High-Density Cell Systems Incorporating Polymer Microspheres as Microenvironmental Regulators in Engineered Cartilage Tissues

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To address the significant clinical need for tissue-engineered therapies for the repair and regeneration of articular cartilage, many systems have recently been developed using bioactive polymer microspheres as regulators of the chondrogenic microenvironment within high-density cell cultures. In this review, we highlight various densely cellular systems utilizing polymer microspheres as three-dimensional (3D) structural elements within developing engineered cartilage tissue, carriers for cell expansion and delivery, vehicles for spatiotemporally controlled growth factor delivery, and directors of cell behavior via regulation of cell–biomaterial interactions. The diverse systems described herein represent a shift from the more traditional tissue engineering approach of combining cells and growth factors within a biomaterial scaffold, to the design of modular systems that rely on the assembly of cells and bioactive polymer microspheres as building blocks to guide the creation of articular cartilage. Cell-based assembly of 3D microsphere-incorporated structures represents a promising avenue for the future of tissue engineering.

Introduction

MATURE ARTICULAR CARTILAGE is a fairly inert tissue with no vasculature, relatively low cellularity, and a slow rate of turnover.¹ For these reasons, cartilage has a limited capacity for repair and any damage leads only to further degeneration.¹ This lack of intrinsic repair capability is one factor in the development of osteoarthritis (OA), a debilitating progressive disease involving the irreversible erosion of articular cartilage. OA affects a large and growing number of people worldwide² and poses a serious clinical problem, as there is no single treatment that can consistently restore normal joint function to all patients.³ Common first-line clinical approaches to OA include nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroid injections with the goal of reducing symptomatic pain and inflammation.^{4,5} Although these treatments aim to manage the symptoms of OA, they have associated risks and neither seeks to address the fundamentally limited regenerative capacity of cartilage.⁶

Since pharmaceutical management of OA symptoms is not always successful as cartilage degeneration progresses,⁵ surgical treatments have been developed with the goal of repair and regrowth of articular cartilage. Current cell-based approaches to the treatment of cartilage injury or disease

include subchondral bone marrow stimulation and autologous chondrocyte transplantation (ACT). Subchondral bone marrow stimulation techniques, such as microfracture and subchondral drilling permit blood and bone marrow from the underlying subchondral bone to fill the cartilage defect site and provide a rich source of signaling factors and stem cells. However, these procedures may result in the formation of fibrocartilage, which lacks the molecular composition, structural organization, and mechanical properties of native articular cartilage.⁷ ACT is another surgical approach involving isolation of healthy cartilage cells from an area of intact articular cartilage, expansion of these chondrocytes in monolayer culture, and transplantation of the cells into a cartilage defect. Although some improvements in pain and joint function have been reported, this expensive procedure has associated problems, such as donor-site morbidity and the patient outcomes are variable.^{8,9} Unfortunately, neither of these cell-based treatment methods can reliably restore normal joint function.³

Other surgical treatment options include mosaicplasty, soft tissue grafts from the periosteum or perichondrium, and allogeneic grafts. Mosaicplasty is an osteochondral autograft procedure involving the transfer of cylindrical plugs from low-weight bearing regions of articular cartilage to the site of a cartilage defect.¹⁰ Although this procedure has shown

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TABLE 1. SPECIFIC MICROSPHERE FORMULATION PARAMETERS FROM SELECTED HIGH CELL DENSITY CARTILAGE TISSUE ENGINEERING APPROACHES

Microsphere polymer type	MW average or range or crosslinking level	Diameter average or range	Cell type	Loaded growth factor	Study type	Measures of cartilage formation	Major findings	Ref
<i>Synthetic</i>								
RGD-modified PLLA	85–160 kDa	100–200 µm	CHS (human)	(none)	<i>in vitro</i>	SEM & biochemical analyses	GRGDSPK surface modification on microspheres increased cell attachment	59
PLGA	10 or 50 kDa	52–68 µm	AC (bovine)	(none)	<i>in vitro</i>	Histology, IHC & biochemical analyses	High MW microspheres aided in maintenance of tissue shape, increased tissue mass, and enhanced GAG and type II collagen production	46
PLGA	14 kDa	80–90 µm	AC (bovine)	(none)	<i>in vitro</i>	SEM & biochemical analyses	Cationic microspheres increased cell attachment, proliferation and GAG production	48
Star-shaped PLLA	69.3 kDa	49 µm	AC (rabbit)	(none)	<i>in vitro</i> , SQ injection (mouse), OC defect (rabbit)	Histology, IHC, SEM, gene expression & biochemical analyses	Hollow nanofibrous microspheres increased cell adhesion, matrix deposition and cartilage repair	42
PLGA	80 kDa	422 µm	AC (rabbit)	(none)	SQ injection (mouse)	Histology & biochemical analyses	Macroporous microspheres increased amount and uniformity of GAG staining	43
PLGA	110 kDa	30–80 µm	AC (rabbit)	(none)	SQ injection (mouse)	Histology & IHC	Microspheres increased lacunae formation and intensity of GAG and type II collagen staining	44
PLGA	10 or 50 kDa	52–199 µm	AC (bovine)	(none)	SQ injection (mouse)	Histology, IHC & biochemical analyses	Microspheres increased tissue mass, GAG production and uniformity of type II collagen staining	45
Fibronectin-coated PLGA	25 kDa	60 µm	MSC (human)	TGF-β3	<i>in vitro</i> , SQ and IM implantation (mouse)	Histology, IHC & gene expression	GF-loaded microspheres induced expression of chondrogenic markers and staining for chondrogenic markers after SQ implantation	81
PLGA	18 kDa	7 µm	MSC (human)	TGF-β1	<i>in vitro</i>	Histology & biochemical analyses	GF-loaded microspheres induced GAG production at levels approaching those of cell pellets with exogenous GF delivery	66
Nanoparticle-coated PLGA	33 kDa	20–80 µm	MSC (rabbit)	TGF-β3	<i>in vitro</i>	Histology, IHC, gene expression & biochemical analyses	GF-loaded microspheres increased chondrogenic morphology and GAG and type II collagen production compared to microspheres without GF	74

(continued)

TABLE 1. (CONTINUED)

Microsphere polymer type	MW average or range or crosslinking level	Diameter average or range	Cell type	Loaded growth factor	Study type	Measures of cartilage formation	Major findings	Ref
Natural Fibrin	Crosslinking level Unknown	63–105 μm	MSC (mouse)	(none)	<i>in vitro</i>	Histology, IHC, SEM, gene expression & biochemical analyses	Microspheres enabled formation of 3D structures containing GAG and type II collagen in GF-containing medium	53
Alginate with various ECM coatings	Unknown	unknown	MSC (human)	(none)	<i>in vitro</i>	Histology, IHC, gene expression & biochemical analyses	CS and type II collagen-coated microspheres increased cell proliferation and GAG and type II collagen production compared to pellet cultures	79
Gelatin	28 or 68%	62 or 53 μm	MSC (human)	TGF- β 1	<i>in vitro</i>	Histology, IHC, mechanical & biochemical analyses	Sheets with GF-loaded microspheres had equilibrium moduli of 9.88 kPa vs. 0 kPa without microspheres and increased thickness, GAG, and type II collagen production	47
Gelatin	Unknown	15 μm	MSC (rabbit)	TGF- β 3	<i>in vitro</i>	Histology, IHC, gene expression & biochemical analyses	Microspheres increased pellet size, cell proliferation, and total GAG and collagen production in cell pellets	65
Gelatin	28 or 68%	62 or 53 μm	MSC (human)	TGF- β 1	<i>in vitro</i>	Histology, IHC & biochemical analyses	GF-loaded microspheres induced GAG and type II collagen production at levels similar to or greater than that from exogenous GF delivery	67
Gelatin	29 or 65%	61 or 54 μm	ASC (human)	TGF- β 1	<i>in vitro</i>	Histology, IHC & biochemical analyses	GF-loaded microspheres induced GAG production at levels similar to exogenous GF delivery	73
Gelatin	93.5% water content	107 μm	MSC (rat)	TGF- β 1	<i>in vitro</i>	Biochemical analyses	Microspheres induced more proliferation and higher viability, and cells produced GAG with GF in media or released from microspheres	77

AC, articular chondrocyte; ASC, adipose-derived stem cell; CHS, chondrosarcoma; CS, chondroitin sulfate; ECM, extracellular matrix; GAG, glycosaminoglycan; GF, growth factor; IHC, immunohistochemistry; MSC, mesenchymal stem cell; MW, molecular weight; OC, osteochondral; PLGA, poly(lactide-co-glycolide); PLLA, poly(L-lactic) acid; SEM, scanning electron microscopy; SQ, subcutaneous; TGF- β 1, transforming growth factor- β 1.

promising short-term results for improved joint functionality, donor-site morbidity can be a problem¹¹ and data on long-term outcomes are limited.¹² Soft tissue grafting techniques have produced variable results and allogeneic grafts pose risks which, although rare, include immune rejection and disease transmission.¹³ Ultimately, progressive cartilage degeneration often necessitates total prosthetic replacement of the affected joint. This invasive procedure carries risks of infection and is typically indicated only for older patients due to the limited lifetime of the prosthetic implant.^{5,14}

Due to the shortcomings of these treatments as well as the lack of intrinsic repair capacity of mature cartilage tissue, tissue engineering strategies may be necessary to address the significant clinical need for cartilage repair and replacement. Many approaches to the engineering of articular cartilage involve the use of chondrogenic cell sources, including mature chondrocytes,^{15,16} mesenchymal stem cells (MSCs),^{17,18} or adipose-derived stem cells (ASCs)^{19,20} as reviewed in detail elsewhere.²¹ Several different systems have been developed for the culture of these cells, many of which take into account the fact that high cell density is an important factor in both the chondrogenic induction of stem cells^{22,23} and the maintenance of differentiation state of mature chondrocytes.²⁴ Common methods of high-density (HD) chondrogenic cell culture include aggregate or pellet culture,^{22,24–29} micromass culture,^{30,31} membrane-based systems,^{23,32–34} and rotary suspension culture.^{35,36} Inherent to the success of these assorted culture systems is the formation of densely cellular constructs that promote cell–cell interactions. However, other environmental cues are often necessary for the production of engineered cartilage tissue, including soluble factors and cell–matrix interactions.²¹ In fact, the coordination of an intricate set of structural elements and microenvironmental signals may be required for the successful engineering of functional tissues, including articular cartilage.

A number of new systems have recently emerged with the common theme of incorporating polymer microspheres into densely cellular constructs. Within these systems, polymer microspheres direct the presentation of specific microenvironmental cues to chondrogenic cells in HD culture to regulate cell behavior and tissue formation. The microspheres may act in a variety of specialized roles to define the three-dimensional (3D) arrangement of neocartilage tissue formation, deliver chondrogenic growth factors in a temporally and spatially controlled manner, and instruct cell–cell and cell–material interactions. This review highlights these roles within specific studies in the literature, focusing on HD cell culture systems that employ microspheres for cartilage tissue engineering applications.

Microspheres in Cartilage Tissue Engineering

Many tissue engineering strategies have been developed using microspheres, which are typically defined as spherical or approximately spherical particles with diameters on the micron scale (ranging from 1 to 1000 μm).³⁷ Polymer microspheres can be formed through methods, including single- or double-emulsion processes³⁸ or spraying techniques³⁹ and have been produced from both natural and synthetic polymers for utilization in a wide variety of applications. In particular, microspheres have been utilized as part of a number of biomaterial-based cartilage tissue engineering

strategies. For example, in one unique system, MSCs were coencapsulated with transforming growth factor- β 1 (TGF- β 1)- and insulin-like growth factor-1-releasing gelatin microspheres within an oligo(poly(ethylene glycol) fumarate) hydrogel as an injectable system for *in vitro* cartilage formation.⁴⁰ In another approach, moldable macroporous scaffolds were formed into specific shapes from poly(lactide-co-glycolide) (PLGA) microspheres by a CO₂ sintering method and seeded with chondrogenic cells, producing constructs that could be shaped to fit individual cartilage defects.⁴¹

Polymer microspheres are a popular choice for tissue engineering applications due to the versatility of available materials and tailorable microsphere properties, including size, porosity, surface characteristics, permeability, cell adhesivity, and degradation rates. Selection of materials and formation methods enables control and tuning of these characteristics, which can be modified to suit the specific needs of the system. For example, porosity may be a desirable characteristic in cell-containing systems, as porous microspheres can allow cell infiltration and may enable cell proliferation and extracellular matrix (ECM) deposition within void spaces of the material.^{42,43} Although broadly used, a major drawback to utilizing polymer microspheres as part of tissue engineering strategies is the difficulty of localizing multiple microspheres to a specific location within the body. This issue has been addressed through a number of approaches, including using microspheres as part of cohesive, 3D cellular constructs, which aid in localizing microspheres to a particular location, while preserving their useful characteristics. The specific microsphere formulation parameters utilized in several systems involving cells in HD culture for cartilage tissue engineering applications are summarized in Table 1.

Microspheres as Matrix Spacers

When incorporated into HD cell culture systems, polymer microspheres can provide a scaffold for tissue-engineered cartilage that enhances matrix production^{42–47} and contributes to the mechanical stability of the constructs.^{46,47} Polymer microspheres incorporated within densely cellular tissues act as spacers, defining 3D space for tissue formation, while accommodating ECM accumulation and cell infiltration as the microspheres degrade.^{42,47,48} This spacing effect within dense cell systems could be advantageous for creating tissues morphologically similar to native articular cartilage, which consists primarily of an organized ECM containing water, type II collagen, and proteoglycans with relatively few, disperse chondrocytes. Several studies have reported that the inclusion of polymer microspheres into HD cell systems enables a more uniform cartilaginous matrix assembly with a morphological structure similar to that of native cartilage tissue when compared to cell-only constructs without microspheres,^{42–47} potentially due to the 3D spacing of cells by microspheres within the developing tissues. There is also evidence that the cell and matrix spacing effect conferred by incorporated microspheres may increase the permeability of cellular aggregates, improving oxygen diffusion within microsphere-incorporated HD cell constructs as indicated by higher aerobic metabolic activity within cell aggregates containing gelatin microspheres when compared to cellular aggregates without microspheres.⁴⁹

In addition to the spacing advantages provided by microspheres within neocartilage matrices, the tissue bulking effect of polymer microspheres within cellular systems lends added volume and improved mechanical properties to the resultant tissues. Mechanical properties are a critical aspect of these constructs, as an ideal engineered cartilage should be mechanically resilient enough to enable handling and implantation without losing its shape,³⁴ and strong enough to endure forces within the joint until the regenerating cartilage gains sufficient mechanical properties to support the tissue.^{50,51} It has been demonstrated that the addition of PLGA⁴⁶ or gelatin⁴⁷ microspheres to cellular systems enables the formation of neocartilage constructs that maintain shape and thickness during *in vitro* culture. In studies describing injectable systems of chondrocytes with PLGA microspheres as well as self-assembled MSC sheets incorporated with gelatin microspheres, the inclusion of polymer microspheres was shown to increase tissue mass^{46,47} and improve compressive mechanical properties⁴⁷ by comparison to cell-only constructs. In the injectable system, the inclusion of 50-kDa PLGA microspheres with chondrocytes resulted in growing tissues that had a three- to fourfold greater mass than tissues formed from cells alone at 2, 4, and 8 weeks.⁴⁶ Within microsphere-incorporated MSC sheets, incorporation of gelatin microspheres with or without a loaded growth factor increased the equilibrium compressive moduli of the sheets when compared to sheets without microspheres, which failed to exhibit any detectable change in the equilibrium force with applied sequential step strains up to 15%.⁴⁷ In both systems, the characteristics of the newly formed cartilage varied with the specific properties of the incorporated microspheres, including the polymer degradation rate.^{46,47} This effect of the degradation rate on cartilage formation is likely due to the relationship between microsphere degradation and ECM deposition by the differentiating cells. As

the polymer degrades, more space is made available for cell migration and cell-derived matrix accumulation. However, if the polymers were to degrade too slowly or too rapidly, it could interfere with neocartilage tissue formation and/or matrix remodeling within these systems.

Microsphere-containing cell systems have also been tested *in vivo* in mouse subcutaneous injection models.^{42–45} It has been demonstrated that after up to 8 weeks *in vivo*, cartilage tissues formed with PLGA microspheres and chondrocytes were more similar to native cartilage in terms of matrix deposition, cell spacing, and matrix uniformity than tissues formed from chondrocytes alone.⁴⁵ As in the *in vitro* studies, the results were dependent on specific polymer properties, and the best results in this case were demonstrated with high molecular weight (MW) PLGA microspheres. Due to the slower degradation rate of these high MW polymer microspheres (6 to 8 weeks for 50-kDa PLGA vs. 2 to 4 weeks for 10-kDa PLGA microspheres), they may be able to provide effective matrix spacing for a longer period, allowing gradual creation of free volume for more ECM elaboration and tissue accumulation.⁴⁵ Another study involving an injectable system of chondrocytes and PLGA microspheres had similarly positive findings, showing that the incorporation of microspheres into chondrocyte suspensions improved *in vivo* cartilage formation by comparison to chondrocytes or microspheres alone.⁴⁴ Studies on neocartilage tissue formed with incorporated macroporous PLGA microspheres⁴³ or hollow nanofibrous microspheres made from star-shaped poly(L-lactic acid) (Fig. 1)⁴² were also conducted, with results indicating that microspheres with a higher total porosity produced more total glycosaminoglycan (GAG) and a more uniform cartilaginous matrix. This outcome may be due to enhanced cellular infiltration of porous microspheres leading to additional GAG deposition in the void spaces, which was observed histologically (Fig. 1c, d).

FIG. 1. *In vivo* cartilage formation 6 weeks after mouse subcutaneous implantation. Safranin O stained histological sections of neocartilage formation by chondrocytes alone (a), chondrocytes and solid PLA microspheres (b), chondrocytes and nanofibrous microspheres (c), and chondrocytes and nanofibrous hollow microspheres (d). Scale bar = 100 μ m. Arrows indicate nanofibrous (c) or nanofibrous hollow (d) microspheres. Modified from Liu *et al.* 2011 with permission from Macmillan Publishers, Ltd. PLA, poly(lactic) acid. Color images available online at www.liebertpub.com/teb

As a whole, these studies demonstrate that the formation and growth of engineered cartilage can be enhanced through the inclusion of polymer microspheres as structural elements within densely cellular systems, and that specific microsphere properties, including polymer formulation, degradation rate, and porosity, can influence cartilage tissue formation. It may be conceivable to design an HD cell system incorporated with microspheres that have a finely tuned degradation rate proportional to the rate of ECM elaboration, enabling the formation of a uniform, structurally appropriate cartilage tissue.

Microspheres as Cell Carriers

In addition to the advantages of using microspheres as spacing and bulking agents in densely cellular constructs to enhance neocartilage tissue formation, microspheres may address other major challenges inherent to cartilage tissue engineering, including expanding cells to sufficient numbers and induction and maintenance of the chondrogenic phenotype.⁵² Polymer microspheres have been formulated as a means of expansion and delivery of MSCs^{53,54} and chondrocytes^{42,48,55–57} for cartilage tissue engineering applications. Microsphere carriers are uniquely suited to cell culture and delivery, as they can be synthesized with various biomaterial formulations to promote cell attachment, and they provide a much larger surface area for cell proliferation compared to conventional two-dimensional tissue culture plastic surfaces. Additionally, these polymer microsphere carriers have been shown to support both differentiation of stem cells and the phenotypic maintenance of mature chondrocytes during *in vitro* culture.^{42,57}

In these systems, cells are typically combined in a stirred solution with carrier microspheres, incubated to allow cell attachment, and cultured in suspension to promote cell proliferation and/or differentiation. The cell-attached microspheres can then be delivered as an injectable system,^{42,48,56–58} or used to form constructs containing aggregated microsphere-adherent cells and matrix components^{42,53,59} (Fig. 2). It is important to note that some microcarrier systems must be optimized to avoid particle aggregation, as this can be a problem, for example, in injectable systems. However, this aggregation phenomenon can also be helpful for creation of larger, cohesive tissues utilizing the cell-carrying microspheres as building blocks for the tissue.^{53,59,60} When cell-carrying microspheres are used to create 3D constructs, the spherical nature of the particles creates space between the cell carriers, enabling cell migration, proliferation, and aggregation among the particles.

Microsphere cell carriers can be composed of various natural and synthetic materials, including fibrin,^{53,54} poly(lactic acid) (PLA),^{42,55,56,59} or PLGA.^{48,55–58} In addition to varied material compositions, multiple surface modifications have been employed to improve cell attachment, including PLA with surface-conjugated RGD,⁵⁹ PLA grafted with collagen⁵⁵ or chitosan,⁵⁶ and amine-functionalized PLGA.⁵⁸ All of these surface modifications have been shown to promote chondrocyte adhesion and/or expansion on carrier microspheres. Commercially available microcarriers, typically consisting of particles made from materials, such as collagen or gelatin, can also be used for chondrogenic cell expansion and differentiation.^{61–63} Cell-carrying microsphere systems represent an innovative way to support the expansion of both chondrocytes and chondrogenic stem cells,

FIG. 2. Chondrocyte adhesion and *in vitro* cartilage formation on PLA microspheres. Chondrocyte adhesion on nanofibrous hollow microspheres (NF-HMS), nanofibrous microspheres (NF-MS), and solid microspheres (SI-MS) (a). Adhesion rate is defined as the fraction of cells adherent to the microspheres at each time point. 3D morphology of chondrocytes on NF-HMS (b), NF-MS (c), and SI-MS (d) indicating that cells are more rounded on the nanofibrous microspheres, with some cells migrating inside the NF-HMS. Cartilage formation by chondrocytes with NF-HMS after 4 weeks *in vitro* culture in a condyle-shaped mold (e). Safranin O (f) and type II collagen (g) staining of *in vitro*-cultured neocartilage tissue. In (f) and (g), scale bar = 100 µm. Reprinted from Liu *et al.* 2011 with permission from Macmillan Publishers, Ltd. Color images available online at www.liebertpub.com/teb

while providing a 3D environment that enables mature chondrocytes to maintain their differentiation state.^{42,57} In addition to their utility in *in vitro* chondrogenic culture, these microsphere cell carriers have the capacity to be used as *in vivo* cell delivery systems, as they can be easily injected into the body, where the microspheres degrade over time, eliminating carrier material from the tissue.

Microspheres as Bioactive Factor Delivery Vehicles

Beyond their utility as structural elements and cell carriers, polymer microspheres are widely used as bioactive factor delivery vehicles within HD chondrogenic cell culture systems. In these systems, polymer microspheres are loaded with growth factors, such as the chondrogenic growth factors of the TGF- β super family. The incorporation of bioactive microspheres in HD cell culture systems could provide a means to decrease growth factor diffusion distance within densely cellular constructs, improving growth factor delivery to central regions of the tissues. Further, these systems allow growth factor release over a sustained period of time as the microspheres degrade, enabling localized delivery of chondrogenic factors to the surrounding cells at controllable rates over a period of days, weeks, or months. The release of bioactive factors can be mediated by, for example, diffusion, polymer degradation, or affinity interactions, and controlled release may be achieved through methods, including modifying polymer MW or crosslinking density to speed or slow degradation, changing the microsphere permeability, or varying the concentration of affinity-binding molecules. Importantly, constructs containing growth factor releasing microspheres have the potential to enable *in vivo* stem cell implantation without extended prior *in vitro* differentiation, allowing cartilage formation to occur within the body.

One common method for the *in vitro* chondrogenic induction of MSCs is aggregate or pellet culture.^{22,25,26,29,64} This culture method involves the centrifugation of cells into free-floating 3D constructs, which can be cultured in a chemically defined medium containing TGF- β for up to 3 weeks, resulting in neocartilage formation as indicated by the production of GAG and type II collagen. This system has recently been modified by the addition of growth factor containing microspheres within the cell pellets, with the goal of eliminating the need for culture in a growth factor-containing medium and improving growth factor delivery to interior regions of the cellular constructs. Biodegradable polymer microspheres containing TGF- β 1 or TGF- β 3 have been shown to promote the chondrogenic differentiation of both MSCs^{65–67} and ASCs^{68,69} in microsphere-incorporated pellet culture. The inclusion of a growth factor containing gelatin microspheres within these stem cell pellets has the potential to induce *in vitro* chondrogenic differentiation at levels equivalent to or greater than pellets cultured by conventional methods in a TGF- β containing medium. The growth factor containing microsphere-incorporated cell pellets have also been shown to undergo chondrogenesis *in vivo* after implantation in a chondral defect model.⁶⁸ The sustained delivery of either TGF- β 1 or TGF- β 3 in implantation models may play a role in maintaining the chondrocytic phenotype of the cells, preventing dedifferentiation or hypertrophic progression, as often occurs after the *in vivo* implantation of *in vitro* differentiated MSC pellets.^{70–72}

Within these microsphere-incorporated pellet systems, adjusting the amount of microspheres alters the spatial presentation of bioactive factors, permitting a degree of spatial control over the cellular microenvironment. Our group demonstrated the effect of varied amounts of incorporated TGF- β 1-loaded PLGA microspheres on pellet chondrogenesis, showing increased amounts of GAG with increased loading of PLGA microspheres per pellet.⁶⁶ We have also shown that MSC and ASC differentiation can be regulated by the incorporation of crosslinked gelatin microspheres with varied microsphere amount, growth factor concentration per microsphere, and polymer degradation rate.^{67,73} Chondrogenesis of MSCs from three donors was evaluated in terms of DNA, GAG, and type II collagen production at 1 and 2 weeks.⁶⁷ Differentiation varied based on the specific conditions of microsphere incorporation, with a larger mass of fast degrading microspheres tending to improve differentiation by comparison to delivery from a smaller mass of microspheres with a higher growth factor concentration per microsphere, even though the total amount of loaded growth factor was the same. Similar results were observed in ASC aggregates incorporated with TGF- β 1-loaded gelatin microspheres.⁷³ A major advantage of using a microsphere formulation with an adjustable degradation rate is the potential to tailor growth factor release kinetics to suit the needs of the system. As a whole, these microsphere-incorporated pellet systems demonstrate the utility of delivering chondrogenic growth factors from an intrinsically incorporated source for the enhancement, regulation, and maintenance of stem cell differentiation.

Besides pellet cultures, the success of chondrogenic induction via growth factor releasing polymer microspheres has been shown in other HD cell systems as well. In one system, PLGA microspheres were coated with TGF- β 3-loaded heparin nanoparticles and mixed with a concentrated suspension of MSCs.⁷⁴ After 4 weeks of *in vitro* culture, the constructs showed increased GAG production when compared to similar systems of coated PLGA microparticles without a growth factor. In the TGF- β 3 releasing systems, MSCs formed aggregates that exhibited a hyaline-like cartilage morphology and stained positive for type II collagen.^{74,75} The group showed similar results in a subcutaneous implantation model, demonstrating neocartilage formation by MSCs and growth factor-loaded microspheres after 6 weeks.⁷⁶ Another study utilized dehydrothermally cross-linked gelatin microspheres loaded with TGF- β 1 as a scaffolding system for MSCs.⁷⁷ They found that cells aggregated on and around the growth factor releasing microspheres, exhibiting chondrogenic differentiation as measured by GAG production equivalent to that occurring in similar microsphere-incorporated cell cultures with TGF- β 1 supplied in the medium over 14 days.

Our group has recently developed a system of stem cell sheets incorporated with TGF- β 1-loaded gelatin microspheres.^{47,73} These sheets are formed by seeding the cells in high density onto a permeable membrane, enabling multiple cell-cell interactions and self-assembly of 3D tissues. Genipin crosslinked gelatin microspheres with different levels of crosslinking were used to enable different rates of cell-mediated microsphere degradation and growth factor release. While MSC sheets containing either fast or slow degrading TGF- β 1-loaded microspheres formed cartilage after 3 weeks

FIG. 3. Safranin O/Fast Green stained cross sections from the central (A–E) and edge (F–J) regions of sheets. Arrows indicate cell- and matrix-filled regions where low genipin (Gp) crosslinked (low Gp) gelatin microspheres degraded (B, G and D, I) or the presence of residual high Gp crosslinked (high Gp) microspheres (C, H and E, J). Modified from Solorio *et al.* 2012 with permission from Elsevier, Inc. Color images available online at www.liebertpub.com/teb

of *in vitro* culture, the sheets containing rapidly degrading microspheres produced more GAG and GAG per DNA, and stained more strongly for type II collagen than either sheets containing more slowly degrading microspheres or sheets with or without unloaded microspheres cultured in a medium containing TGF- β 1 (Fig. 3).⁴⁷ The applicability of this system to cartilage tissue engineering with another promising chondrogenic stem cell source was demonstrated through the formation of TGF- β 1-loaded microsphere-incorporated ASC sheets, which produced GAG at levels equivalent to those of control sheets without microspheres cultured in a medium with TGF- β 1.⁷³ The incorporation of TGF- β 1-loaded microspheres within stem cell sheets eliminates the need for extended culture in the growth factor-containing medium, potentially enabling more rapid implementation as part of engineered cartilage therapies. Additionally, these self-assembling microsphere-incorporated sheets form larger constructs with much greater surface areas than cell aggregates, resulting in constructs of a more appropriate size for treating clinical cartilage defects.

These studies demonstrate that bioactive factors released from polymer microspheres can induce chondrogenic differentiation of stem cells in HD culture both *in vitro* and *in vivo* in the absence of external growth factor supplementation. Within these systems, it is important to note that the delivery kinetics of bioactive factors may be complicated by the presence of cells and ECM surrounding the microspheres, particularly within systems in which release is predominantly based on diffusion. This should be a consideration for the design of engineered cartilage constructs, in which abundant ECM production could restrict or delay diffusion of factors from microspheres to the surrounding cells. However, in systems such as those in which release is mediated by proteolytic degradation of the polymer, the presence of enzyme-secreting cells at the microsphere surface could be beneficial, promoting degradation and facilitating release of the bioactive factor. Using microspheres within HD cell constructs, it may be possible to engineer a cell microenvironment with spatiotemporally regulated cues, by controlling the spatial position of bioactive microspheres, the rate of microsphere degradation

and growth factor release, and the biomaterial surface with which the cells interact.

Surface-Active Microspheres

Besides growth factor release capabilities, polymer microspheres can have a considerable influence on cell behavior within HD systems based on the properties of the cell-biomaterial interface. Polymer microspheres with surface coatings or modifications designed to interact specifically with cells to direct chondrogenic differentiation are defined here as “surface-active.” Native articular cartilage contains a complex 3D network of molecules, including type II collagen and GAG, as well as cells, which interact with both each other and the surrounding microenvironment.⁷⁸ Cellular interactions with ECM components and insoluble signaling factors are crucial to both directing chondrogenesis of stem cells and maintaining the differentiation state of mature chondrocytes,²¹ and have been examined in several microsphere-incorporated HD cell systems.

In one unique system, various ECM components, including chondroitin sulfate (CS), HA, and type II collagen were coated onto the surface of alginate microspheres and mixed with MSCs to examine the effects of each coating on cell adhesion, proliferation, and chondrogenic differentiation.⁷⁹ When cultured in the chondrogenic medium containing TGF- β 3, MSCs aggregated on and between the matrix-coated microspheres to form irregular 3D structures. Constructs containing particles coated with CS and type II collagen exhibited increased GAG and type II collagen production by comparison to MSC pellet cultures without microspheres after 4 weeks. Interestingly, the type II collagen surface coating induced MSC hypertrophy not seen in the CS group, indicating that the presence of CS may reduce or delay the onset of hypertrophic differentiation in this particular system. A separate study found that nondegradable RGD-conjugated poly(ethylene glycol) microspheres altered the gene expression profiles and protein localization in chondrogenic 3D MSC aggregates cultured with TGF- β 3, potentially due to altered growth factor diffusion, cell-material interactions, and/or dilution of the HD cell aggregates by microspheres.⁸⁰

Another microsphere-incorporated cell system was formulated to enable both the isolation of MSCs from bone marrow and the formation of 3D porous cartilage structures utilizing a single set of microspheres.⁵³ Fibrin microspheres were used to isolate multipotent progenitor cells from whole bone marrow by separating the microsphere-adherent stem cell population from nonadherent hematopoietic cells. The cells on fibrin microspheres were cultured in a slowly rotating suspension, and micron-sized collagen pieces were subsequently added. Within 2 days, aggregates of cells, collagen pieces, and fibrin microspheres formed in the rotating suspension, creating 3D cellular structures incorporated with collagen pieces and fibrin microspheres. These microsphere-incorporated cell structures underwent chondrogenic differentiation over a period of 3 weeks in a medium containing TGF- β 3. In similar control cultures without growth factor supplementation, the cells cultured without the growth factor formed soft and easily dissociated aggregates, while the cells supplemented with TGF- β 3 formed stable microsphere-incorporated structures with a well-formed ECM. However, these cartilage structures were not phenotypically stable *in vivo*, and appeared histologically equivalent to undifferentiated control tissues by 2 weeks after ectopic implantation. This was likely due to cell dedifferentiation as a result of termination of exogenous TGF- β 3 supplementation at the end of *in vitro* culture. As mentioned earlier, one common problem with using *in vitro* differentiated MSCs for cartilage tissue engineering is that they tend to have an unstable cartilaginous phenotype when implanted *in vivo*, and they may dedifferentiate or undergo hypertrophy, resulting in the formation of fibrous or calcified tissue.⁷⁰⁻⁷² Potentially, this system could benefit from the integration of growth factor releasing components, which could enable the cells within the cartilage structures to maintain a chondrogenic phenotype after *in vivo* implantation.

Other groups have developed unique microsphere systems that combine bioactive surface modifications with growth factor release capabilities. One such system utilizing surface-active, growth factor releasing microspheres consisted of TGF- β 3-loaded PLGA microspheres coated with a cell-adhesive layer of fibronectin and poly(D-lysine).⁸¹ When combined with MSCs, the microspheres induced an upregulation of chondrogenic markers at both the gene and protein level compared to MSCs combined with particles without a growth factor. After 2 weeks *in vivo* implantation, the microspheres promoted neocartilage tissue formation that had a cartilage-like histological morphology and stained positively for both aggrecan and type II collagen.

Overall, systems incorporating surface-active microspheres within HD cell systems demonstrate the regulation of the cellular microenvironment through controlled interactions with specific surface modifications or coatings, which may instruct cell behavior. These systems differ from cells seeded onto biomaterial scaffolds or mixed into hydrogels, as the cells are allowed to interact heavily with one another in addition to interacting with the biomaterial surface, and the degree and type of cell-material interaction can be regulated by the presentation of the surface modification and the density of microspheres present in the cellular constructs.

Conclusions

Although the need for a successful engineering approach to hyaline cartilage regeneration is apparent, current systems

have failed to achieve the complete restoration of damaged or degenerating cartilage. However, the use of incorporated microsphere elements has shown promise in terms of improving the *in vitro* and *in vivo* properties of engineered neocartilage and has imparted additional control over the microenvironment presented to cells within engineered tissue constructs, which may help to counteract common difficulties in cartilage tissue engineering. The incorporation of microspheres into HD cell culture systems has been shown to improve different aspects of cartilage tissue formation in several diverse systems. Beyond the use of microspheres as matrix-enhancing spacers, which has been demonstrated to improve ECM production and structural organization of engineered cartilage tissues, the inclusion of cell-carrying microspheres in suspension culture has the additional capacity to enhance cell proliferation and promote the formation of highly cellular 3D aggregate structures by combining multiple cell-carrying microspheres. The addition of growth factors and surface modifications to microspheres further enhances the capacity of the microspheres within HD constructs to provide specific soluble and insoluble signaling to the cells with the potential for spatial and temporal control over their delivery in the cell microenvironment. Growth factor-loaded microspheres have the capacity to enhance both chondrogenic induction and the phenotypic maintenance of stem cells through controlled growth factor release. These drug-releasing microspheres have the capability not only to overcome diffusion limitations posed by traditional culture techniques, but also to lend tailorable release kinetics for temporal control of differentiation and extended maintenance of differentiation state *in vivo*. Surface-active microspheres have the potential to mimic elements of the ECM components of native cartilage, and may enhance cell adhesion, proliferation, and chondrogenic differentiation within HD culture systems. Through all of these mechanisms, the incorporation of bioactive microspheres into densely cellular engineered cartilage constructs may enhance microenvironmental regulation of cell behavior as well as ultimate composition, organization, and functional properties of the resultant tissues.

The diverse systems described here represent a shift from the more traditional top-down (predominantly scaffold-based) tissue engineering approach, which typically involves seeding cells in or on a biomaterial scaffold with bioactive factors and/or mechanical stimulation to regulate cell behavior and induce ECM formation⁸² to bottom-up approaches that rely on the assembly of micron-scale structural elements as the basis for providing defined signals to drive the formation of tissues that can be scaled up to create larger tissues.⁸³ The cartilage tissue engineering systems described here are examples of the bottom-up (predominantly cell-based) approach, using cells and bioactive polymer microspheres as building blocks to guide the production of articular cartilage. Specific microsphere formulations are designed to provide chondrogenic microenvironmental cues to the cells, instructing cell behavior and neocartilage tissue growth. In addition to the field of cartilage tissue engineering, microsphere-incorporated cell systems have also been pursued for neural tissue engineering,⁸⁴ bone tissue engineering,⁸⁵ and embryonic development models.⁸⁶ The cell-based assembly of 3D structures utilizing microspheres as microenvironmental regulators represents an exciting avenue for the future of regenerative medicine.

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Disclosure Statement

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