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Original Article

A Newly Developed Chemically Crosslinked Dextran–Poly(Ethylene Glycol) Hydrogel for Cartilage Tissue Engineering

Jojanneke M. Jukes, M.Sc.,[1] Leonardus J. van der Aa, M.Sc.,[2] Christine Hiemstra, Ph.D.,[2] Theun van Veen, B.Sc.,[1] Pieter J. Dijkstra, Ph.D.,[2,3] Zhiyuan Zhong, Ph.D.,[3] Jan Feijen, Ph.D.,[2] Clemens A. van Blitterswijk, Ph.D.,[1] and Jan de Boer, Ph.D.[1]

Cartilage tissue engineering, in which chondrogenic cells are combined with a scaffold, is a cell-based approach to regenerate damaged cartilage. Various scaffold materials have been investigated, among which are hydrogels. Previously, we have developed dextran-based hydrogels that form under physiological conditions via a Michael-type addition reaction. Hydrogels can be formed *in situ* by mixing a thiol-functionalized dextran with a tetra-acrylated star poly(ethylene glycol) solution. In this article we describe how the degradation time of dextran–poly(ethylene glycol) hydrogels can be varied from 3 to 7 weeks by changing the degree of substitution of thiol groups on dextran. The degradation times increased slightly after encapsulation of chondrocytes in the gels. The effect of the gelation reaction on cell viability and cartilage formation in the hydrogels was investigated. Chondrocytes or embryonic stem cells were mixed in the aqueous dextran solution, and we confirmed that the cells survived gelation. After a 3-week culturing period, chondrocytes and embryonic stem cell–derived embryoid bodies were still viable and both cell types produced cartilaginous tissue. Our data demonstrate the potential of dextran hydrogels for cartilage tissue engineering strategies.

Introduction

Because of the poor self-healing capacity of cartilage, surgical intervention is generally required when the tissue is damaged or diseased. Some techniques rely on the formation of fibrocartilage, such as microfracturing or subchondral bone drilling; other techniques use grafts to replace the damaged cartilage, such as mosaicplasty. However, the fibrocartilage has poor mechanical properties and only results in temporary relief and there is a limited availability of chondral autografts.[1,2] Cartilage tissue engineering is a cell-based therapy aimed at regenerating the damaged articular cartilage. Chondrocytes can be combined with a scaffold material, such as a hydrogel, to achieve high cell density and homogeneous seeding and to retain the cells in the defect.

Hydrogels are hydrated networks of crosslinked hydrophilic polymers. Due to their high water content, many hydrogels are compatible with cells and proteins. Cells can be combined with the hydrogel precursors before gelation, and functional groups or growth factors, such as transforming growth factor,[3,4] can be incorporated into the hydrogel to enhance tissue formation. A wide variety of hydrogels based on natural materials and synthetic polymers[5–9] have been developed and studied in recent years.

The aqueous polymer solution can be turned into a gel by physical or chemical crosslinks.[10] The noncovalent bonds of physically crosslinked hydrogels, for example, in stereocomplexed hydrogels,[11] result in mechanically weak hydrogels. The physical interactions are reversible, resulting in disruption of the gel upon a change in, for example, temperature or pH. An advantage is that these gels can generally be formed under mild conditions. Chemical crosslinking results in more stable hydrogels due to the covalent bonds formed. The properties of chemically crosslinked hydrogels can be varied by the amount of crosslinks introduced and the hydrophilic–hydrophobic ratio. Further customization can be achieved by varying the concentration of polymer and the polymer length. Reactive crosslinkers or initiators, or crosslinking conditions can be toxic for included cells or may lead to modification of biological compounds. Examples of chemical crosslinking methods include photopolymerization of

Departments of [1]Tissue Regeneration and [2]Polymer Chemistry and Biomaterials, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Enschede, The Netherlands.
[3]Biomedical Polymers Laboratory and Jiangsu Key Laboratory of Organic Chemistry, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou, P.R. China.

acrylated polymers by UV or visible light[12] and redox polymerization.[13] Recently, more mild conditions for chemical crosslinking have been developed, such as the Michael reaction.[14–17] The addition of a nucleophile to an α,β-unsaturated carbonyl compound, a type of conjugate addition, results in the formation of covalent bonds. Using Michael-type addition, we have developed dextran hydrogels.[18,19]

Dextran is a bacterial-derived polysaccharide that is commercially available. Dextran has been used as a blood plasma expander, and dextran hydrogels have been investigated for drug delivery applications.[20] Dextran-based hydrogels are highly hydrophilic and biocompatible. Degradation products can be excreted through the kidneys as long as the molecular weight of the original dextran components is below the filtration threshold of the kidney. The hydroxyl groups of dextran allow the introduction of functional groups by conjugation.[21,22] When dextran is functionalized with reactive groups such as thiols, chemically crosslinked hydrogels can be formed via Michael-type addition between thiols and polymers containing acrylate or vinyl sulfone groups. The reaction is catalyzed by a weak base, which is present under physiological conditions. The precursor solutions can be injected at the defect site and the hydrogel will form *in situ*. The crosslinking reaction is self-selective: the polymer components react with each other, and side reactions with proteins are minimal.[14]

We developed a range of dextran hydrogels with various characteristics.[18,19,23] Based on gelation and degradation time, we decided to investigate hydrogels of dextran functionalized with thiol (Dex-SH) crosslinked with tetra-acrylated star poly(ethylene glycol) (PEG-4-Acr) for cartilage tissue engineering using both chondrocytes and stem cells, and compared the results with agarose gels.

Materials and Methods

Bovine chondrocyte culture

Articular cartilage was harvested from the femoral patellar groove of a freshly slaughtered calve and dissected into 1-mm pieces. Chondrocytes were isolated via overnight digestion in 0.15% type II collagenase (Worthington Biochemical, Lakewood, NJ). Freshly isolated chondrocytes were encapsulated in the hydrogels and cultured in a chondrocyte medium, which was composed of Dulbecco's modified Eagle's medium (DMEM)–high glucose (Invitrogen, Carlsbad, CA), 10 mM HEPES (Invitrogen), 10% fetal bovine serum (FBS; Cambrex, Walkersville, MD), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 0.2 mM ascorbic acid 2-phosphate (Sigma, St. Louis, MO), 0.1 mM nonessential amino acids (NEAA; Sigma), and 0.4 mM proline (Sigma).

Mouse embryonic stem cells culture

Mouse embryonic stem cell (ESC) line IB10 was plated at a density of 5000–10,000 cells/cm^2 on gelatin-coated tissue culture flasks. Mouse ESCs were cultured in a 50% mouse ESC proliferation medium consisting of DMEM (Biowhittaker, Basel, Switzerland) containing 4.5 mg/mL D-glucose, 10% FBS (selected batch for mouse ESC culture; Greiner, Kremsmünster, Austria), 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine (Invitrogen), 0.1 mM NEAA, and 50% of a Buffalo rat liver cell–conditioned mouse ESC proliferation medium.[24] Before use, 1000 U/mL leukemia inhibitory factor (Esgro; Chemicon International, Billerica, MA) and 50 μM 2-mercaptoethanol (Invitrogen) were added to the medium. Cells were grown at 37°C in a humidified 5% CO_2 incubator and passaged with 0.05% trypsin–ethylenediaminetetraacetic acid (EDTA) (Invitrogen) before reaching confluence.

A common first step in differentiation protocols of ESCs is the formation of embryoid bodies (EBs). EBs formed spontaneously when mouse ESCs were cultured in suspension in nontissue culture-treated six-well plates (Greiner) at 20,000 cells/cm^2 in the 4 mL EB medium consisting of DMEM, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine, 0.1 mM NEAA, and 50 μM 2-mercaptoethanol for 4 days. To obtain a single-cell suspension, the free-floating EBs were dissociated with 0.25% trypsin–EDTA. ESC-derived EB cells and EBs were used for differentiation experiments.

Further differentiation of stem cells into the chondrogenic lineage was performed in the chondrogenic medium consisting of DMEM–high glucose (Invitrogen), 100 nM dexamethasone (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.2 mM ascorbic acid 2-phosphate, insulin-transferrin-selenite (ITS+1; Sigma), 100 μg/mL sodium pyruvate (Sigma), 40 μg/mL proline, and 10 ng/mL transforming growth factor-β3 (Biovision, Mountain View, CA).[25,26]

Gel preparation

Dex-SH, based on dextran (31 kDa; Fluka, Buchs, Switzerland), and the crosslinker PEG-4-Acr, based on PEG-4 (Mw = 2000; Nektar Therapeutics, Huntsville, AL), were synthesized as described previously.[18] The degree of substitution (DS, defined as the number of substituents per 100 anhydroglucosidic rings of dextran) was either 16 or 22 (the resulting hydrogels are denoted as Dex16-PEG and Dex22-PEG). We prepared gels with a final polymer concentration of 10 w/v% (total dry weight of both dextran and PEG per volume of buffer). Dextran hydrogels were formed via Michael-type addition between Dex-SH and PEG-4-Acr in phosphate-buffered saline (PBS) at pH 7.2–7.4 at room temperature (Fig. 1). The molar ratio of thiol to unsaturated groups was kept at 1.1, to compensate for thiol groups that have formed disulfide bonds upon exposure to air and to ensure complete reaction with the available acrylate groups. In addition, Dex-SH was weighed and dissolved in PBS under nitrogen flow to reduce contact with O_2, which would result in a lower effective concentration of free thiol groups. PEG-4-Acr can crosslink under the influence of light or O_2 and was kept in the dark and also weighed under nitrogen flow until mixing of the gel components.

Cell encapsulation

To encapsulate cells in the gel, chondrocytes or EB cells were washed with PBS, and the pelleted cells were subsequently resuspended in the Dex-SH solution. A 96-well plate was used as a mold to prepare gels of 150 μL, with a diameter of 6 mm and height of 5 mm. Aliquots of the Dex-SH solution, with or without cells, were transferred to the mold, and aliquots of a PEG-4-Acr solution were added and mixed

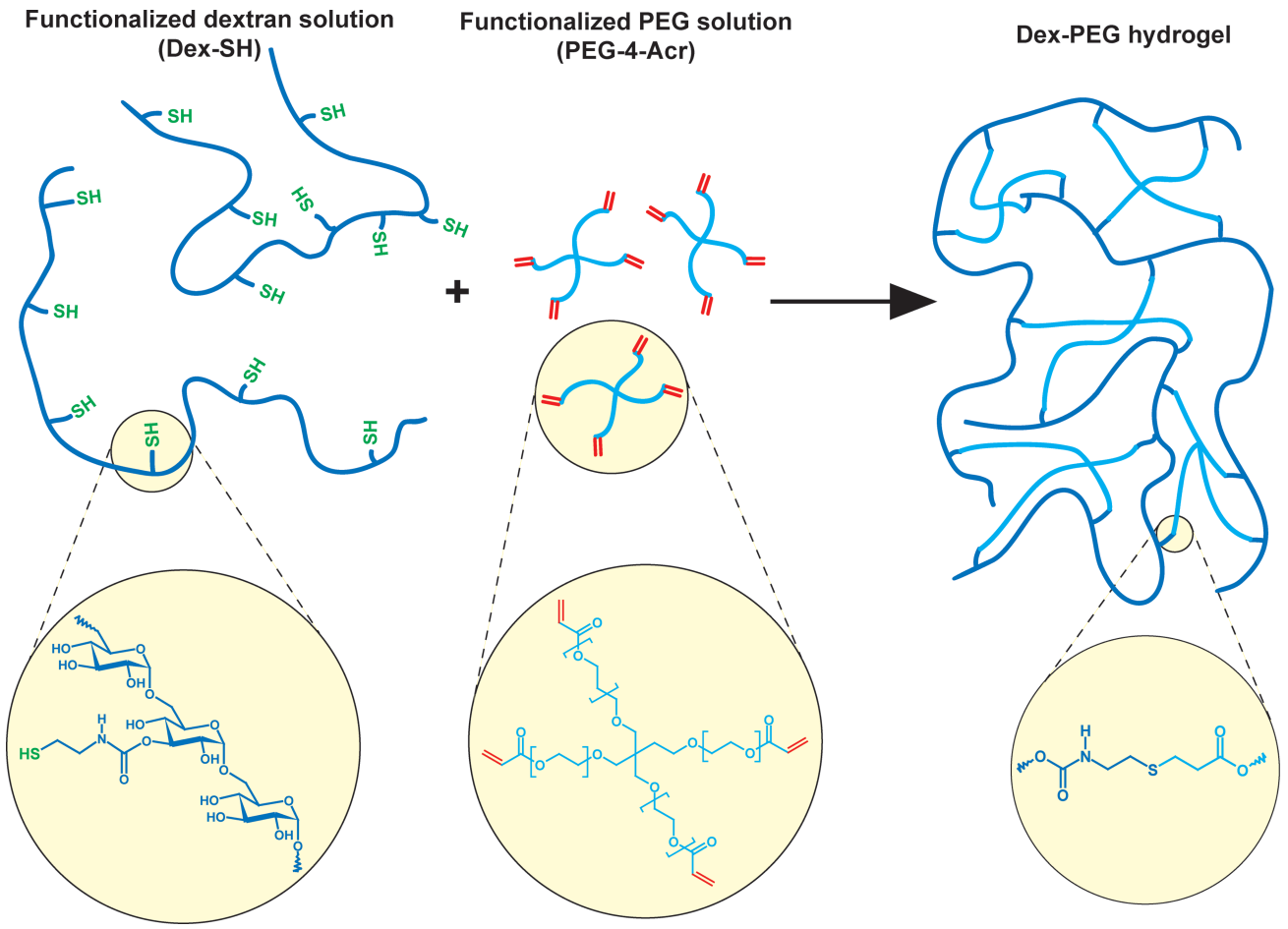

FIG. 1. Dex-PEG hydrogel formation. Schematic representation of *in situ* hydrogel formation by Michael-type addition between aqueous solutions of dextran functionalized with thiol and tetra-acrylated star poly(ethylene glycol). Dex-PEG, dextran-poly(ethylene glycol). Color images available online at www.liebertonline.com/ten.

thoroughly by pipetting. The final chondrocyte or EB cell concentration was 5 million cells/mL. Intact EBs, with a cell number equivalent to EB cells, were washed with PBS and encapsulated in the hydrogel as described for chondrocytes and EB cells. After gelation and hardening for an additional 5 min, gels were transferred to a nonadhering 25-well plate using a spatula. Three milliliters of chondrocyte or chondrogenic medium was added and refreshed twice a week. The plates were placed in a CO_2 incubator at 37°C. As a control, agarose gels were prepared by mixing 0.5% agarose (Sigma) with PBS. Chondrocytes could be mixed in the agarose solution that was cooled down from 40°C before forming a gel at 37°C.

Swelling tests

Dex16-PEG and Dex22-PEG gels, both with and without chondrocytes, were compared in a swelling test. The swelling experiment was performed in triplicate. The hydrogels were removed from the medium and weighed after 0, 5, 7, 11, 14, 17, and 21 days. The swelling ratio of the gel was calculated by dividing the weight of the swollen hydrogel after exposure to medium (W_t) by the initial hydrogel weight after preparation (W_0): swelling ratio $= W_t/W_0$.

Cell viability assay

A live/dead assay (Molecular Probes, Carlsbad, CA) was used to analyze cell viability according to manufacturer's specifications. Briefly, 1-mm-thick sections were cut from the center of the hydrogels and incubated in PBS containing 6 μM ethidium homodimer-1 and 2 μM calcein AM for 30 min at 37°C. Sections were immediately examined in an inverted fluorescent microscope (Nikon Eclipse E400, Nikon, Melville, NY) using an FITC Texas Red filter. Calcein AM is enzymatically converted, producing green fluorescence in living cells. Ethidium homodimer-1 is able to enter cells with damaged membranes and bind to nucleic acids, thereby producing red fluorescence in dead cells.

Histology

Safranin-O staining was used to analyze tissue morphology and glycosaminoglycan (GAG) expression. Gels were washed with PBS and fixed overnight in 4% paraformaldehyde (Merck, Darmstadt, Germany) and dehydrated using sequential ethanol series. Hydrogels were embedded in glycol methacrylate (Merck) and cut using a microtome to yield 5-μm-thick sections. Sections were stained with

hematoxylin (Sigma), and fast green (Merck) to observe cells/cell nuclei (blue staining) and Safranin-O (Sigma), which stained sulfated extracellular GAGs pink.

Quantitative GAG and DNA assay

Hydrogels for quantitative analysis of GAG expression and cell number were washed with PBS and frozen at −80°C. Subsequently, the contents of the gels were digested with 1 mg/mL proteinase K (Sigma) in Tris/EDTA buffer (pH 7.6) containing 185 mg/mL iodoacetamide and 1 mg/mL pepstatin A (Sigma) for >16 h at 56°C. GAG content was spectrophotometrically determined with 9-dimethylmethylene blue chloride (Sigma) staining in phosphate buffered solution containing EDTA (PBE) buffer (14.2 g/L Na_2HPO_4 and 3.72 g/L Na_2EDTA, pH 6.5) with a microplate reader (Bio-Tek instruments, Richmond, VA) at an absorbance of 520 nm. Values were corrected for background by staining of empty gels. Cell numbers were determined via quantification of total DNA with a CyQuant DNA kit according to the manufacturer's description (Molecular Probes) using a fluorescent plate reader (Perkin Elmer, Boston, MA). A cell number standard curve was used to convert sample fluorescence values into numbers of cell/gel. Data ($n = 6$, measured in duplicate) are expressed as mean ± standard deviation. Statistical significance was determined by one-way analysis of variance with SPSS 14.0 software (Chicago, IL).

Results

Gel formation and cell encapsulation

We investigated the potential of dextran–poly(ethylene glycol) (Dex-PEG) hydrogels for cartilage tissue engineering. To achieve homogeneous seeding in the hydrogel, mixing the cells into the polymer solution before gelation is a prerequisite. In a previous experiment, gelation times of 50 and 22 s were determined for Dex16-PEG and Dex22-PEG hydrogels, respectively (data not shown). These gelation times might be too fast to add cells after mixing the two gel components. Therefore, we decided to mix the chondrocytes or ESC-derived EB cells or intact EBs in the Dex-SH solution before adding the crosslinker PEG-4-Acr. Gels formed homogeneously within 1 min and the cell distribution was homogeneous. The presence of cells did not seem to affect the gelation process.

Swelling and degradation of Dex-PEG hydrogels

Dex-PEG gels are degradable under physiological conditions through hydrolysis of the ester bonds between the thioether and PEG. Upon hydrolysis, the gels swell, until the hydrogel network disintegrates and all the degradation products finally dissolve (Fig. 2A). The swelling ratio W_t/W_0 was determined by weighing the gels at various time points. The time when no gel was left was taken as the degradation time. Figure 2B shows the swelling profiles of the Dex16-PEG and Dex22-PEG gels, both with and without cells. The gels could swell to approximately 3.5 times their original weight (W_0) and the diameter increased from 6 mm to almost 2 cm. Next, the gels dissolved completely. The gels with a higher DS had an increased degradation time. The Dex16-PEG gels degraded in approximately 17 to 22 days. The Dex22-PEG gels degraded slower (Fig. 2B), and all gels, both with and without cells, were still present after 21 days. Dex22-PEG gels with cells gradually swelled for more than 7 weeks, before they finally become a solution (data not shown). In all experiments, it was observed that the presence of cells in the gel resulted in slower swelling and degradation (Fig. 2B). In the 21-day period, no signs of swelling or degradation were observed for the agarose samples.

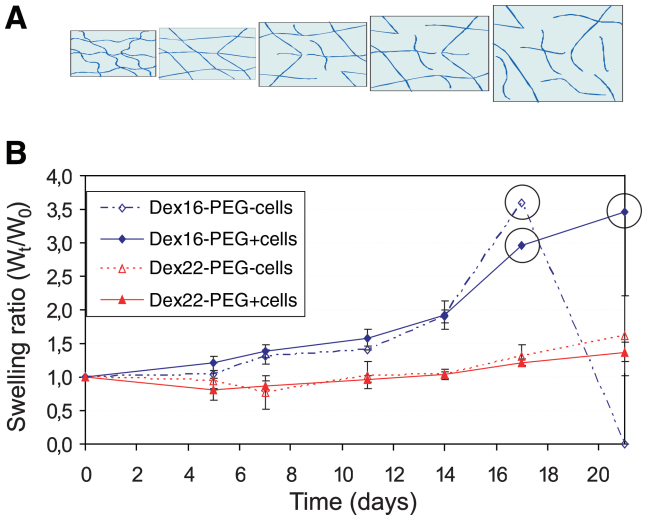

FIG. 2. Swelling profiles of Dex16-PEG and Dex22-PEG hydrogels. (**A**) Schematic representation of the swelling and degradation of the hydrogel. (**B**) Hydrogels with two different degrees of substitution were prepared with and without chondrocytes. Swelling ratios (W_t/W_0) are shown as mean ± SD ($n = 3$). A circle indicates that one or more of the three samples had dissolved completely. Therefore, error bars were omitted for these samples. SD, standard deviation. Color images available online at www.liebertonline.com/ten.

Viability of chondrocytes in the Dex-PEG hydrogel

We investigated whether cells would survive the gelation reaction and whether, in time, sufficient nutrients would diffuse into the gels to maintain the viability of the cells in the center of the gels. The viability of the bovine chondrocytes after 1 h and 1, 2, 7, and 21 days was analyzed. As a control, we encapsulated chondrocytes into a 0.5% agarose gel. The viability of chondrocytes in both Dex16-PEG and Dex22-PEG gels remained above 95% at all time points, similar to cells encapsulated in agarose gels (Fig. 3). Cells were homogeneously distributed in the hydrogels. In time, the Dex-PEG gels took up water and swelled. This resulted in more distance between the chondrocytes, especially observed at day 21 with the Dex16-PEG gel, which was reaching its maximum size, before it would dissolve (Fig. 3). Thus, bovine chondrocytes survived both gelation and prolonged culture times in the Dex-PEG hydrogels.

Cartilage formation by chondrocytes in Dex-PEG hydrogels

After 21 days, proliferation of chondrocytes was observed in the agarose gels, but not in the Dex-PEG hydrogels. Multicellular aggregates were observed in the agarose samples, whereas chondrocytes are still mainly distributed as single cells in the Dex-PEG gels (Fig. 4A). This was con-

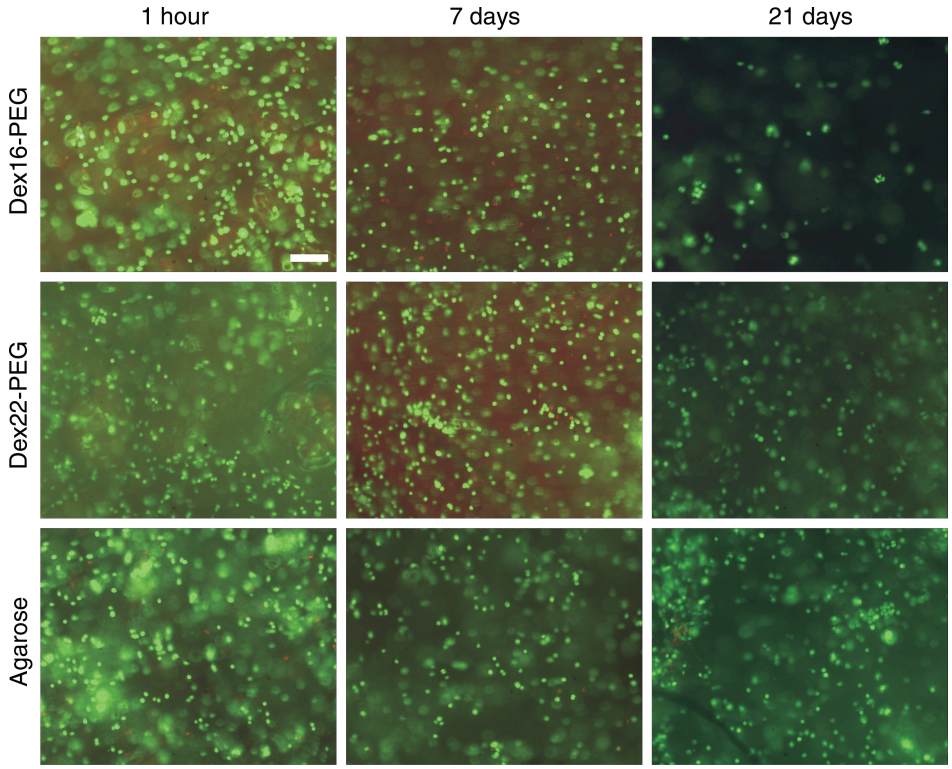

FIG. 3. Viability of bovine chondrocytes encapsulated in Dex-PEG hydrogels. The viability of chondrocytes in the hydrogels was measured in time, and compared with the viability of the cells in agarose. Cells are homogeneously distributed in the gels. After 21 days, chondrocytes in the Dex16-PEG gel are more dispersed because of swelling. Green cells indicate viable cells; red cells indicate dead cells. Scale bar represents 100 μm; all pictures were taken at the same magnification (100×). Color images available online at www.liebertonline.com/ten.

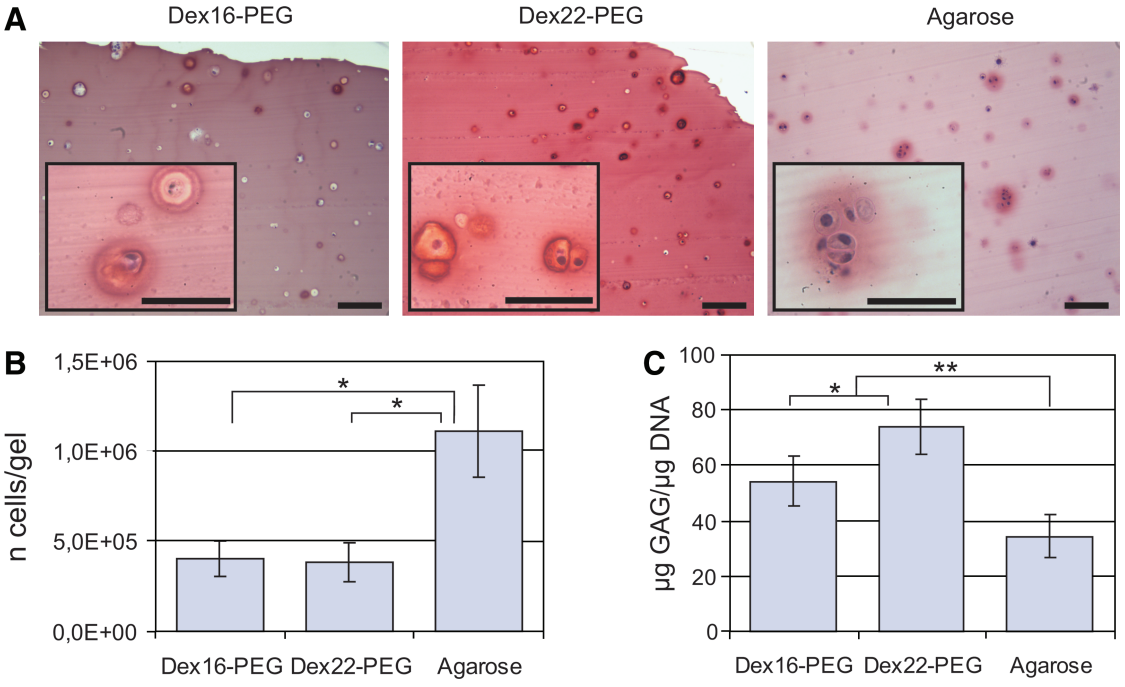

FIG. 4. GAG production by bovine chondrocytes in Dex-PEG hydrogels and agarose. (**A**) Chondrocytes in the three gels (scale bar represents 100 μm). The insets show higher magnification images (scale bar represents 50 μm) of multicellular aggregates in the agarose gels. GAG production was analyzed by safranin-O staining, which stains GAGs surrounding the cells pink. Note that the hydrogels display background staining, but in a different shade of pink. (**B**) Total chondrocyte number in the hydrogel constructs after 21 days. Data are shown as mean ± SD ($n = 6$), *$p < 0.001$. (**C**) Quantitative GAG formation by bovine chondrocytes in the three gels, normalized to DNA (μg GAG/μg DNA). Data are shown as mean ± SD ($n = 6$); *$p < 0.05$ and **$p < 0.01$. GAG, glycosaminoglycan. Color images available online at www.liebertonline.com/ten.

firmed by a significant difference in the amount of DNA after 21 days. Two to three times more DNA was measured in the agarose samples than in both Dex-PEG gels (Fig. 4B).

We analyzed GAG production both qualitatively and quantitatively as an indication of the formation of cartilage-like tissue by the chondrocytes encapsulated in the hydrogels. Chondrocytes were surrounded by a ring of positively stained GAGs, which diffused into the hydrogel (Fig. 4A). During histological processing, the hydrogels shrunk to their original size. As such, we did not observe the spacing of cells as seen in the viability assay samples and the GAG distribution as shown in Figure 4A might not reflect the distribution in the swollen hydrogels. The highest GAG levels were measured in the agarose samples (average of 1.80 μg GAG/Dex16-PEG gel, 2.31 μg GAG/Dex22-PEG gel, and 3.08 μg GAG/agarose gel, data not shown). However, when we corrected GAG production for cell number (GAG/DNA) ($n = 6$), we observed significantly higher levels for both Dex-PEG gels when compared with agarose, and also higher levels for Dex22-PEG than Dex16-PEG (Fig. 4C).

Viability and cartilage formation by mouse ESCs

The potential of Dex-PEG hydrogels for cartilage tissue engineering using another chondrogenic cell type, mouse ESCs, was investigated. We first analyzed whether mouse ESCs are able to attach to the hydrogel. A well plate was coated with the Dex-PEG hydrogel by mixing the precursor solutions and allowing a thin layer to gel on the bottom of the well. When mouse ESCs in the medium were applied on top of the gel, we did not observe any cell attachment (data not shown). Above the gel, the mouse ESCs formed EBs, as also seen when cultured on nonadhering culture plastic. The thin layer of hydrogel degraded fast, and the degradation products in the medium did not interfere with the formation of EBs (data not shown).

We have previously demonstrated that EB formation is an essential step for successful chondrogenic differentiation of mouse ESCs (unpublished data). Therefore, the next experiments were performed with mouse ESC–derived EB cells or intact EBs. First, the viability of mouse EB cells in the Dex-PEG hydrogel was investigated. Mouse EB cells were encapsulated into the hydrogels using a similar approach as described above for bovine chondrocytes. Two hours after encapsulation, the EB cells were still viable. Within 1 day, the viability of the EB cells decreased rapidly (Fig. 5A), similar to previous results with mouse ESC–derived EB cells in all other gels we tested so far, such as agarose, alginate, Matrigel, and Puramatrix.[27] The viability of intact EBs in the dextran hydrogels was also analyzed. The EBs displayed better survival in the Dex-PEG hydrogels than single EB cells (Fig. 5A), as also seen in other hydrogels analyzed.[27] We also analyzed the formation of cartilage tissue by the EBs. In this experiment, during the 21 days of observation, the Dex16-PEG hydrogels with encapsulated EBs degraded slightly faster than those with encapsulated chondrocytes. Therefore, we analyzed cartilage formation in both Dex-PEG gels after 16 days rather than 21 days. We observed some cartilaginous tissue formation by the EBs, both in the Dex16-PEG and Dex22-PEG gels (Fig. 5B), indicating that chondrogenic cells other than chondrocytes could also form cartilage tissue in the Dex-PEG hydrogel.

Discussion

In this article, the potential of a newly developed dextran-based hydrogel for cartilage tissue engineering is demonstrated. Chondrocytes or ESC-derived EBs survived encapsulation and incubation over several weeks, and formed cartilaginous tissue in the Dex-PEG hydrogel.

Dex-PEG hydrogels, prepared with Dex-SH and PEG-4-Acr as crosslinker, were chosen based on their gelation and degradation time. The gelation time should not be too short to allow homogeneous mixing of cells and gelation. On the other hand, too long gelation times would result in settling of the cells at the bottom of the gels, cell death due to a lack of medium nutrients and longer exposure to reactive groups, and leakage out of the defect site when administered *in situ*. The Dex-PEG gels formed within 1 min, with homogeneous cell or EB distribution in the hydrogel and homogeneous gelation.

A prerequisite of our design was that the gels had to be stable for at least 3 weeks, to allow less committed stem cells to differentiate into the chondrogenic lineage and to allow the formation of cartilage extracellular matrix before the gel degrades. We could influence the swelling profile and degradation time of the Dex-PEG hydrogel by changing the DS of dextran, resulting in degradation times of 3 and 7 weeks for Dex16-PEG and Dex22-PEG, respectively. Previous studies have shown that further fine-tuning of the mechanical and degradation properties could be achieved by varying the molecular weight of the polymer, the concentration of polymer, or choosing another crosslinker.[18,19] This demonstrates the versatility of the dextran hydrogels prepared via Michael-type addition, and it can ultimately lead toward controlled degradation, allowing the hydrogel to be replaced by newly formed tissue. The degradation times of 9 and 17 weeks for the Dex16-PEG and Dex22-PEG hydrogels, respectively,[18] could not be compared to the results presented in this article, because of a different experimental setup.

The cells in the hydrogel seem to stabilize the gel, and the degradation time increases accordingly. Whether there is a correlation between cell density and degradation time, the type of cells or cell aggregates encapsulated and degradation time or the formed extracellular matrix and degradation time has not been determined yet. It has been reported that serum proteins can influence the degradation behavior of hydrogels.[28] Further investigation about the influence of serum-containing chondrocyte medium and serum-free chondrogenic medium on the degradation profiles of the dextran-based hydrogels is required. In addition, the *in vivo* degradation profile of these hydrogels has to be further investigated. The surrounding tissue and confined space in the defect site will be important factors that influence the hydrogel stability and its potential application in tissue engineering.

In situ forming hydrogels are highly interesting, as the cell-laden hydrogels can be injected into the defect site by a minimally invasive surgical procedure.[9] The hydrogel can be designed for the desired application, for example, articular chondrocyte transplantation.[29] The Dex-PEG hydrogels are self-assembling[9] and can form *in situ*. With the help of a double syringe with a mixing chamber,[21] the components are mixed upon injection. There is no need for an external stimulus, as seen for photopolymerizable hydrogels. Transdermal photopolymerization resulted in hydrogels,[30] but a

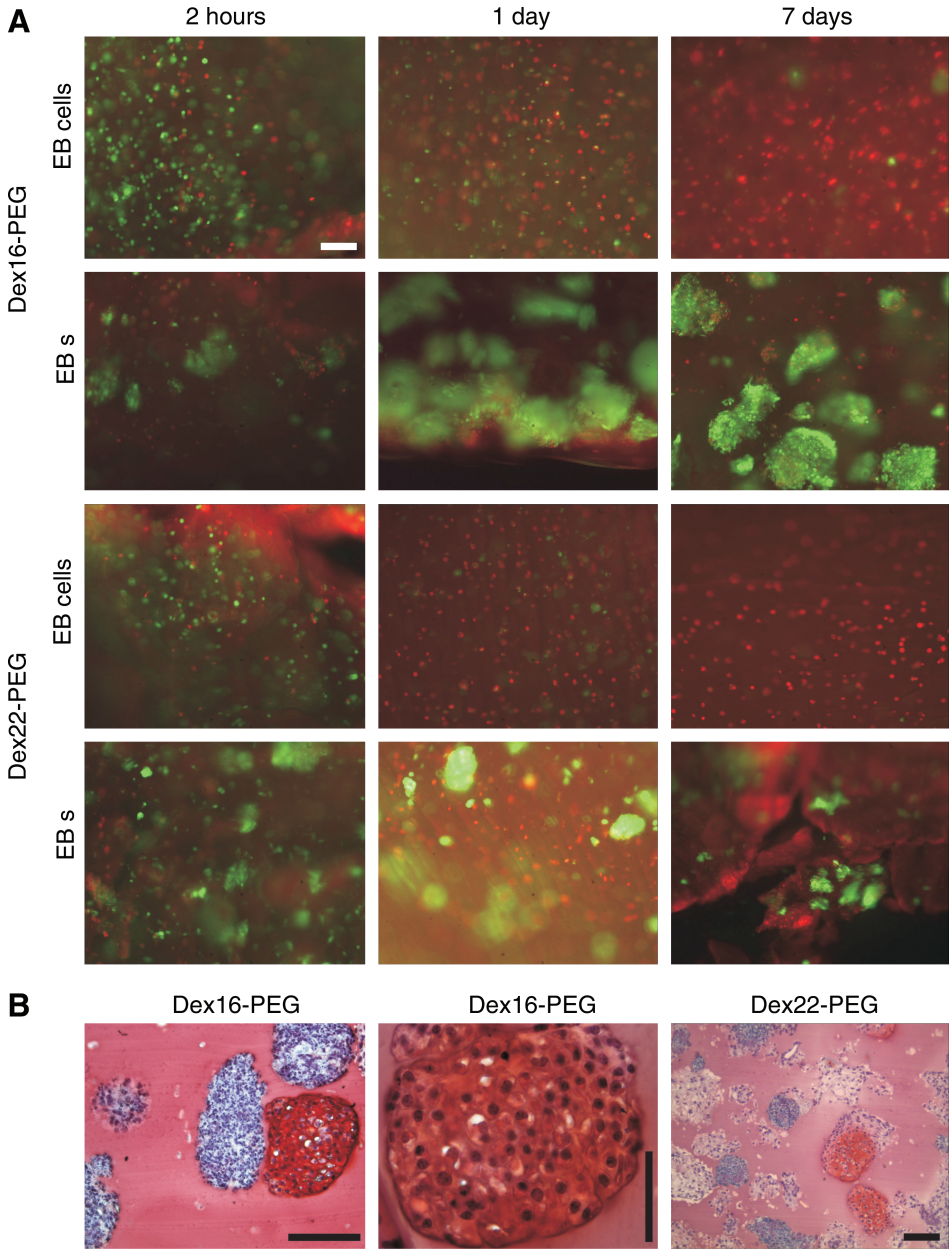

FIG. 5. Viability and chondrogenic differentiation of mouse embryonic stem cell–derived EB cells and EBs in Dex-PEG hydrogels. (**A**) Viability of EB cells and intact EBs after 2 h, 1 day, and 7 days. EB cells did not survive; intact EBs were still viable. Some red background staining of the gel was observed. Scale bar represents 100 μm. (**B**) Chondrogenic differentiation of intact EBs after 16 days of culture in the serum-free chondrogenic medium. Pink staining indicates the formation of GAGs by mouse embryonic stem cell–derived EBs. In the higher magnification image in the middle panel, cells in lacunae surrounded by extracellular matrix positively stained for GAGs, which are characteristics of cartilage, can be recognized. Horizontal scale bar represents 100 μm; vertical scale bar represents 50 μm. EB, embryoid bodies. Color images available online at www.liebertonline.com/ten.

more invasive surgical procedure might be required for polymerization in a cartilage defect in a joint. The Dex-PEG hydrogel can be formed in any desired shape to match the defect's contours, unlike gels as alginate that rely on ionic interactions resulting in inhomogeneous hydrogels, which are limited to beads of a defined size.[31]

Chondrocytes are not the only cell source investigated for cartilage tissue engineering. Stem cells are another cell source that upon differentiation into the chondrogenic lineage can form cartilage tissue. Both ESCs[32–34] and adult stem cells, for example, mesenchymal stem cells isolated from bone marrow[26] or fat tissue derived from liposuction procedures,[35] can form cartilage. We have previously investigated the potential of ESCs for cartilage and bone tissue engineering.[27,36,37] ESCs have been cultured in hydrogels that supported long-term self-renewal,[38] but ESCs have also been successfully differentiated into the chondrogenic lineage in hydrogels.[27,39–41] Therefore, we also investigated chondrogenic differentiation of mouse ESCs in dextran-based hydrogels. The viability of mouse ESC–derived EB cells and intact EBs encapsulated in the Dex-PEG gels was investigated first. Where EB cells survived and differentiated into the chondrogenic lineage when cultured in pellets[27] or on scaffolds,[36] cell death was observed when single EB cells were encapsulated in the hydrogel. However, intact EBs did survive the encapsulation and subsequent incubation period. Cell–cell contact seemed to increase the survival rate of ESC-derived EB cells. Similar results were obtained with all gels we have investigated earlier.[27] Next, the chondrogenic capacity of intact EBs in the Dex-PEG hydrogels was analyzed. Cells in the EBs differentiated into the chondrogenic lineage, as indicated by the presence of large cells in lacunae surrounded by GAGs. The formation of GAGs was mainly observed in the differentiated EBs, which is comparable to the

behavior of EBs in other nonadhering gels such as agarose and alginate.[27] In gels to which cells could adhere, such as Matrigel or Puramatrix, a more intense superficial layer of cartilage was observed, which is not only restricted to the EBs.[27]

The many hydroxyl groups on the dextran backbone allow further functionalization of the Dex-PEG hydrogel. The addition of adhesion molecules might enhance cartilage formation by ESC-derived EBs as reported for ESC-derived mesenchymal-like cells.[42] In contrast, the nonadhesive property of the dextran-based hydrogels may benefit tissue engineering using articular chondrocytes. We previously observed that the integrin-mediated attachment of chondrocytes to polymeric films is associated with dedifferentiation of the chondrocytes and reduced cartilage formation.[43] It is important to functionalize the hydrogel for the appropriate cell type with the appropriate adhesion molecules. When bovine chondrocytes were seeded in the hydrogel, cartilaginous tissue was formed. Even though the total amount of GAG was less than in control agarose samples, the amount of GAG per cell was higher than in agarose. Therefore, we conclude that there is potential for Dex-PEG hydrogels for tissue engineering applications. Future work will include additional biocompatibility tests and *in vivo* performance of the gel and encapsulation of clinically more relevant passage-expanded human chondrocytes from healthy and diseased cartilage.

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Disclosure Statement

No competing financial interests exist.

References

1. Bobic, V., and Noble, J. Articular cartilage—to repair or not to repair. J Bone Joint Surg Br **82,** 165, 2000.
2. Buckwalter, J.A., and Mankin, H.J. Articular cartilage repair and transplantation. Arthritis Rheum **41,** 1331, 1998.
3. Park, H., Temenoff, J.S., Tabata, Y., Caplan, A.I., Raphael, R.M., Jansen, J.A., and Mikos, A.G. Effect of dual growth factor delivery on chondrogenic differentiation of rabbit marrow mesenchymal stem cells encapsulated in injectable hydrogel composites. J Biomed Mater Res A **88,** 889, 2009.
4. Yun, K., and Moon, H.T. Inducing chondrogenic differentiation in injectable hydrogels embedded with rabbit chondrocytes and growth factor for neocartilage formation. J Biosci Bioeng **105,** 122, 2008.
5. Lee, K.Y., and Mooney, D.J. Hydrogels for tissue engineering. Chem Rev **101,** 1869, 2001.
6. Nicodemus, G.D., and Bryant, S.J. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. Tissue Eng Part B Rev **14,** 149, 2008.
7. Vinatier, C., Guicheux, J., Daculsi, G., Layrolle, P., and Weiss, P. Cartilage and bone tissue engineering using hydrogels. Biomed Mater Eng **16,** S107, 2006.
8. Baroli, B. Hydrogels for tissue engineering and delivery of tissue-inducing substances. J Pharm Sci **96,** 2197, 2007.
9. Van Tomme, S.R., Storm, G., and Hennink, W.E. *In situ* gelling hydrogels for pharmaceutical and biomedical applications. Int J Pharm **355,** 1, 2008.
10. Hennink, W.E., and van Nostrum, C.F. Novel crosslinking methods to design hydrogels. Adv Drug Deliv Rev **54,** 13, 2002.
11. Hiemstra, C., Zhong, Z., Li, L., Dijkstra, P.J., and Feijen, J. *In-situ* formation of biodegradable hydrogels by stereocomplexation of PEG-(PLLA)8 and PEG-(PDLA)8 star block copolymers. Biomacromolecules **7,** 2790, 2006.
12. Nguyen, K.T., and West, J.L. Photopolymerizable hydrogels for tissue engineering applications. Biomaterials **23,** 4307, 2002.
13. Temenoff, J.S., Park, H., Jabbari, E., Conway, D.E., Sheffield, T.L., Ambrose, C.G., and Mikos, A.G. Thermally crosslinked oligo(poly(ethylene glycol) fumarate) hydrogels support osteogenic differentiation of encapsulated marrow stromal cells *in vitro*. Biomacromolecules **5,** 5, 2004.
14. Elbert, D.L., Pratt, A.B., Lutolf, M.P., Halstenberg, S., and Hubbell, J.A. Protein delivery from materials formed by self-selective conjugate addition reactions. J Control Release **76,** 11, 2001.
15. Metters, A., and Hubbell, J. Network formation and degradation behavior of hydrogels formed by Michael-type addition reactions. Biomacromolecules **6,** 290, 2005.
16. Lutolf, M.P., and Hubbell, J.A. Synthesis and physicochemical characterization of end-linked poly(ethylene glycol)-co-peptide hydrogels formed by Michael-type addition. Biomacromolecules **4,** 713, 2003.
17. Lutolf, M.P., Tirelli, N., Cerritelli, S., Cavalli, L., and Hubbell, J.A. Systematic modulation of Michael-type reactivity of thiols through the use of charged amino acids. Bioconjug Chem **12,** 1051, 2001.
18. Hiemstra, C., Aa, L.J., Zhong, Z., Dijkstra, P.J., and Feijen, J. Rapidly *in situ*-forming degradable hydrogels from dextran thiols through Michael addition. Biomacromolecules **8,** 1548, 2007.
19. Hiemstra, C., van der Aa, L.J., Zhong, Z.Y., Dijkstra, P.J., and Feijen, J. Novel *in situ* forming, degradable dextran hydrogels by Michael addition chemistry: Synthesis, rheology, and degradation. Macromolecules **40,** 1165, 2007.
20. Van Tomme, S.R., and Hennink, W.E. Biodegradable dextran hydrogels for protein delivery applications. Expert Rev Med Devices **4,** 147, 2007.
21. Jin, R., Hiemstra, C., Zhong, Z., and Feijen, J. Enzyme-mediated fast *in situ* formation of hydrogels from dextran-tyramine conjugates. Biomaterials **28,** 2791, 2007.
22. Van Tomme, S.R., van Steenbergen, M.J., De Smedt, S.C., van Nostrum, C.F., and Hennink, W.E. Self-gelling hydrogels based on oppositely charged dextran microspheres. Biomaterials **26,** 2129, 2005.
23. Hiemstra, C., Zhong, Z., van Steenbergen, M.J., Hennink, W.E., and Feijen, J. Release of model proteins and basic fibroblast growth factor from *in situ* forming degradable dextran hydrogels. J Control Release **122,** 71, 2007.
24. Smith, A.G., and Hooper, M.L. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev Biol **121,** 1, 1987.
25. Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., and Yoo, J.U. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res **238,** 265, 1998.
26. Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. Chondrogenic differenti-

ation of cultured human mesenchymal stem cells from marrow. Tissue Eng **4,** 415, 1998.
27. Jukes, J.M., Moroni, L., van Blitterswijk, C.A., and de Boer, J. Critical Steps toward a tissue-engineered cartilage implant using embryonic stem cells. Tissue Eng Part A **14,** 135, 2008.
28. Martens, P.J., Bryant, S.J., and Anseth, K.S. Tailoring the degradation of hydrogels formed from multivinyl poly(ethylene glycol) and poly(vinyl alcohol) macromers for cartilage tissue engineering. Biomacromolecules **4,** 283, 2003.
29. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med **331,** 889, 1994.
30. Sharma, B., Williams, C.G., Khan, M., Manson, P., and Elisseeff, J.H. *In vivo* chondrogenesis of mesenchymal stem cells in a photopolymerized hydrogel. Plast Reconstr Surg **119,** 112, 2007.
31. Kuo, C.K., and Ma, P.X. Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: part 1. Structure, gelation rate and mechanical properties. Biomaterials **22,** 511, 2001.
32. Evans, M.J., and Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature **292,** 154, 1981.
33. Martin, G.R. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA **8,** 7634, 1981.
34. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. Embryonic stem cell lines derived from human blastocysts. Science **282,** 1145, 1998.
35. Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., and Hedrick, M.H. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng **7,** 211, 2001.
36. Jukes, J.M., Both, S.K., Leusink, A., Sterk, L.M., van Blitterswijk, C.A., and de Boer, J. Endochondral bone tissue engineering using embryonic stem cells. Proc Natl Acad Sci USA **105,** 6840, 2008.
37. Jukes, J.M., Both, S.K., van Blitterswijk, C.A., and de Boer, J. Potential of embryonic stem cells for *in vivo* bone regeneration. Regen Med **3,** 783, 2008.
38. Gerecht, S., Burdick, J.A., Ferreira, L.S., Townsend, S.A., Langer, R., and Vunjak-Novakovic, G. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. Proc Natl Acad Sci USA **104,** 11298, 2007.
39. Hwang, N.S., Kim, M.S., Sampattavanich, S., Baek, J.H., Zhang, Z., and Elisseeff, J. The effects of three dimensional culture and growth factors on the chondrogenic differentiation of murine embryonic stem sells. Stem Cells **24,** 284, 2006.
40. Hwang, N.S., Varghese, S., and Elisseeff, J. Derivation of chondrogenically-committed cells from human embryonic cells for cartilage tissue regeneration. PLoS ONE **3,** e2498, 2008.
41. Tanaka, H., Murphy, C.L., Murphy, C., Kimura, M., Kawai, S., and Polak, J.M. Chondrogenic differentiation of murine embryonic stem cells: effects of culture conditions and dexamethasone. J Cell Biochem **93,** 454, 2004.
42. Hwang, N.S., Varghese, S., Zhang, Z., and Elisseeff, J. Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine-glycine-aspartate-modified hydrogels. Tissue Eng **12,** 2695, 2006.
43. Mahmood, T.A., de Jong, R., Riesle, J., Langer, R., and van Blitterswijk, C.A. Adhesion-mediated signal transduction in human articular chondrocytes: the influence of biomaterial chemistry and tenascin-C. Exp Cell Res **301,** 179, 2004.

Address correspondence to:
Jan de Boer, Ph.D.
Department of Tissue Regeneration
MIRA Institute for Biomedical Technology
and Technical Medicine
University of Twente
Drienerlolaan 5
7522NB Enschede
The Netherlands

E-mail: j.deboer@tnw.utwente.nl

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