rows). The central regions of the defects treated with PLGA colloidal gel were filled with residual implant materials and fibrous tissue without new bone, suggesting that complete bone healing was not achieved in 8-mm critical-sized cranial defects even in the presence of the colloidal gel implants. A combination of osteoconductive biomaterials and osteoinductive factor(s)/osteogenic cells may be required to achieve complete bone regeneration of critical-sized rat cranial bone defects. No cartilage formation was observed in the PLGA colloidal gel-treated or untreated defects. These results suggest that PLGA colloidal gels are non-cytotoxic *in vivo* and highly osteoconductive for the repair of rat cranial bone defects.

4. Conclusions

Cohesive colloidal gels made by mixing oppositely-charged PLGA nanoparticles were investigated as potential bone defect fillers. The strength of the colloidal gels resulted from electrostatic interparticle interactions. The pseudoplastic, shear-thinning behavior of the colloidal gels was desired for applications as an injectable scaffold for tissue repair. The PLGA colloidal gel also exhibited recovery of viscosity after shear thinning suggesting that the material may stiffen after placement *in vivo*. Drug release tests revealed that DEX was slowly released at a constant rate for more than two months. *In vivo* results demonstrated that PLGA colloidal gels were osteoconductive fillers capable of controlled release for the repair of rat cranial bone defects.

CHAPTER IV

PLGA-chitosan/ PLGA-alginate Nanoparticle Blends as Biodegradable Colloidal Gels for Seeding Human Umbilical Cord Mesenchymal Stem Cells

1. Introduction

Injectable scaffolds have received attention due to their potential for avoiding the invasive surgery typically required for tissue implantation.[461] From a clinical perspective, the use of injectable materials is an attractive alternative to surgery as it reduces the risk of infection, scar formation, patient discomfort and the cost of treatment.[462] Injectable scaffolds may be applied to fill tissue defects of irregular size and shape. Next generation injectable scaffolds should exhibit modest viscosity upon administration to ensure retention at the defect site, and may benefit from on increase in viscosity upon placement. Recently, many scaffolds that stiffen or solidify *in vivo* have been applied as injectable tissue scaffolds.[463] Usually, injectable scaffolds are polymerized or chemically crosslinked to stiffen the material. In this way, scaffolds may form via an *in situ* reaction induced by the presence of water, heat, light or other stimuli. During the process of solidification, however, toxic chemical agents are sometimes employed, which may adversely affect the scaffolds, destabilize encapsulated biomolecules, or pose toxicity concerns. So, new materials that may stiffen through interactions such as electrostatic forces, van der Waals attraction and steric hindrance are desirable in the applications.

Naturally occurring polymers exhibit multiple desirable properties as scaffolds in tissue engineering.[476] Depending on the material and the source, certain natural polymers have been identified as biodegradable, non-antigenic, non-toxic and biofunctional.[477] The hydrophilic properties of many of these molecules and ample functional groups for further modification also make natural polymers excellent

candidates for biomedical appications.[478-479] Chitosan and alginate are two major naturally occurring polysaccharides which have been widely used as drug delivery systems and tissue scaffolds.[480-483] Chitosan is a natural cationic polymer obtained by deacetylating chitin comprising copolymers of β (1 → 4)-glucosamine and N-acetyl-D-glucosamine. When chitosan is dissolved in a dilute acid solution, the amino groups become protonated and introduce positive charge to the polymer.[484] Alginate is a natural anionic polymer derived from brown sea algae. Alginate has carboxyl groups which may introduce negative charge to the polymer at appropriate pH.[485] These two natural polyelectrolytes can facilitate the formation of oppositely charged biomedical materials.

Colloidal gels with three-dimensional (3-D) microperiodic structures comprised of biodegradable materials were manufactured to overcome some of these limitations.[66,442] These colloidal systems were composed of oppositely-charged nanoparticles at high concentration and stiffen through interparticle interactions such as electrostatic forces and van der Waals attraction.[438] Colloidal gels exhibiting pseudoplastic behavior resulting from interparticle interactions can facilitate the fabrication of shape-specific macroscale materials with 3-D architectures.[428,441] Recent research in materials science has aimed to leverage these phenomena to achieve unique bulk material properties for many different applications.[6,439-440,444,446-447] The application of moldable colloidal gels towards generating tissues has also been proposed.[448-449]

Here, natural polymers were integrated into biodegradable colloidal gels and

examined for their potential as injectable tissue scaffolds. Poly (D, L-lactic-co-glycolic acid) (PLGA) is a biocompatible and biodegradable polymer, which has been approved in pharmaceutical products and used in tissue engineering scaffolds.[451,464-466] Chitosan and alginate were employed as surface modifiers to make oppositely-charged PLGA nanoparticles. Cohesive colloidal gels were created by simply mixing the oppositely-charged PLGA nanoparticles at different ratios. These materials exhibited desirable rheological properties for facile injection as tissue scaffolds. Colloidal gels were also highly compatible with human umbilical cord mesenchymal stem cells (hUCMSCs), which further supported possible translation of these materials.

2. Materials and Methods

2.1. Materials

All materials were purchased from Fisher Scientific Inc. unless otherwise stated. PLGA (75:25) (7525 DLG 2.5E) was purchased from Lakeshore Biomaterials. Chitosan (448869, DD (degree of deacetylation) was 85%, M_n was 612 kDa) was obtained from Sigma-Aldrich Co., Alginate sodium (Mv= 1.6×10^5, viscosity was 39 MPas in 1.0% solution at 20 $^{\circ}$C) was purchased from FMC BioPolymer.

2.2. Preparation of Charged PLGA Nanoparticles

Oppositely-charged PLGA nanoparticles were prepared by a solvent diffusion method. 100 mg of PLGA was dissolved in 10 mL acetone and then the solution was

added into chitosan (dissolved in 0.2% wt/vol acetic acid solution) or alginate (dissolved in deionized water) surfactant solution with different concentration (0.1%, 0.2%, 0.5% and 1.0%) through a syringe pump at constant rate (20 mL/h, 40 mL/h and 60 mL/h) under stirring at 200 rpm overnight to evaporate acetone. Nanoparticles were collected by centrifugation (Beckman Co., Avanti 30) (14,000 rpm, 15 min). The nanoparticles were resuspended using deionized water and centrifuged three times to remove excess chitosan or alginate. A fine powder of charged nanoparticles was obtained by lyophilization for ~2 days.

2.3. Preparation of Colloidal Gels

Lyophilized nanoparticles (PLGA-Chitosan or PLGA-Alginate) were dispersed in deionized water at the reported concentrations. These dispersions were mixed in different proportions to obtain the different weight ratios studied. Homogeneous colloid mixtures were prepared in a bath sonicator for 3 minutes and stored at 4 $^{\circ}$C for 2 h to allow particles to be structurally organized before use. Several colloidal gels, with different mass ratios of PLGA-chitosan nanoparticles to PLGA-alginate nanoparticles, were designated as C100, CA37, CA55, CA73 and A100 (C: PLGA-chitosan nanoparticles; A: PLGA-alginate nanoparticles; the weight ratios of PLGA-chitosn nanoparticles to PLGA-alginate nanoparticles were 100:0, 30:70, 50:50, 70:30 and 0:100, respectively).

2.4. Characterization of Nanoparticles and Colloidal Gels

The sizes and zeta potentials of the different PLGA nanoparticles were determined using a ZetaPALS dynamic light scattering system (Brookhaven, ZetaPALS). All samples were analyzed in triplicate. Scanning electron microscopy (SEM) was performed using a Jeol JSM-6380 field emission scanning electron microscope at an accelerating voltage of 10kV.

2.5. Rheological Experiments

Rheological experiments were performed using a controlled stress rheometer (AR2000, TA Instrument Ltd.). 2° cone steel plates (20 mm diameter) were used and the 500 μm gap was filled with tested colloidal gel. A solvent trap was used to prevent evaporation of water. The viscoelastic properties of the sample were determined at 20 °C by forward-and-backward stress sweep experiments. The viscosity (η) was monitored while the stress was increased and then decreased (frequency = 1 Hz) in triplicate with 10 minutes between cycles. The gel recoverability was assessed using defined time breaks between cycles. All samples were analyzed in triplicate.

2.6. Cytotoxicity

hUCMSCs were harvested and cultured until passage 2 as previously described[452] for cell seeding in culture medium, which included low glucose Dulbecco's Modified Eagle's Medium**,** 10% FBS, and penicillin /streptomycin (PS). Then, hUCMSCs were seeded at a density of 1×10^4 cells per cm^2. Cells were grown to near confluence in the individual wells of a 12-well tissue culture-treated plate and then exposed to 100, 200,

and 300 µl of colloidal gel. The working concentrations of the nanoparticles used were as follows: PLGA 2 mg/ml; chitosan 30 mg/ml; and alginate 30 mg/ml. Cells were cultured in the presence of the materials for 48 hours and 2 weeks, the media being carefully changed every 2-3 days without disturbing the settled gels at the bottom. Subsequently, the cells were stained with LIVE/DEAD reagent (dye concentration 2 mM calcein AM, 4 mM ethidium homodimer-1; Molecular Probes) and incubated for 45 mins, before being subjected to fluorescence microscopy (Nikon TS 100 with Epifluoresence Attachment).

3. Results and Discussion

3.1. PLGA Nanoparticles were Coated with Natural Biopolymers

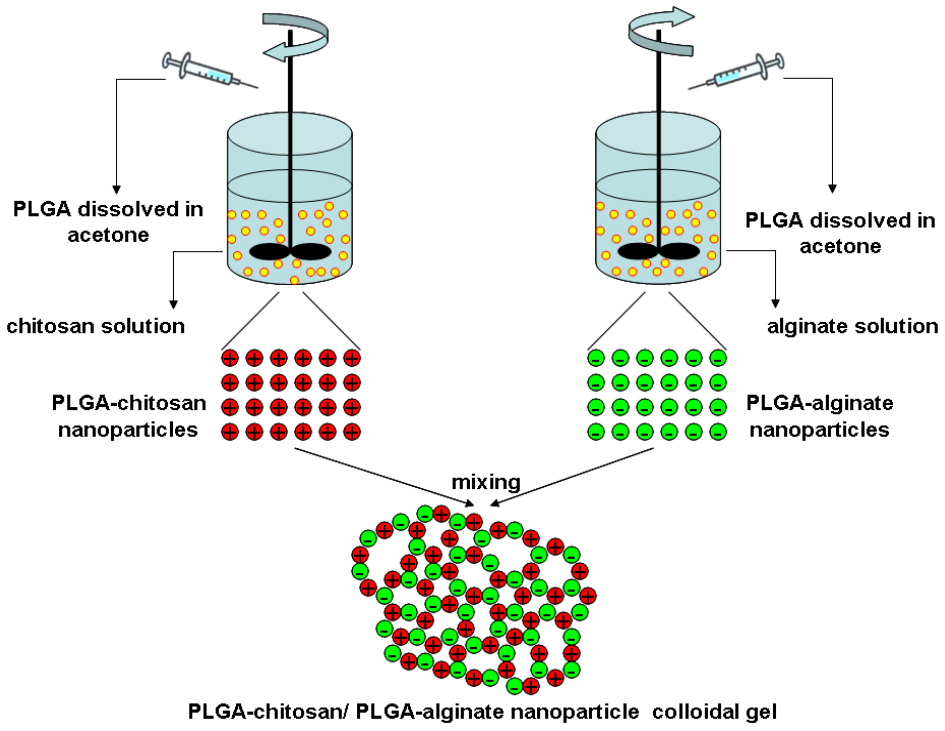

Figure 4.1. Schematic representation of the process for fabrication of oppositely charged PLGA nanoparticles and formation of colloidal gel.

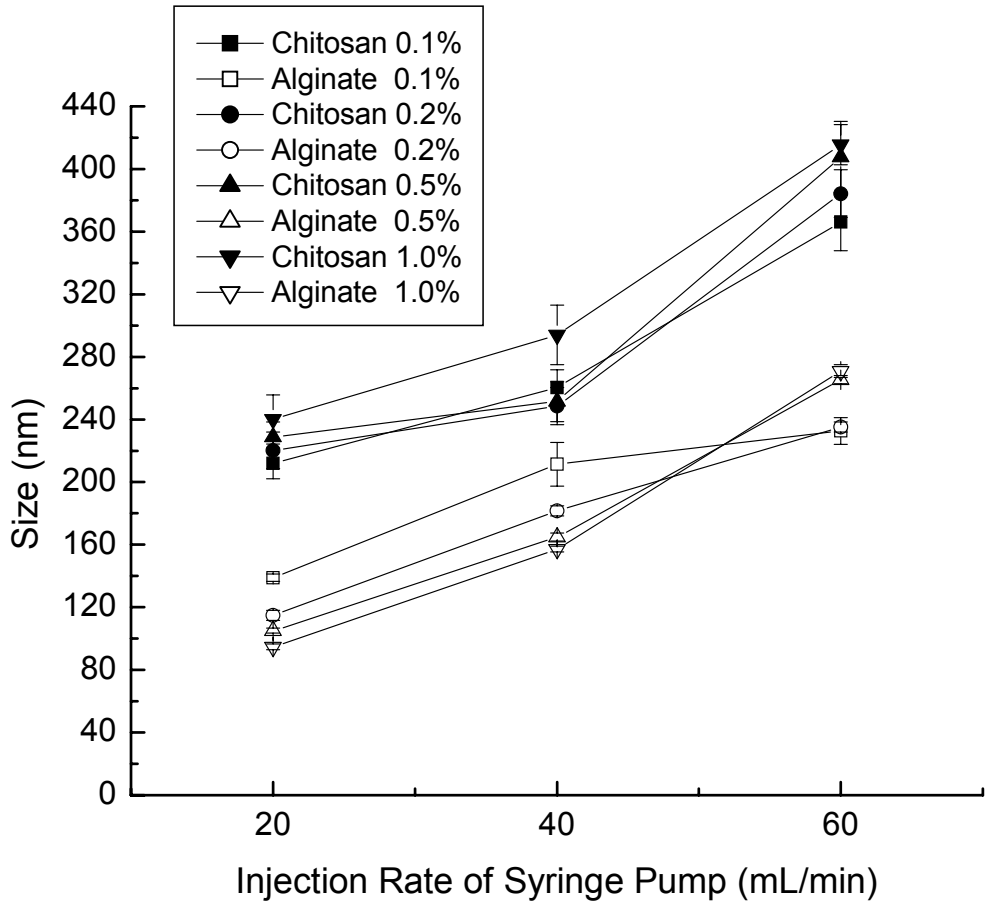

Figure 4.2. Sizes of PLGA nanoparticles made in chitosan or alginate solution with different concentrations (0.1%, 0.2%, 0.5% and 1.0%) at different injection rates (20 mL/h, 40 mL/h and 60 mL/h) by syringe pump.

The entire process for the fabrication of PLGA nanoparticle colloidal gels is illustrated in Figure 4.1. In the first step, PLGA nanoparticles were prepared by a solvent diffusion method. The precipitation of PLGA nanoparticles occurred in the respective polyelectrolyte solution, chitosan or alginate, resulting in a coating of either polymer on the nanoparticle surface. Chitosan introduced positive charge and alginate introduced negative charge on the surface of PLGA nanoparticles. The particle sizes (Figure 4.2) and zeta potentials (Figure 4.3) of each particle type

depended on the synthesis conditions. The injection rate of the dissolved PLGA and the concentration of the biopolymer solution were two key factors influencing the sizes and zeta potentials of the nanoparticles.

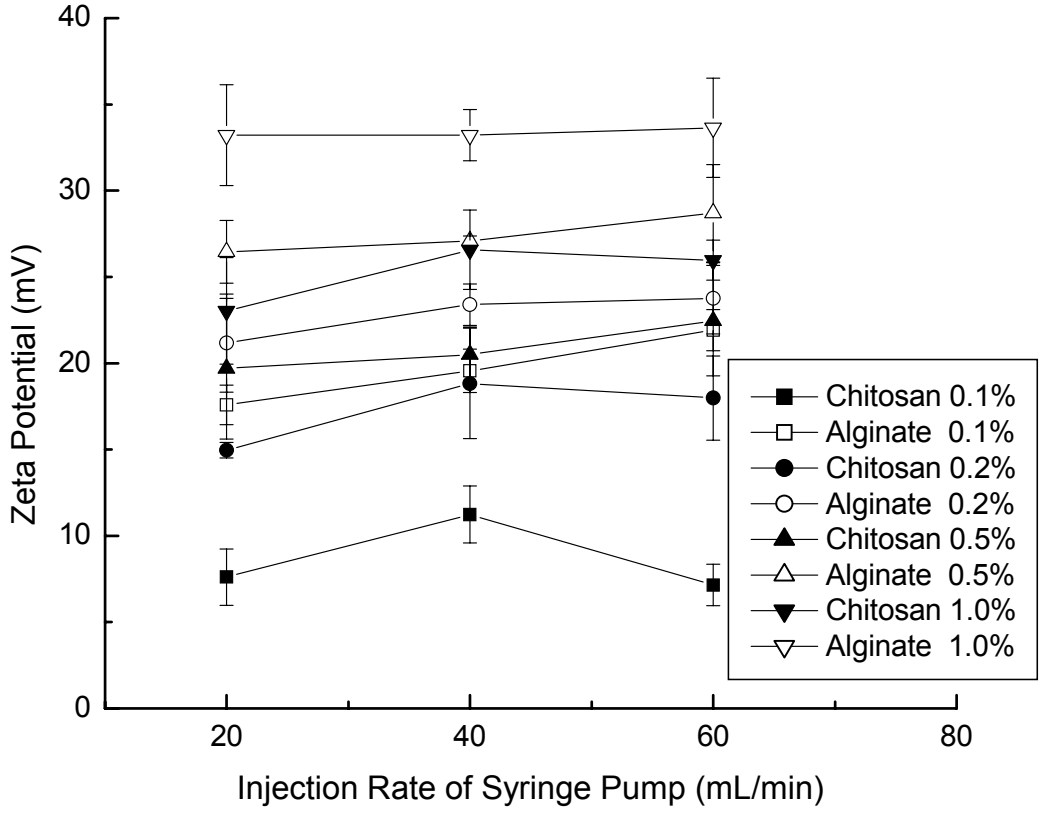

Figure 4.3. Zeta potentials of PLGA nanoparticles made in chitosan or alginate solution with different concentration (0.1%, 0.2%, 0.5% and 1.0%) at different injection rates (20 mL/h, 40 mL/h and 60 mL/h) by syringe pump (chitosan embued positive charge and alginate embued negative charge).

During the fabrication process, PLGA precipitated to form nanoparticles as the organic solvent mixed with water. The faster injection rate resulted in a higher local concentration of PLGA at the needle tip, therefore, larger nanoparticles were produced (Figure 4.2). In addition, the concentration of biopolymer solution (chitosan or alginate) determined the magnitude of the nanoparticle surface charge. Increasing the

concentration of biopolymer solution induced a higher charge on the nanoparticles, since presumably more polyelectrolyte was associated with the nanoparticle surface (Figure 4.3). In order to balance the particle sizes and zeta potentials of the two oppositely charged particles, 248.5±11.9 nm PLGA-chitosan nanoparticles with a zeta potential of +18.8±3.2 mV and 181.6±3.3 nm PLGA-alginate nanoparticles with a zeta potential of -23.4±1.2 mV were prepared for colloidal gel experiments by injecting PLGA solution into 0.2% polyelectrolyte solution at a constant rate of 40 mL/h. The optimized nanoparticles were collected and used to prepare colloidal gels.

3.2. PLGA Colloidal Gels Exhibited Microporous Structures and Shape Retention

For initial studies, cationic or anionic nanoparticles were suspended in deionized water at 20% (w/w) at room temperature. SEM pictures of dried colloidal networks revealed little difference in the structure of dried gels containing different mass ratios of oppositely-charged nanoparticles (Figure 4.4). After drying, all mass ratios (3:7, 1:1, and 7:3; PLGA-chitosan: PLGA-alginate) exhibited a loosely organized, microporous structure. The oppositely-charged nanoparticles were linked together to form micrometer-scale, ring-like structures with microchannels, which interconnected to form the bulk porous structure observed. Domains of more tightly packed nanoparticle agglomerates were evident, suggesting that the cohesive nature of colloidal gels results from the equilibrium of interparticle attractions (tight agglomerates) and repulsions (pores).

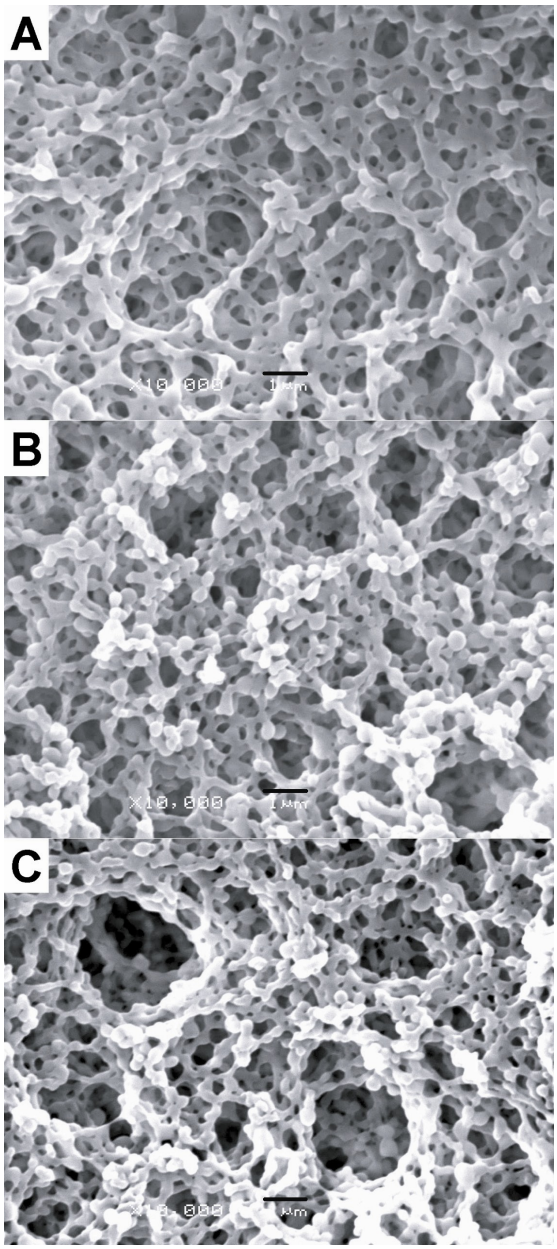

Figure 4.4. SEM images of colloidal gels (A) CA37, (B) CA55 and (C) CA73 (scale bar = 1μm).

Colloidal gels composed of oppositely-charged nanoparticles at high concentration exhibit unique pseudoplastic properties facilitating the fabrication of shape-specific microscale materials. In this project, the pseudoplastic behavior of colloidal gels was leveraged to construct tissue engineering scaffolds of the desired

shape (Figure 4.5). The colloidal gels with different compositions showed slight differences in moldability due to the different ratios of oppositely charged PLGA nanoparticles. Colloidal gel composed of a more equal overall charge balance (CA55 with 1:1 mass ratio, Figure 4.5.B) exhibited less fluidity and better shape stability. Pure nanoparticles at the same concentration showed no moldability (pure PLGA-chitosan nanoparticles, Figure 4.5.D). Results confirmed that the overall charge ratio determined the bulk and microscopic structures and properties of the colloidal system.[436] In this research, the small size and high charge of the PLGA nanoparticles were leveraged to form stable colloidal gels.

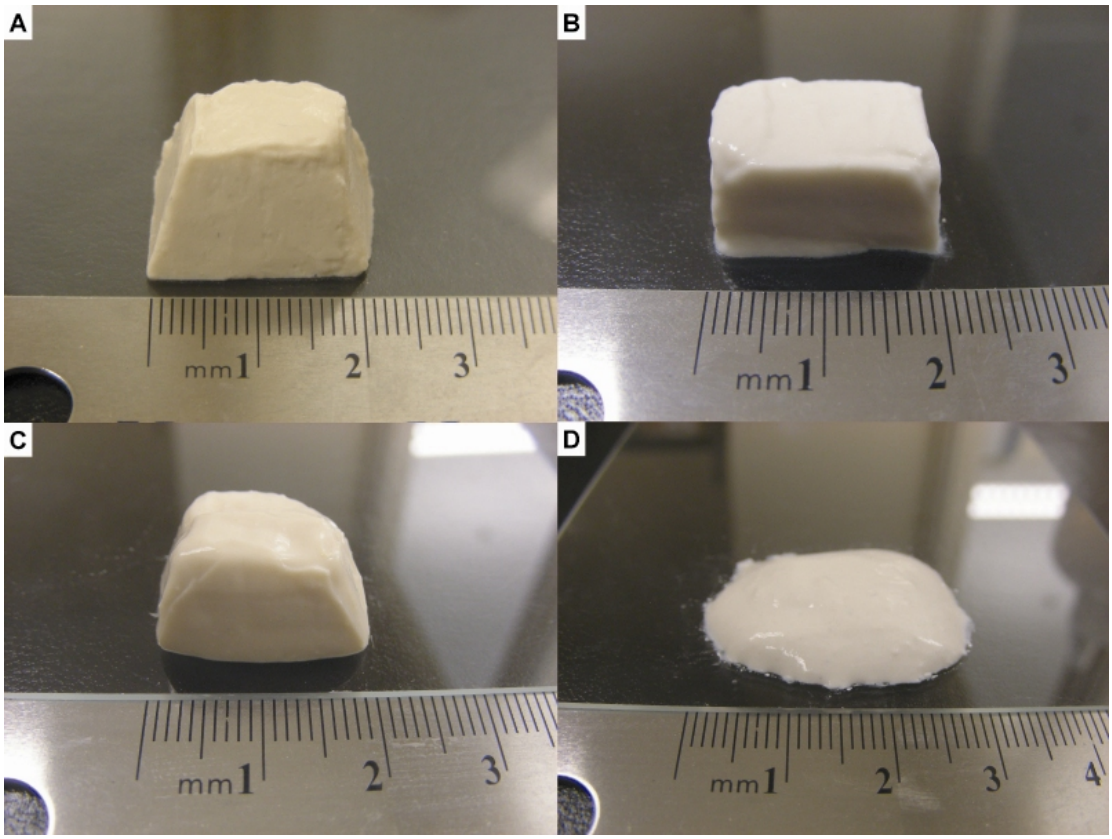

Figure 4.5. Shaped tissue scaffolds made by colloidal gels (A) CA37, (B) CA55, (C) CA73 and (D) pure PLGA-chitosan nanoparticles.

3.3. PLGA Colloidal Gels were Shear Thinning with Recoverable Stiffness

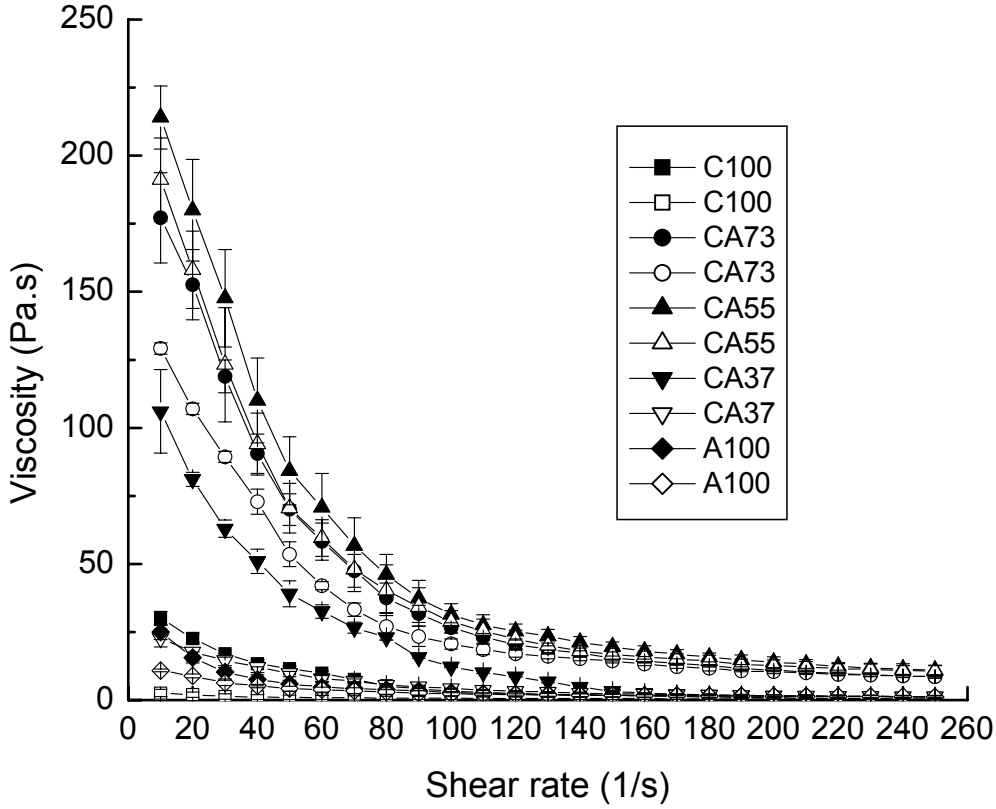

Figure 4.6. Viscosity and shear-thinning behavior of colloidal gels (20% concentration) mixed at different ratios for accelerating (solid symbols) and decelerating (open symbols) shear force.

Rheological studies were employed to probe the differences in viscoelasticity of colloidal gels. Equal mass ratios of nanoparticles yielded the highest viscosity gel and improved reversibility compared to other ratios (Figure 4.6). As expected from previous research,[466] colloidal gels containing more positively-charged particles (CA73, 7:3 mass ratio, 20% concentration) exhibited higher viscosity, while colloidal gels composed of excess negatively-charged particles (CA37, 3:7 mass ratio, 20% concentration) exhibited more fluidity. The larger zeta potential of negatively charged

nanoparticles resulted in a more equal overall charge balance when positively charged particles were in excess, thus, providing a probable explaination for the stronger cohesion and enhanced reversibility observed in the 7:3 mass ratio compared to the 3:7 mass ratio. Pure nanoparticle suspensions exhibited minimal shear-thinning behavior (C100 and A100).

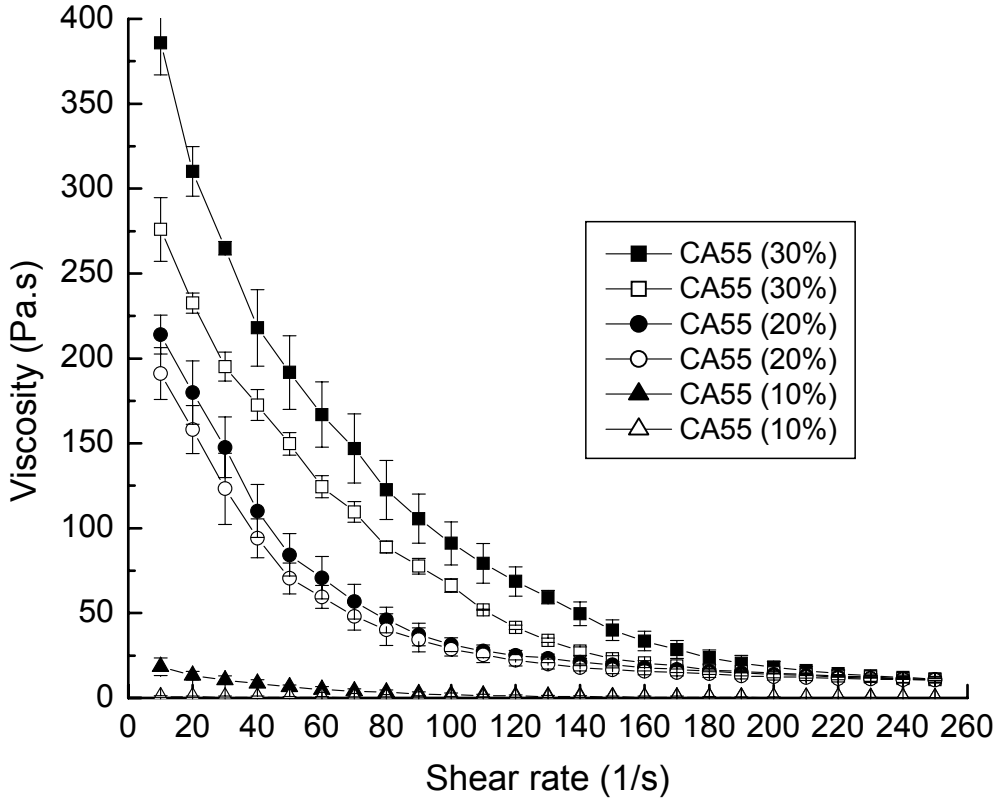

Figure 4.7. Viscosity and shear-thinning behavior of CA55 colloidal gel at different concentrations for accelerating (solid symbols) and decelerating (open symbols) shear force when no recovery time was allowed between shear cycles.

The viscosity of colloidal gel was enhanced and shear-thinning more pronounced as the concentration of nanoparticles increased (Figure 4.7). Consecutive acceleration/deceleration cycles of the shear force applied to CA55 colloidal gels (20% concentration) revealed that these materials did not rapidly recover (Figure 4.8).

Delaying shear cycles for more time may enhance the recovery of gel viscosity. All the results suggested that the colloidal gels were desirable for injectable applications.

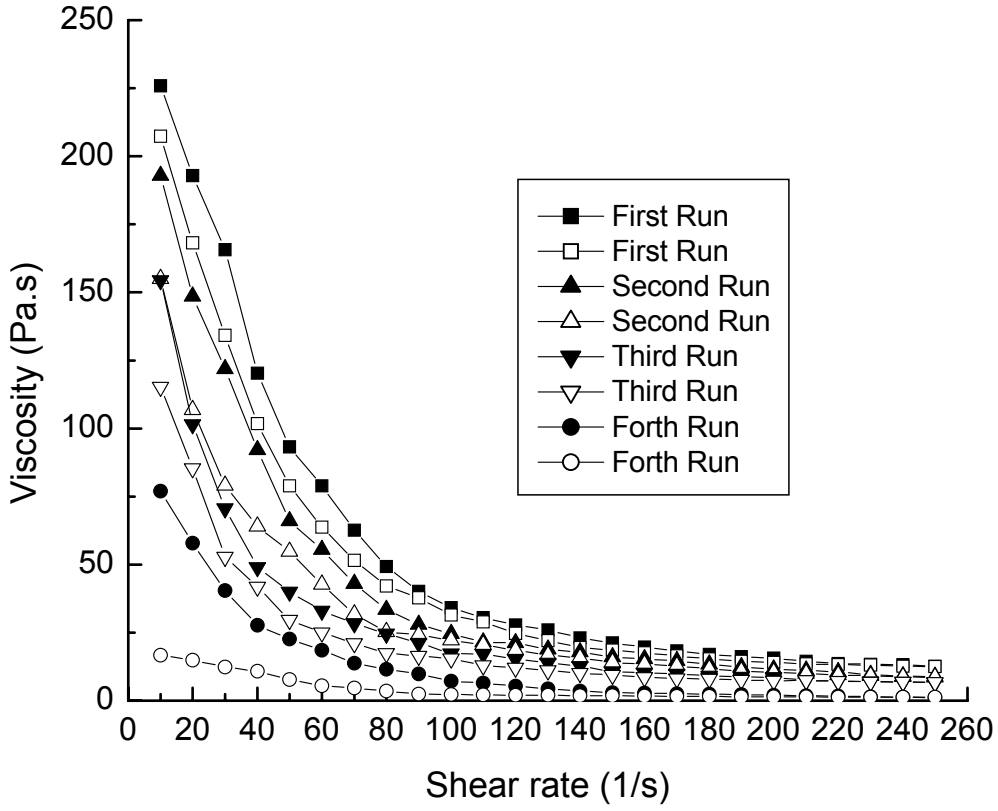

Figure 4.8. Viscosity and shear-thinning behavior of CA55 colloidal gel (20% concentration) for repeated accelerating (solid symbols) and decelerating (open symbols) shear force when no recovery time was allowed between shear cycles.

For colloidal gels, the strength of the cohesion depends upon the interparticle interactions such as electrostatic forces and van der Waals attractions.[442] These interparticle interactions were controlled by the composition of the colloidal gels, such as concentration and ratio of the two oppositely-charged particles. PLGA-chitosan and PLGA-alginate nanoparticles self-assembled through interparticle interactions resulting in a stable 3-D porous network. Under static conditions, the viscosity and structure of colloidal assemblies leading to a stable structure exhibiting

high viscosity at equilibrium (Figure 4.5, A-C). If the particle-particle equilibrium is disrupted, e.g. by external force applied to disrupt the interparticle interactions, the colloidal system will demonstrate shear-thinning behavior. Once the external force is removed, the strong cohesive property of the colloidal gel is recovered and the 3D porous structure is reconstructed. This reversibility makes the gel an excellent material for applications in molding, extrusion, or injection of tissue scaffolds and drug delivery systems.

3.4. PLGA Colloidal Gels had Negligible Cytotoxicity to hUCMSCs

Stem cell based tissue engineering has the potential to revolutionize biomedicine with the ability to repair or regenerate the damaged or diseased tissue.[486-487] hUCMSCs are pluripotent, able to differentiate into adipocytes, osteoblasts, chondrocytes, neurons, and other cells.[488-491] Comparing to other stem cells, hUCMSCs are advantageous because umbilical cords can be collected at a low cost and represent an inexhaustible stem cell source. hUCMSCs can be harvested from discarded umbilical cords, expanded in culture, induced to differentiate and combined with a scaffold to repair tissue defects.[492-493] However, pre-formed carriers for cell delivery have drawbacks including the difficulties in seeding the cells on the scaffold, biocompatibility between the cells and the scaffolds, and placement in minimally invasive surgeries.[494] In this research, biodegradable PLGA colloidal gels were used to overcome these difficulties.

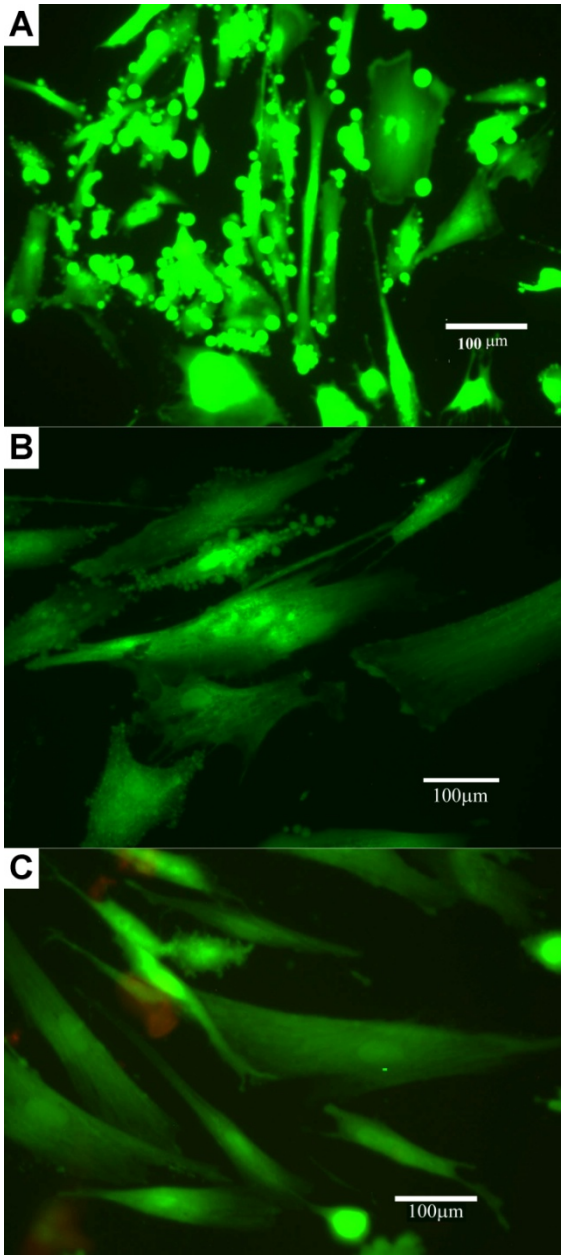

Figure 4.9. Human umbilical cord matrix stem cells cultured on colloidal gels CA 55 (B) and CA37 (C) demonstrated high viability (green) and minimal cell death (red) comparing to reference (A, treated without colloidal gels).

hUCMSCs were harvested and cultured on CA55 and CA37 colloidal gels for up to two weeks, following which, viability assays were conducted. The Live/Dead Cytotoxicity Assay Kit from Molecular Probes was employed, which uses two probes, namely Calcein AM and Ethidium Bromide homodimer, to identify two critical

matrices of cell viability: (a) protease activity and (b) membrane integrity. The membrane-permeable dye Calcein/AM (Ex495nm/Em515nm) was hydrolyzed by cellular proteases, ubiquitous in living cells, to yield a green fluorescent product; on the contrary, ethidium homodimer (Ex495/Em635nm) only entered the cell membranes of dead or dying cells to bind the DNA, undergoing a manifold enhancement in red fluorescence (Figure 4.9). Very little cell death was observed in cells treated with PLGA nanoparticles for 48 hours and for 2 weeks. On the other hand, when 70 % ethanol was used as a negative control, appreciably higher numbers of dead cells were observed. The live/dead experiments qualitatively demonstrated that PLGA colloidal gels possessed little cytotoxicity towards hUCMSCs, which suggested that the material may be used as a scaffold for seeding stem cells.

4. Conclusions

In this chapter, a new colloidal gel made by oppositely-charged PLGA nanoparticles coated with naturally occurring polyelectrolytes was reported. The cohesive strength of the colloidal gels resulted from the interparticle interactions between the oppositely-charged nanoparticles. The shear sensitivity to external force and recoverable pseudoplastic property make it an excellent injectable biomaterial. Cytotoxicity tests of the colloidal gels also demonstrated negligible toxicity to hUCMSCs. Thus, injectable PLGA colloidal gels represent a promising new material for tissue engineering. In further research, integration of controlled release of active ingredients (*e.g.* growth factors) will allow for advanced combination strategies for

tissue engineering coupled with drug release.

CHAPTER V

Conclusions and Future Directions

Nanotechnology provides the tools and methods to characterize and manipulate materials at the nanoscale, further elucidate nanoscale phenomena, and equip researchers and developers with the ability to fabricate novel materials and structures. Nanomaterials have unique physicochemical properties due to the large surface area to mass ratio, which can be different from bulk materials of the same composition. These properties can be used to overcome some of the limitations found in traditional research areas. Drug delivery systems and tissue engineering are two major areas which have been deeply influenced by nanotechnology.

Injectable fillers have emerged as an alternative to the invasive surgery often required to repair non-load bearing skeletal defects such as cranial defects. Achieving controlled release from these materials is desired for accelerating healing. In this thesis, positively-charged PLGA-PVAm nanoparticles and negatively-charged PLGA-PEMA nanoparticles at high concentration were used to create a cohesive biodegradable colloidal gel as an injectable drug-loaded filler to promote healing in bone defects. The colloids were successfully created and self-assembled through interparticle interactions, such as electrostatic forces and van der Waals attraction, resulting in a stable 3-D porous network that was easily molded to the desired shape. The colloidal gel demonstrated shear-thinning behavior due to the disruption of interparticle interactions as the applied shear force was increased. Once the external force was removed, the cohesive property of the colloidal gel was recovered. These cohesive colloidal gels also exhibit pseudoplastic behavior facilitating the fabrication of shape-specific microscale materials.

Similar reversibility and shear-thinning behavior were also observed in colloidal gels loaded with dexamethasone. Near zero-order dexamethasone release was observed over two months when the drug was encapsulated in PLGA nanoparticles and simply blending the drug with the colloidal gel showed similar kinetics for one month. Surgical placement was facilitated by the pseudoplastic material properties and *in vivo* observations demonstrated that the PLGA colloidal gels stimulated bone formation in rat cranial bone defects.

The natural polymers chitosan and alginate represent an attractive material choice for biodegradable implants. In order to enhance the biocompatibility of the biodegradable colloidal gels, chitosan and alginate were used as coating materials to make positively and negatively charged PLGA nanoparticles, respectively. The resulting colloidal gels showed the typical properties observed for the other colloidal gel systems. Viability tests of human umbilical cord mesenchymal stem cells (hUCMSCs) seeded on the colloidal gels also demonstrated the negligible cytotoxicity of the materials.

Comparing to colloidal gels made by synthetic surfactants, PLGA colloidal gels made by natural surfactants, chitosan and alginate, should be more biocompatible. Since synthetic surfactants were more highle charged than natural ones, PLGA-PEMA and PLGA-PVAm nanoparticles had smaller sizes but larger zeta potentials than PLGA-alginate and PLGA-chitosan nanoparticles. Moreover, colloids made using natural surfactants were not as stable as synthetic surfactants. PLGA nanoparticles made using natural surfactants could not be stored as long as synthetic ones. So PLGA

nanoparticles made by natural polymers should be used without longtime storage.

Right now, I am cooperating with other labs to expand the applications of achieved colloidal gels. I work together with Dr. Siahaan's lab to release immunomodulating peptides *in vivo* to treat autoimmune disease. I also cooperate with Dr. Detamore's lab to use colloidal gels for liver tissue regeneration.

Future research will focus on improving the overall properties of these colloidal systems and expand the applications of this novel material in different areas. For example, charged hydroxyapatite nanoparticles can be used as a component to make colloidal gels for bone tissue engineering. As an ingredient of bone, hydroxyapatite is expected to enhance the biocompatibility and mechanical strength of colloidal gels (mechanical strength will be checked by yield and compressive stress tests). Since low temperature can make these systems stiffer, scaffolds made by colloidal gels may even be molded and freeze dried to create more rigid structures or directly injected as *in situ* forming scaffolds. Introduction of porogens to these materials may ultimately be leveraged to promote in-growth of cells and enhance the interconnected porous 3-D structure. Growth factors could be encapsulated into the PLGA nanoparticles or blended into the colloidal gel for growth factor delivery to accelerate the regeneration process of injured tissue. In addition, charged microparticles could be incorporated into this system to adjust the drug release rate to get the desirable drug release profiles for different controlled release applications.

In summary, colloidal gels were fabricated using oppositely-charged, biodegradable PLGA nanoparticles that interact to form stable 3-D porous structures.

The shear-thinning behavior and reversibility make the gel an excellent material for molding, extrusion, or injection of tissue scaffolds and drug carriers. These gels also have good biocompatibility and ability for drug controlled release. All results supported the potential application of biodegradable colloidal gels as an injectable scaffold in tissue engineering and drug release.

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