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Investigation of potential injectable polymeric biomaterials for bone regeneration

Michael B. Dreifke, Nabil A. Ebraheim, and Ambalangodage C. Jayasuriya

Department of Orthopaedic Surgery, The University of Toledo, College of Medicine, Toledo, Ohio 43614

Abstract

This article reviews the potential injectable polymeric biomaterial scaffolds currently being investigated for application in bone tissue regeneration. Two types of injectable biomaterial scaffolds are focused in this review, including injectable microspheres and injectable gels. The injectable microspheres section covers several polymeric materials, including poly(L-lactide-co-glycolide)-PLGA, poly(propylene fumarate), and chitosan. The injectable gel section covers alginate gels, hyaluronan hydrogels, poly(ethylene-glycol)-PEG hydrogels, and PEG-PLGA copolymer hydrogels. This review focuses on the effect of cellular behavior *in vitro* and *in vivo* in terms of material properties of polymers, such as biodegradation, biocompatibility, porosity, microsphere size, and cross-linking nature. Injectable polymeric biomaterials offer a major advantage for orthopedic applications by allowing the ability to use noninvasive or minimally invasive treatment methods. Therefore, combining injectable polymeric biomaterial scaffolds with cells have a significant potential to treat orthopedic bone defects, including spine fusion, and craniofacial and periodontal defects.

Keywords

injectable; bone regeneration; biocompatibility; gel; polymer; microspheres; biodegradation; stem cells

INTRODUCTION

Bone is a crucial element within the body as it provides numerous functions that are essential for survival. It is responsible for the protection of internal organs and tissues, which aid in maintaining homeostatic calcium levels and provide scaffolding for the body necessary for movement and support.¹ Bone marrow provides a source of multipotent hematopoietic stem cells.² While extremely durable and capable of self-regeneration, elements, such as aging, disease, and trauma, can cause devastating effects that may severely threaten the integrity of bone. In some cases, intervention is necessary for the recovery and regeneration of severe bone defects.

Despite autografts consider as the gold standard for treatment of bone defects, several drawbacks exist.³ This procedure involves the removal of bone tissue from one area of the patient's body and the graft used to repair a defect in another area. There are only a few areas of the body conducive for harvesting tissue as well as the fact that minimal amounts of bone tissue may be obtained from any one source. An alternative approach to autograft transplantation is the allograft and xenograft.^{4,5} These methods utilize the same basic concepts; allografts are derived from another patient or cadaver. Xenografts are obtained from the animal bone tissues. The problems associated with these approaches involve the host's immune system rejecting what it identifies as foreign tissue.³⁻⁵ Immune system complications are not the only difficulty with these procedures, however. Depending on the fracture type, bone grafts, such as allograft, xenograft, and autograft transplantation, may involve internal fixation which, like any other invasive procedure, bares the risk of infection.

Bone morphogenetic proteins (BMPs) have been shown to be osteoinductive to mesenchymal stem cells (MSCs).⁶ The use of BMPs to treat the bone defects currently increased. Recent research indicates that post surgery morbidity may be reduced by increasing bone matrix synthesis by utilizing both BMPs and MSCs.⁷ Tissue engineering is a multifaceted field that focuses on utilizing biological surrogates to enhance regeneration of failing or damaged tissues.⁸ The most common form of tissue engineering incorporates the use of stem cells to regenerate new tissue in damaged areas. The cells aid in enhancing regeneration of lost or damaged tissues while the scaffolds of varying makeup provide the attachment sites for the cells as well as a protected environment for the attached cells to proliferate and differentiate.⁹

For successful bone tissue regeneration to occur, it is important to find scaffolds that can not only temporarily act in the place of the damaged tissue, but are also compatible with the biological environment of the host tissue and have optimal porosities that allow for the transport of sufficient numbers of cells. In addition, it is necessary that the scaffold biodegrade at an appropriate rate within the host tissue.¹⁰ The proposed biomaterials must be able to achieve an interaction with the surrounding tissues and must minimize the patients' immune response.⁵ The scaffolds should provide sufficient mechanical strength to the bone defect site. In addition, these scaffolds should be able to incorporate the drugs or proteins and release as predicted.

Ample research has been conducted in the area of suitable biodegradable 3D scaffolds that include a variety of natural and synthetic polymers. The ability of various polymer scaffolds to allow cellular attachment, proliferation, differentiation, and extracellular matrix formation is being observed in a variety of studies to determine whether they may be used as an injectable material for bone replacement therapies.¹⁰ MSCs are a viable option for increasing bone regeneration because they are capable of differentiating into osteoblasts and synthesizing new bone matrix when placed in a controlled environment.¹¹ The ability of the cells to stay in the region of damaged tissue and their ability to survive and differentiate into various bone matrix secreting cells is a fundamental aspect of their use. The main advantage of injectable polymeric materials compared to 3D scaffolds is that they can be applied to repair the bone defects using noninvasive or minimally invasive surgeries. In addition,

injectable polymeric materials can fill irregularly shaped bone defects easily. Orthopedic procedures can be performed using the needle gauge of 10–16.¹²

Characterization of weight loss, swelling properties, water absorption, cellular attachment, proliferation, differentiation, and changes in molecular weight are important aspects for future use of injectable polymeric biomaterials in bone regeneration. This review will focus on specific aspects of various synthetic, natural, and hybrid polymer materials as suitable injectable materials for bone tissue regeneration.

INJECTABLE POLYMERIC BIOMATERIALS

Regenerative medicine has turned to new noninvasive measures to heal severe bone defects without the risk of infection and rejection. With that said, synthetic injectable scaffolds must be able to provide structural stability and mechanical support to a defect site. The scaffolds must degrade at a sufficient rate since they are only temporary structures.¹³ The degradation rate of scaffolds is vital because it must maintain its integrity long enough for new tissue to replace damaged tissue, while simultaneously providing mechanical strength¹⁴ to help absorb some, but not all, of the stress. The polymer microparticles were fabricated using conventional oil/water emulsification method and can inject by a needle with gauge size range of 10–16.¹²

Some of the advantages of gel polymers, compared to injectable scaffolds, are gels ability to take the shape of any size defect and ability to integrate numerous agents easily within a gel¹⁵. Typically two classes of gel-forming polymers exist, chemical and physical. Chemical gels are formed by covalent interactions, while physical gels are formed by heat and pH changes¹⁶. Each type of injectable scaffold and gel contains its own set of advantages and disadvantages. Select constituents of each and their applications will be addressed within this review.

Injectable microparticles

PLGA microspheres—Poly(L-lactide-co-glycolide) (PLGA) is a copolymer of poly(lactic acid) and poly(glycolic acid). PLGA is an attractive scaffold due to its biocompatibility, controllable degradation properties, and ability to encapsulate osteogenic inducing factors.^{17,18} PLGA has been used for sutures, pins, screws, and nails for repair of the bone defects.

The PLGA microspheres were fabricated by conventional oil/water emulsification method and coated using a simulated body fluid for 5 days at 37°C. The apatite-coated PLGA microspheres with osteoblasts were injected into a subcutaneous dorsum of the mice and tested for bone formation at 6 weeks. The new bone formation was significantly enhanced for the apatite-coated PLGA microsphere group compared to the plain PLGA microsphere group¹⁹. In another study, PLGA-hydroxyapatite (HA) microsphere composites were loaded with the bisphosphonate-based osteoporosis preventing drug, alendronate (AL)²⁰. These microspheres were prepared with solid/oil/water (s/o/w) or water/oil/water (w/o/w) technique. The AL release from the PLGA/HA-AL system showed a sustained releasing tendency, except a minimal burst at the very beginning over a 30-day period. Their results

indicated that the PLGA/HA-AL system was able to improve osteoblast proliferation and also enable upregulation of ALP.

Along with degradation properties, encapsulation efficiency, cellular proliferation, and MSC differentiation are important parameters when studying the utilization of PLGA microspheres. Although BMP-2 is one of the most significant osteoinductive substances, used in bone tissue regeneration, it has some limitations. BMP-2 requires high dosages to initiate differentiation and has a relatively short half-life *in vivo* due to proteolysis.²¹

New approaches focus on extending the half-life of BMP-2 and the utilization of other osteoinductive substances, like dexamethasone and ascorbic acid (vitamin D3) because of their stability.²² The dexamethasone release from PLGA microspheres exhibited burst release in the first 5 days (32%) followed by the approximately >90% release at day 36. In contrast, ascorbic release from PLGA microspheres appeared 89% at day 5.²² PLGA microspheres encapsulated with dexamethasone were investigated to study its cellular compatibility. They were able to encapsulate the spheres via a single and double emulsion-solvent evaporation technique, which yielded >80% encapsulation efficiency. The biodegradation properties of PLGA microparticles can be tailored from a few weeks to several months by varying the amount of poly(lactic acid) to poly(glycolic acid).

In vitro studies found that human MSCs (hMSCs) were able to attach and proliferate on the encapsulated microspheres over a 21 day study with larger numbers being observed via fluorescent microscopy at 21 days. In addition, ALP assays and alizarin red assays over 21 days yielded positive results, suggesting MSC differentiation into osteoblasts. Specifically, ALP secretion was significantly higher on day 14 than day 7; however, a slight decrease was noted on day 21.

The size and spherical nature of the microspheres are important properties for cell culture. The spherical shape gives PLGA microspheres the highest surface area for cellular attachment and proliferation and is thus one of the most important parameters for its use. The PLGA microspheres with diameters between 50 and 150 μm were spherical, did not clump, and had a high efficiency for BMP-2 encapsulation. Microspheres with diameters >150 μm were not spherical and tended to clump together, thus making poor scaffolds for cellular attachment.²¹

While PLGA scaffolds have yielded very positive results as a possible tissue engineering application, there are some disadvantages. First, PLGA has the possibility of causing an aseptic inflammatory response due to its acidic byproducts.²³ The inflammatory response negatively reacts with the osteoinductive properties of BMP-2²¹ leading to its inability to induce MSC differentiation. In addition, the drop in pH, caused by the breakdown products of PLGA, may increase PLGA degradation, leading to the untimely loss of mechanical support.²⁴ One possibility of reducing the risk of an inflammatory response is co-manufacturing PLGA with bone particulates and collagen. The particulates and collagen, in this case, would act as a barrier from the acidic byproducts; thus, protecting BMP-2 from the toxins and extending its *in vivo* half-life.²¹

Poly(propylene fumarate) microspheres—Poly(propylene fumarate) (PPF) is a synthetic polymer that can be covalently cross-linked due to its one unsaturated double bond (Fig. 1).²⁵ The PPF scaffold can be applied for bone tissue engineering due to its ability to be broken down into non-toxic waste products of fumaric acid and propylene glycol as well as its ability to be used as an injectable scaffold.^{26,27} Interestingly, PPF, like many synthetic polymers, has been shown to provide mechanical strength, such as elastic modulus ranging from 20 to 40 MPa, while maintaining its biocompatibility and osteoconductive properties.

Poly(propylene fumarate)/poly(lactic-co-glycolic acid) (PPF/PLGA) blend microspheres were fabricated using a double emulsion-solvent extraction technique.²⁸ In this process, poly(vinyl alcohol) (PVA) was used as the internal aqueous phase. The average microsphere diameter ranged between 19.0 and 76.9 μm for all the fabricated PPF/PLGA blends. The size distribution of blend microsphere was affected mostly by the external PVA concentration and vortex speed.

However, a potential problem with utilizing synthetic polymers is its incapability to induce osteogenesis. Therefore, PPF must either be modified with peptides that encourage host cell attachment or osteoinductive factors encapsulating within the material.²⁹ Research centers upon utilizing PLGA microspheres incorporated with osteogenic agents to make PPF scaffolds osteoinductive. Kempen et al. compared the compressive modulus and release properties of PPF scaffolds encapsulated with both PPF microspheres and PLGA microspheres.²⁹ For their release kinetic studies, they used model drug, Texas red dextran, because of its similar molecular weight to a bone morphogenetic protein. Their studies found that PPF scaffolds incorporated with PLGA microspheres had considerably less mechanical strength, but a relatively fast drug release, while those scaffolds incorporated with PPF microspheres had higher mechanical strength with a significantly lower amount of drug being released over 28 days. Importantly, they found that increasing microsphere loading from 100 to 250 mg/g significantly reduced the difference in release rates being observed between the PLGA- and PPF-loaded microspheres. They suggested that enhanced mechanical behavior and lower burst release is due to the covalent bonding of PPF microspheres to the PPF scaffold.

Local injection of thrombin-related peptide (TP508) in PPF/PLGA microparticles enhanced bone formation in a rabbit model of distraction osteogenesis.³⁰ Their results have shown that the most advanced bone formation and remodeling occurred with PPF/PLGA microspheres compared to the control groups, with microcomputed tomography (microCT) at 2 weeks.

Unfortunately, loading PPF scaffolds with PLGA micro-particles creates new issues that center on the scaffolds degradation properties. These must be addressed carefully because the space created by degradation is utilized by the new tissue and because gradual degradation allows for the controlled shift in pressure to the new matrix.³¹ Beginning at 2 weeks, experiments with PPF/PLGA composite scaffolds showed a decrease in pH when placed in a buffered solution.¹² Figure 2 shows the histological sections of PPF/PLGA scaffolds.¹³ This would suggest that degradation products of the scaffold are acidic enough to sufficiently alter the pH of its surroundings. The drop in pH may cause problems with cell viability and increased degradation rates. Specifically, it was observed that scaffolds

consisting of higher concentrations of PLGA microspheres yielded a lower pH than those scaffolds loaded with a lower concentration of PLGA.¹² Similarly, only those scaffolds containing higher amounts of PLGA microspheres yielded a significant loss of mass, indicating degradation, over a 26-week period. This seems to suggest that PLGA concentration affects the controlled degradation of PPF scaffolds.

Based on this information, it is obvious that many parameters must be carefully taken into account when attempting to produce an optimal PPF injectable scaffold. While the above studies focused on utilizing an osteoconductive peptide, the decrease in pH caused by the PLGA could affect subsequent experiments that incorporate MSCs with an osteoconductive agent. The decrease in pH caused by the degradation products may prove to decrease the cell viability, an issue that will continue to be addressed throughout this review.

Chitosan microspheres—Chitosan, second most abundant natural polymer, is the *N*-deacetylated product of the polysaccharide chitin. It is a linear polysaccharide made up of both glucosamine and *N*-acetyl glucosamine. The degree of deacetylation is the deacetylated units (glucosamine) in the polymer chain, an important parameter for properties of chitosan.³² Chitosan has a high molecular weight and commonly found in the shells of marine crustaceans and cell walls of fungi. Another interesting property of chitosan is its intrinsic antibacterial activity. Importantly, there are a number of methods for synthesizing chitosan microspheres in the laboratory depending upon the desired chemical makeup of the final product.³⁴ The molecular weight and degree of *N*-deacetylation are important properties to consider when manufacturing chitosan scaffolds as their biocompatibility, biodegradability, and swelling properties are influenced by these parameters. These behaviors are significant because they can affect cell growth, tissue regeneration, and the host response.³³ The bone regeneration was observed when microparticles were implanted in an animal bone defect suggesting the biocompatibility of chitosan microparticles.^{35,36} A strong interaction between the mucin and chitosan was observed due to a more specific adsorption process where electrostatic interaction between the positively charged chitosan and negatively charged mucin is involved.^{37,38}

Chitosan has been used in a wide variety of studies, including organ regeneration, epidermal surface repair, antibiotic transportation, nerve regeneration, anti-cancer therapy and most importantly, in bone regeneration. Chitosan's ability to have specific interactions with components of the extracellular matrix and growth factors are of great interest for using it as a bone tissue engineering agent.³⁹ Additionally, it is important to understand the biodegradable behavior of chitosan to recognize what will ultimately happen to the microspheres once they have successfully served as a scaffold for MSCs.

It is known that chitosan is naturally degraded *in vivo* by the lysozyme enzyme.³³ However, the degree of deacetylation has profound influences on the degradation behavior of chitosan and on cell adhesion to its surface. The cell adhesion to chitosan is directly dependent upon the degree of deacetylation. Specifically, the higher the degree of deacetylation, the greater the number of MC3T3-E1 mouse preosteoblasts⁴⁰ and L929 cell adhesion⁴¹ to the surface. This pattern of cell adhesion is probably due to the large amount of positive charges

distributed on the chitosan surface, which allows negative molecules on the cell surface to interact with the chitosan molecule.^{32,41}

While the degree of deacetylation has been found to be directly related to cell adhesion, it has been determined to be inversely related to the rate of degradation.³² Chitosan with a 71.7% or higher degree of deacetylation underwent a very slow degradation.³³ Conversely, chitosan scaffolds containing less than a 71.7% degree of deacetylation observed significantly rapid rates of degradation. While the degree of deacetylation has a profound impact on degradation and cell adhesion, porosity is also crucial to its function. Chitosan, like other scaffolds, requires progenitor cell and osteoinductive materials to be encapsulated to induce new bone synthesis.

Jayasuriya et al. fabricated the organic/inorganic microspheres that were based on chitosan and consisted of inorganic components, such as dibasic calcium phosphate (CaHPO_4) or calcium carbonate (CaCO_3).^{42,43} The microspheres were cross-linked using tripolyphosphate, which is an ionic cross-linking agent. Figure 3 shows the scanning electron microscopy (SEM) image of chitosan microspheres. Hybrid organic/inorganic microspheres were noncytotoxic and supported the MSC attachment, spreading, proliferation, and differentiation into the osteoblast phenotype.^{44,45} The MSC-seeded microspheres were implanted into partial thickness bone defects in the rat femurs at 4 and 8 weeks. The control group of rats did not receive any implant material except the stainless steel plate to support the defect. A new bone formation was observed in the experimental group at 8 week implantation.⁴⁶

The degradation properties of the microspheres is important to the long-term benefits of the use of the chitosan-based hybrid microspheres in bone tissue engineering because the degradation kinetics, slow or fast degradation, could affect a multitude of processes within the cell, such as cell growth, tissue regeneration, and host response. The degradation data suggested that the hybrid microspheres were stable at least up to 25 weeks and maintained the physiologically relevant pH.⁴⁷

MTS assay was used to observe the attachment and proliferation of cells on nontreated chitosan microspheres over a 7- and 14-day period.⁴⁸ They demonstrated that chitosan naturally contains free amine groups on its surface that enhance cell attachment. Specifically, they found that when these amine groups are taken up by cross-linking agents, there is less opportunity for attachment and thus fewer cells adhere to the particles. Therefore, while cross-linking density is important because of chitosan's solubility in body fluids, they need to be carefully designed in such a manner that they do not obstruct significant cell attachment.

Injectable gels

Alginate gels—Alginate gels have been utilized in tissue engineering experiments due to their biocompatibility, biodegradability, ability to form gels easily, and very low toxicity.^{14,49–51} They are a suitable polymer for generating autograft-like tissue noninvasively due to their ability to encapsulate MSCs⁴⁹ and take the shape of any 3D defect. Alginate is a naturally occurring, soluble polysaccharide in aqueous solution, but can

form a gel via ionic contact with divalent cations. These ionic interactions are made possible through the guluronate residues located within the polysaccharide.⁵⁰ Chemically cross-linked alginate gels, however, are heavily restricted in their mechanical properties and ability to disintegrate.⁵¹ Therefore, it is common to utilize alginate derivatives to form hydrogels that have a much more broad range of mechanical and degradation properties.

Hydrogels containing poly(aldehyde guluronate) (PAG) and calcium cross-linked sodium alginate gels are commonly used in bone tissue engineering. PAG gels are formed via a chemical acid hydrolysis reaction. Their structural integrity and broad range of degradation behavior depend upon the degree of cross-linking. The degradation property of PAG gels can range from days to weeks depending upon the degree of cross-linking with adipic acid dihydrazide.⁵¹ Figure 4 shows the gelatin of 4% alginate using incident light.⁵² Degradation of alginate derivative gels also depends upon the concentration of alginate within the gel. Alginate gels containing lower concentrations of sodium alginate degrade at a much faster rate compared to those containing a higher concentration.⁵³ Specifically, gels containing 3% alginate were found to degrade within 4 weeks, while 8% alginate gels remained in their original shape after 4 weeks.

In vitro cell adhesion experiments were performed using alginate derivative gels to determine whether the degree of ionic cross-linking and concentration of alginate affects matrix synthesis.^{52–56} Rat bone marrow cells cultured on 3% alginate gels survived significantly better than cells cultured on 8% sodium alginate hydrogels.⁵³ It is thought that 8% sodium alginate gels may release byproducts at high enough levels to inhibit proliferation and thus matrix synthesis. The composition of alginate and alginate derivative gels are critical to degradation, cell proliferation, and matrix synthesis. Interestingly, the geometry of alginate gels is also important to cell proliferation. Alginate gels produced in 2D discs were found to have significantly less cellular colonization than 3D alginate tubes; however, there was no difference in the amount of differentiation as determined by gene expression.⁵³

Arginine-glycine-aspartic acid (RGD)-modified alginate hydrogels were cross-linked with bioactive strontium, zinc, and calcium ions.⁵⁷ Calcium and strontium containing gels have shown similar stiffness, but different stabilities over time. The gels containing alginate with high percentage of guluronic acid residues (high G) and strontium showed slow degradation compared to those gels containing alginate rich in mannuronic acid (high M). Saos-2 cultured within alginate gels upregulated the osteoblast phenotypic marker genes RUNX2, collagen I (COL1A1), and bone sialoprotein (BSP), and ALP protein activity was highest in alginate gels cast with strontium ions.

Alginate gel containing calcium phosphate-DNA nanoparticles and MC3T3-E1 preosteoblasts were examined for the bone formation.⁴⁹ MC3T3-E1 cells mixed with alginate gels containing DNA nanoparticles encoding for BMP-2 were injected subcutaneously in the backs of mice. This cell-matrix combination has shown the bony tissue in mice within two and half weeks of post implantation. In a recent study, porcine adipose-derived stem cells (ADSC) and bone-marrow derived stem cells (BMSC) were examined for the differentiation potential toward osteogenic and adipogenic lineages *in vitro*

when cells were cultured in a 3D alginate hydrogel.⁵⁸ They found that osteogenic gene expression markers such as ALP, COL1A1, SPARC, and SPP1 were less in the beginning of the culture and then upregulated during the culture period up to 28 days.

While there are promising results being observed with the utilization of alginate and alginate derivative gels,^{57,59–61} there are still some major concerns. Only the outer one third of the implantation site had significant bone formation leading to the assumption that there was not an ample nutrient supply to the inner two-thirds of the site.⁵³ Lack of significant bone formation may also be due to the low efficiency with which single cultures of MSCs are induced to differentiate.⁶⁰ In addition, alginate gels have significant limitations in load-bearing areas due to extremely high pressures affecting their integrity. Addressing these issues is crucial to the future use of alginate gels as an agent for tissue engineering.

Hyaluronan hydrogels—Hyaluronan is a natural glycosaminoglycan found in all connective tissues and the synovial fluid of joints.⁶² Its natural composition and abundance make it the subject of intense research for clinical application. Hyaluronan's biodegradable properties, natural make up, stability to hydrolysis, cell adhesion, cell migration, cell proliferation, and cellular differentiation characteristics are important for its potential use as a tissue engineering construct.^{10,62,63} Hyaluronan derivatives were also prepared with methacrylic esters via photopolymerization for the preparation of the hydrogels.⁶⁴

Previous studies with hyaluronan have shown that the material is immunologically inert and 100% biodegradable both *in vitro* and *in vivo*.^{65,66} Similarly, *in vivo*, hyaluronan has the ability to aid in the construction of a pericellular matrix, which produces a suitable and structurally protected microenvironment for progenitor cell differentiation.⁶⁶ The ability to replicate a natural environment for progenitor cells is important, particularly due to the ease with which differentiated cells lose their functional attributes.⁶⁵ Hydrogels are allowing seeded cells to spread throughout the material.

Unfortunately, natural hyaluronan displays relatively weak mechanical strength thus calling its use as a possible scaffold into question.⁶⁷ Aldehyde-modified hyaluronan is currently being studied as a potential substitute for natural hyaluronan. Such hyaluronan derivatives are capable of forming viscous and stable hydrogels at high enough concentrations. Increased concentrations of hyaluronan, however, may prove to be cytotoxic to MSCs.⁶⁸ In a recent study, aldehyde modified hyaluronic acid (HA) was prepared by incorporating an amino-glycerol side chain via amidation reaction and selective oxidation of the pendent group. This reaction was able to form hydrazone-crosslinked hydrogel within 30 s that was stable at physiological pH.¹⁰ They found that the hydrogel developed are biocompatible performing *in vitro* cytotoxicity assay.

Previous studies have found that modified hyaluronan has the ability to cause the expression of its own receptor on MSCs.⁶⁷ The resulting interaction between the MSC receptor and hyaluronan leads to migration and adhesion of MSCs to the polymer. Thus, hyaluronan gels have the capacity to act as both an attractive force and as a protective carrier for MSCs.

Hyaluronan-based injectable hydrogels have been used as a depot for small molecule drugs, such as bisphosphonate,⁶⁸ and DNA plasmids.⁶⁹ In addition, hyaluronan gels were used as a carrier for growth factors, such as BMPs delivery to promote bone augmentation.^{10,70,71} Hyaluronan gels were also used as an antibacterial carrier for gentamicin to treat osteomyelitis in a rabbit model.⁷²

Hyaluronan hydrogels containing BMP-2 were injected over the rat calvarium and showed bone formation in 8 weeks in correlation with the amount of BMP-2 loaded (0, 1, and 30 μ g) within the gel.¹⁰ In another study cranial defects in minipigs were treated with hyaluronan containing BMP-2.⁶⁷ Computed tomographic scans of the animals treated with hydrogel and BMP-2 revealed a substantial formation of compact bone and complete healing of the defects (Fig. 5).

Acrylated HA was used as a scaffold for bone morphogenic protein-2 (BMP-2) and human mesenchymal stem cells (hMSCs) for rat calvarial defect regeneration.⁶⁴ For *in vivo* calvarial defect regeneration study, five different samples (i.e., control, hydrogel, hydrogel with BMP-2, hydrogel with MSCs, and hydrogel with BMP-2 and MSCs) were implanted for 4 weeks. The histological results demonstrated that the hydrogels with BMP-2 and MSCs had the highest expression of osteocalcin and mature bone formation with vascular markers, such as CD31 and vascular endothelial growth factors, compared with the other samples. This study demonstrated that HA base hydrogel can be used for cell and growth factor carriers for tissue regeneration.

Allogeneic hMSCs loaded onto human demineralized bone matrix (hDBM) and hyaluronan hydrogel were used to repair critical sized bulla defects in the guinea pig model.⁷³ All the hMSCs-loaded hDBM at 7 weeks post-implantation showed greater amounts of bone filling in the bulla space, as compared to the hMSCs-free hDBM implanted group. In the hMSCs-free hDBM group, microCT showed incomplete new bone formation, as compared to the hyaluronic acid gel-hMSCs-treated group.

Poly(ethylene-glycol) hydrogels—Poly(ethylene-glycol) (PEG) gels are important vehicles for tissue engineering since this material has been approved by FDA for medical use.⁷⁴ Importantly, tissue-specific proteins, like BMP-2, are able to be incorporated within PEG gels because they do not interact with the PEG-based constituents.⁷⁵ Specifically, prior studies have found that PEG hydrogels are able to support encapsulated bone marrow stromal cells and deliver BMP-2 to support differentiation and bone matrix deposition.⁷⁶

PEG-based hydrogels are highly permeable to water, oxygen, and nutrients due to their hydrophilic nature making them excellent cell carriers.⁷⁷ The hydrophilicity of PEG-based hydrogels, however, creates issues with its stability in water. Specifically, PEG-based hydrogels do not degrade at a sufficient rate in body fluids. A proposed strategy to alter the composition and thus increase the rate of degradation of these gels is to add degradable monomers such as PPF. This, however, creates new issues because of the acidic byproducts produced upon its degradation. Therefore, novel approaches focus on incorporating degradable terminal cyclic acetals with the PEG polymer. The mechanical properties of PEG

was improved by co-polymerizing or blending with other polymers, such as polycarbonates,⁷⁸ methacrylates,⁷⁹ and poly(propylene fumarate).⁸⁰

Kaihara et al. have developed PEG-based polymer hydrogels containing terminal acetal groups like poly[poly(ethylene glycol)-co-cyclic acetal] (PECA) and 5-ethyl-5(hydroxymethyl)- β,β -dimethyl-1,3-dioxane-2-ethanol diacrylate (EHD).^{77,81} These cyclic terminal acetal groups are promising because their degradation products are alcohol and carbonyl groups, both of which are nonacidic. The hydrophobicity of EHD groups, however, changes some of the properties of EHD/PEG-based gels. Most notably, it was found that higher concentrations of EHD lead to lower degrees of swelling. Interestingly, the water contact angle, which is the angle at which water contacts the surface of the scaffold, was not affected enough to alter the optimal angles for cell attachment (50–75°).

The survival of encapsulated cells on the modified PEG-based hydrogel is also of importance. The viability of bone marrow stromal cells incubated with PECA showed no significant dependence on PECA concentration leading to the assumption that intact PECA is not cytotoxic. Figure 6 shows the cell viability of different gels⁷⁷. Lastly, degradation experiments at three different molecular weights PECA, 600, 1000, and 2000, found that cell viability remained high after 4 days with PECA degradation products.

Furthermore, cell proliferation and extracellular matrix deposition are dependent upon environmental factors, which can be easily controlled in PEG-based hydrogels. For example, the degree of compression and nutrient flow can be organized based on the density of cross-linking, among other factors.⁸² Nicodemus et al. studied the impact that environmental factors, specifically mechanical loading, had on gene expression in chondrocyte-encapsulated PEG-based gels. It was observed that collagen type I and aggrecan levels increased, while collagen type II levels slightly decreased in the absence of mechanical load over a 4-day period. Similarly, when placed under a dynamic load, but over the same culture period, aggrecan and collagen type I levels remained steady, while collagen type II levels significantly decreased.⁶⁸ These results reveal that environmental factors do indeed affect the cell–hydrogel interaction, leading to fluctuations in gene expression and thus tissue deposition.

Cell adhesion is a necessary prerequisite for survival and thus collagen or bone deposition. Introducing cell attachment peptides onto PEG-based hydrogels has become the focus of many studies to enhance cell adhesion, proliferation, and differentiation.^{83–85} Cell attachment was studied after modifying the gels by adding attachment peptides arginine-glycine-aspartic acid (RGD) residues to the PEG-based scaffolds. These residues are used because they are an extracellular matrix protein that supports integrin-receptor binding, thus enhancing cell to gel adhesion.⁸³ Importantly, number of MSCs attached to the modified hydrogel was directly dependent on RGD density. Specifically, gels containing higher levels of RGD saw more cellular adhesion and proliferation.

A recent study investigated the effect of biomolecular clues in chondrogenic and osteogenic differentiation of hMSCs when encapsulated in PEG hydrogels, which fabricated with cell adhesion moieties, RGD. They evaluated the cell-laden hydrogels subjected to 4 h daily

intermittent dynamic compressive loading (0.3 Hz, 15% amplitude strain) for up to 14 days for gene expression and matrix deposition for chondrogenic and osteogenic markers. They observed upregulation of gene expression for osteogenic markers with normal hydrogels. However, under the dynamic conditions, the bone-related markers RUNX2, Col I, and ALP were down-regulated and positive staining for type I collagen and mineralization was reduced.⁸⁴ Similar results were observed when hMSCs encapsulated in oligo(poly (ethylene glycol) fumarate) hydrogels were cultured for 21 days under cyclic strain (10%, 1 Hz, 3 h of strain followed by 3 h without) or at 0% strain.⁸⁶

Cell attachment and proliferation of MSCs on poly(ethylene glycol) (PEG) sebacic acid diacrylate (PEGSDA) hydrogels, with or without RGD peptide modification, was similar to the control tissue culture polystyrene. ALP expression of MSCs, as well as mineralized calcium content, was significantly higher on PEGSDA-based hydrogels than those on the control or unmodified PEG diacrylate (PEGDA) hydrogels. In addition, PEGSDA hydrogel scaffolds can be used as local drug carriers for prolonged sustained release of osteoinductive molecules.⁸⁵

PEG–PLGA copolymer hydrogels—The biodegradation and hydrophilicity of PEG–PLGA copolymers can be tailored by choosing the ratio of its hydrophilic and hydrophobic constituents.^{87–89} These co-polymers have unique properties compared to that counterpart polymers, including microphase separation, crystallinity, water-solubility, and biodegradability.^{87,88}

Promising new research focuses on the biodegradable triblock copolymer consisting of PEG and PLGA because of the ease with which its composition can be controlled. It is manufactured without an organic solvent and has been shown to undergo a similar solution-to-gel transformation at body temperature⁸⁹. It is crucial to develop polymers in such a way that the sol-to-gel transition occurs at temperatures <37°C so that cells and osteoinductive factors can be encapsulated within the structure⁹⁰.

Interestingly, previous studies found that PEG–PLGA polymers maintained their structural integrity within the body for >24 h, a trait that poloxamers failed to do.¹⁶ Further, PEG–PLGA polymers were shown to form a gel extremely efficiently, which was indicated by its ability to maintain a three-dimensional profile instead of a sheet at the implantation site. Additionally, studies indicate that inside the body, the polymer breaks down into constituents consisting of PEG, glycolic acid, and lactic acid, all of which are nontoxic⁹¹. Finally, degradation rates are extremely important because the room created by dissolution is necessary for new tissue deposition. The degradation rate of PLGA–PEG samples was significantly increased upon lengthening of PEG segments located within the structure.^{87,88,91} Furthermore, the degradation rates for these copolymers can be controlled by altering the structural makeup of the polymers backbone. Specifically, the structural integrity of polymers consisting of a PLGA backbone was found to stay intact for over 2 months *in vivo* whereas polymers with a PEG backbone dissolved within 2 weeks. The drastic difference in degradation times between the two structures can be attributed to the hydrophilic nature of PEG⁸⁷.

A recent study reported preparation of injectable thermosensitive gel incorporating acellular bone matrix (ABM) into the triblock copolymer poly(ethylene glycol)-poly (ϵ -caprolactone)-poly(ethylene glycol)-(PEG-PCL-PEG, PECE) hydrogel.⁹³ The composite prepared was able to pursue thermal transition from solution, below or at room temperature, to nonflowing hydrogel around 37°C in several minutes. These hydrogel composite showed mild cytotoxicity to rat bone marrow stromal cells *in vitro*. The biocompatibility of this composite was evaluated by dorsal subcutaneous injections into mice. Although some inflammatory reaction was observed in week 1 and 2 after injection, the number of inflammatory cells reduced dramatically at 4 weeks as analyzed by H&E staining.

A three-component biomimetic hydrogel composite was prepared using triblock PEG-PCL-PEG copolymer (PECE), collagen, and nano-hydroxyapatite. The rheological measurements for *n*-HA/PECE nanocomposites were shown that the gelation temperature was approximately 36°C⁹⁴. This hydrogel composite was evaluated for osteogenic potential by implanting the composite material in cranial defects of New Zealand white rabbits at 4, 12, and 20 weeks. *In vivo* results confirmed that the composite has biocompatibility and biodegradability, and suitability to use in guided bone regeneration than the self-healing process.⁹⁵

One of the best known examples of a temperature-controlled gel is a class of copolymers known as poloxamers, which are composed of poly(ethylene oxide-*b*-propylene oxide-*b*-ethylene oxide). This copolymer turns from dissociated unimers to gelatin micelles as the temperature increases¹⁶. The ability of poloxamers to change from a solution to a gel based on temperature is crucial to their use as an injectable agent. Low viscosity at room temperature allows the solution to be transported subcutaneously into a patient where the rise in body temperature then causes the solution to gel transition. However, there are some limitations in utilizing poloxamers as tissue engineering agents. When the poloxamer is degraded several products are formed such as formic and acetic acid formation, acetaldehyde, and formaldehyde formation. In addition, poloxamer degradation led to pH changes and molar mass changes.^{96,97}

CONCLUSIONS

Injectable polymer scaffolds, including injectable microspheres and injectable gels, have the potential for limiting invasive orthopedic and craniofacial procedures. In addition, the injectable polymer scaffolds can fit into any size and shape of the bone defect compared to the 3D conventional (size fixed) scaffolds. To apply these injectable polymeric scaffolds for bone regeneration, one must take into account the polymers composition, by-products, method, and materials for synthesis to obtain favorable biodegradation and biocompatibility properties. A thorough understanding of each surrogate polymer is crucial to their potential use. There are situations that give some polymers distinct advantages over others. The polymeric microspheres are mechanically strong compared to the polymeric gels. The polymeric gels were modified using other polymers to improve the strength of the mechanical properties. On the other hand, natural polymers provide better biocompatibility when they implanted in the animals compared to the synthetic polymers. Furthermore, the chemical and physical makeup of the scaffold should be conducive to cell adhesion and

matrix deposition. As discussed in the review, it is important to understand the different parameters of each polymer and how they affect cell adhesion, tissue deposition, and the host's response. Tissue engineering is an exciting and expanding field that encompasses many disciplines. An injectable polymer for orthopedic use is of particular importance because of the immediate clinical impact that it would serve.

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FIGURE 1.
Poly(propylene fumarate) reaction scheme.²⁵

FIGURE 2.

Histological sections of PPF/PLGA scaffolds. (a, b) Degradation was characterized by micro-fragmentation of the cross-linked network and was associated with an increase in inflammatory response; (c) multinucleated giant cells were present in the pores of the polymer scaffold; (d) tissue ingrowth was evident in circular pores previously occupied by PLGA microparticles.¹³ [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3.
SEM Image of cross-linked chitosan microparticles at week 0 (A) and chitosan microparticles immersed in PBS at week 25 (B).⁴⁵

FIGURE 4.

Gelation of 4% alginate using incident light. (A) 1 drop of alginate on microscope slide into which 40 μ l IFV has been injected (cloudy region at centre). (B) Irradiation of specimen from a single UV LED at 385 nm to stimulate trans-cis isomerisation of the photosensitive lipid. (C) Following 1 min irradiation, a gelled lump of material could be separated from the remainder of the non-cross-linked alginate. (D) Manipulation of the gelled material with a spatula demonstrated that the material produced was self-supporting.⁵² [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Cranial defect in a minipig was treated with hyaluronan gel and gel containing BMP-2. Three-dimensional computed tomography (*above*) and sagittal computed tomography (*center*) with *red rectangle* and *arrows* indicate the location of the defect in representative animals of groups 1 through 3. Sagittal cross-sections (*below*) show the macroscopic appearance of the reconstructed area.⁶⁷ [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 6.

An investigation of the effects of PECA upon cell viability. (A) Fraction of live cells that had been cultured in media augmented with PECA 600, 1000, and 2000 (0.1 mg/mL), using PEGDA as a control. (B) Representative images of BMSCs visualized with fluorescence light.⁷⁷ [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]