

Cell Delivery Using an Injectable and Adhesive Transglutaminase–Gelatin Gel

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In this study, we developed an injectable gelatin–transglutaminase (TGase) gel for cell delivery. The procedure provides a minimally invasive approach to deliver cells into tissue in a manner that improves localization. The results indicate gelatin–TGase to be noncytotoxic and to have adhesive properties that help localize and prevent the scattering of the cells after delivery. The *in situ* crosslinking between gelatin chains and endogenous collagen can create a strong attachment between the gel and tissue extracellular matrix, preventing cells from dissipation. The gelatin–TGase was also shown to maintain the carried cells to be viable and proliferative. Finally, through the adjustment of the enzymatic crosslinker concentration, the release rate of the cells into the surrounding tissue after injection was demonstrated to be controllable.

Introduction

CELL DELIVERY TECHNOLOGY has shown promising results in the repair of lost, damaged, and degenerated tissue,¹ especially in areas of cell-poor environments which require a large number of site-specific cells for repair.^{2,3} Mesenchymal stem cells (MSCs) are used for their pluripotent nature which can convert them into site-specific cells that can repair damaged tissue. With some manipulation of cytokines and growth factors, these pluripotent cells can differentiate toward its proper lineages.^{4,5} Once differentiated, the cells can regulate regeneration through secretion of signaling molecules^{4,6} or by directly participating in the building of tissue.³ MSCs can be deployed to the repair site by injection or by implantation onto a scaffold. The use of injections has become a favorable approach over implantation because of its minimally invasive procedure.

Direct injection of cells causes scattering of the cells.⁷ To localize and concentrate cells at the injection site, different forms of hydrogels have been tested in recent studies with *in situ* gelable hydrogels being the most favorable.^{8–12} *In situ* gelation allows the injectable material to form into any desired shape at the administered repair site and holds the potential to adhere to tissue during gel formation.¹² The subsequent crosslinking of the hydrogel not only prevents dissolution of materials in aqueous environments but also decreases the biodegradation rate *in vivo*, minimizing cell scattering and improving localization.

Methods of *in situ* gelation methods include photopolymerization, gel polymer modification, and enzymatic crosslinking. Photocurable crosslinking methods take advantage of radical polymerization where photosensitive materials are

mixed with initiators such as camphorquinone¹³ and Iragure 2959.^{14,15} Most problems lie with the initiators which have the potential to be toxic to cells.¹⁶ In Hoshikawa *et al.*, although they were able to successfully encapsulate and proliferate chondrocytes with camphorquinone, only 26% of encapsulated cells survived the initial seeding.¹³ The initiator, Iragure 2959, was partially cytocompatible to certain cell types and was toxic toward cells with high proliferation rates.¹⁷ Further, for all photocurable hydrogels, the poor light penetration, especially by ultraviolet,⁸ limits the injection sites to mostly transdermal applications. Modified polymers use no crosslinkers or initiators interspersed in the gel. Instead, these polymers are modified to have acrylates, thiols,³ or aldehyde¹⁰ side chains to make them reactive. Increased cell death has been observed with high concentration of exposed unreacted side chains after gelation.¹⁸ Because cells are usually added during the liquid phase before or during the gelation process, the reagents and the byproducts of cross-linked hydrogels must have no detrimental effect on the cells. Thus, the use of enzymatic crosslinkers with natural polymers can provide *in situ* gelation with a high transmission of viable cells.

The objective of this study was to explore the potential use of a gelatin gel crosslinked with microbial transglutaminase (TGase, EC 2.3.2.13) as an injectable cell delivery vehicle. TGase is a naturally occurring enzyme found in almost all living organisms and in a variety of tissues.^{19,20} It plays a role in a wide variety of cellular function, such as blood clotting and liver detoxification. As a cell adhesion protein, TGase provides the possibility of increasing cell attachment.^{21,22} Its bacterial analogue, microbial TGase, is also known to be innocuous and is widely used in food processing for human

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consumption.^{23,24} TGase functions by covalently binding the ϵ -amino group of a lysine residue and a γ -carboxamide group of glutamine²⁵ to create intramolecular covalent links within and between polymers, thereby creating a lasting structure that will polymerize when injected.

Gelatin was chosen as the TGase substrate based on its biocompatible hydrogel properties.²⁶ Because it is derived from collagen, a common structural protein, gelatin retains most of the amino acid sequence segments of collagen as well as most of the integrin-binding domain sequences.^{27–29} Gelatin's less-ordered structure, when compared with collagen, exposes more integrin-binding domains for cell attachment.³⁰ Many studies have shown that the addition of biological components or controlled modification of a biological component can prolong cell survival and prevent dedifferentiation. Almany and Seliktar¹⁴ combined the synthetic polyethylene glycol (PEG) with fibrinogen to compensate for the lack of bioactive signals in the extracellular matrix which support cell attachment. For polysaccharide gels such as chitosan and alginate, Arg-Gly-Asp (RGD), an integrin-binding domain peptide sequence,³¹ or gelatin¹¹ has been added to promote cell attachment. The TGase enzyme covalently binds the intra- and intermolecular structures, increasing the resulting gel's melting temperature, preventing gelatin's natural tendency to dissolve at 37°C, and enhancing its resistance to protease degradation.²⁴ However, unlike chemical crosslinking, enzymatic crosslinking is site specific, leaving many functional groups unaltered. Barbetta *et al.* found that the gelatin-TGase hydrogel had a better sustainment of hepatocyte phenotypes than a methacrylated gelatin,³² signifying that lower amounts of modifications on the gelatin's functional groups may hinder dedifferentiation.

This study was designed to evaluate an injectable gelatin-TGase gel for cell delivery. We investigated the gel's cytotoxicity and examined its ability to adhere to the surrounding tissue and to prevent dissipation of cells after delivery. To demonstrate feasibility of cell delivery, the gel's controllable cell release was tested *in vivo*.

Materials and Methods

Material and reagents

Microbial TGase (ACTIVA TI; Ajinomoto, Tokyo, Japan) from *Streptomyces mobaraense* was purified using S Sepharose Fast Flow column.³³ Briefly, 3 g of crude TGase was dissolved in buffer A (20 mM phosphate and 2 mM ethylenediaminetetraacetic acid, pH 6.0) and then mixed with 3 mL of preequilibrated S Sepharose FF beads. After incubation at 4°C overnight with occasional vortexing, the protein solution and bead mixture were batch loaded into a column. After washing with 4 volumes of buffer A, TGase was eluted with buffer B (buffer A with 800 mM NaCl). Protein concentration was monitored by the method of Bradford (Bio-Rad, Hercules, CA), utilizing bovine serum albumin as a standard.

Culture media (denoted as D10 medium) were composed of Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L) (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic/antimycotic solution (Mediatech). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Bone marrow stromal cell maintenance

Rat bone marrow stromal cells (MSCs) were harvested from euthanized, 6-week-old Fisher 344 rats (Harlan Labs, San Diego, CA) using the following described methodology.³⁴ Briefly, the femoral midshaft bone marrow was aspirated into DMEM containing penicillin (100 U/mL) and streptomycin (100 mg/mL). Bone marrow cells were collected and plated on 50-mm tissue culture plates. The harvested stromal cells were isolated using adhesion-depletion methods. Nonadherent cells were removed after a media change 3 days later. The MSCs were maintained with D10 medium in 100-mm tissue culture plates (BD Falcon, Franklin Lake, NJ) and subcultured every 2 days.

Gelatin-TGase gel preparation

Gelatin-TGase was prepared by mixing different amounts of gelatin solution (1–10%, bovine skin type B, 225 bloom) and TGase (5.8, 11.7, 23.5, and 47 μ g/mL). Before experimental use, gelatin was incubated at 37°C to obtain a liquid consistency for easier mixing. Gelation times were measured for different combinations of gelatin concentration and TGase. Gelation time is defined as the time needed for the gelatin to transform from semisolid form to solid form when placed in a syringe at room temperature. Because gelatin can gel at room temperature even without TGase to form reversible solid gels by a thermal cooling effect, a confirmation step was added by placing the solidified gel at 37°C for 30 min to verify that the crosslinked gel is irreversible.

For cell encapsulation, the gelatin-TGase cocktail was composed of 10% gelatin, 10 \times phosphate-buffered saline, and TGase in a ratio of 9:1:0.025 (v/v/v). The concentration of TGase was varied depending on the experiment. Gelatin-TGase was incubated at 37°C for 1 h before *in vitro* and *in vivo* studies.

Burst test

A burst test was conducted to determine the adhesion of the gelatin-TGase to the collagen matrix. An in-house-made apparatus was used for this purpose (Fig. 1A). Fresh bovine pericardium (Sierra, Whittier, CA) was used as a collagen matrix. The sheet was measured and trimmed to the diameter of the mouth of the collecting cup. A hole measuring 4.0 mm in diameter was made in the middle of the sheet. Ten percent gelatin mixed with different amounts of TGase (11.7, 23.5, and 47 μ g/mL) was used to create the sealant. About 150 μ L of the gel sealant was applied to fill the hole and was cured at room temperature for 40 min. The filled hole of collagen sheet was aligned to a 4-mm-diameter hole located on the center of the collecting cup cap. The collagen matrix sheet was fixed and held between the water column and collecting cup. The gel sealant was pressurized by adding water gradually to the column. The height of the water at which the sealant failed and the mode of failure were noted. Pressure (mmHg) was determined by height of water (mm) divided by mercury density (13.6 g/mL).

Cell culture on two-dimensional TGase gel

Cell proliferation and attachment studies were conducted by directly overlaying cells on TGase-crosslinked gelatin gel (gelatin-TGase). In each well of a 24-well plate, 200 μ L of gelatin-TGase (gelatin concentration of 1%, 5%, and 10% and

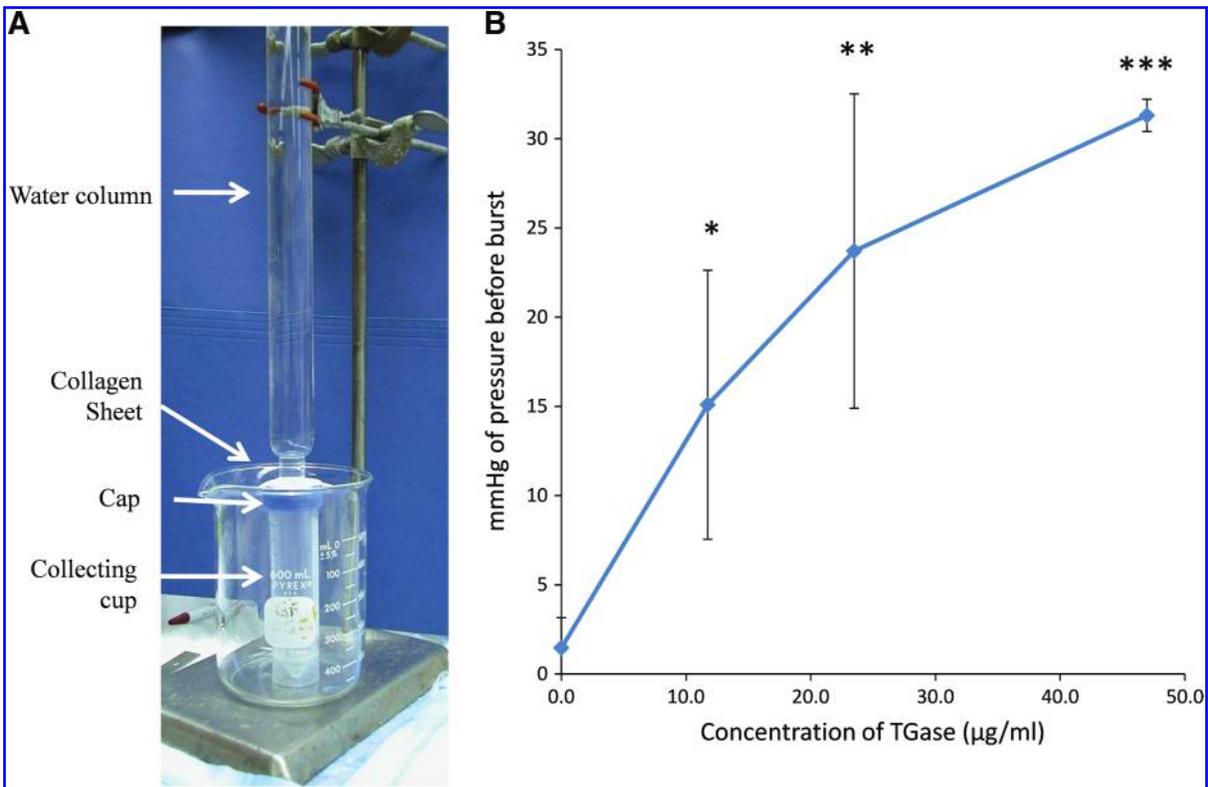


FIG. 1. (A) The burst test apparatus. (B) The resistance to pressure (measured in mmHg) by gelatin–TGase with different concentrations of TGase. Water was incrementally added, 5 mL at a time, before bursting. Water pressure was derived from the height of the water and converted to mmHg. The error bars represent the standard deviation ($n = 4$). Statistical significance: * $p < 0.008$ for 11.7 µg/mL versus gelatin only; ** $p < 0.002$ for 23.5 µg/mL versus gelatin only; *** $p < 0.0001$ for 47 µg/mL versus gelatin only; $p < 0.004$ for 47 µg/mL versus 11.7 µg/mL. TGase, transglutaminase. Color images available online at www.liebertonline.com/ten.

TGase concentration was kept at 23.5 µg/mL) was added to the bottom of the well. After incubating at 37°C for 1 h, the plate was left open in the fume hood for air drying. The wells were rinsed twice with cold phosphate-buffered saline before cell plating. In each well, 5×10^4 of MSCs in 1 mL of D10 medium were seeded. The cells seeded on noncoated plastic surface and 10% gelatin-coated well served as controls. The cell morphology was observed at 1, 3, and 5 days under 10× magnification (Leitz inverted microscope) and the total cell number was counted after trypsinization (0.25%; Mediatech) using a hemocytometer.

Cell culture embedding in three-dimensional TGase gel

MSC morphology and migration rate in/out three-dimensional (3D) matrices (gelatin–TGase) were compared in different gel composites. Cells were detached by trypsin, and cell density was adjusted to 2×10^7 /mL. Cell suspension was mixed with gelatin–TGase cocktail at 1:20 volume ratio. The final cell density was 1×10^6 /mL. An aliquot of 200 µL was loaded to the bottom of the wells of a 24-well plate and incubated at 37°C for 30 min. After forming lattices, 500 µL of 10% fetal bovine serum/DMEM were added on top and incubated at 37°C in 5% CO₂. The medium was changed every 2–3 days. Cell viability was tested by a trypan blue exclusion method and cell morphology was monitored daily up to 10

days. For imaging purposes, green fluorescent protein (GFP)-labeled MSCs were used (provided by Hong Yu, Ph.D., Miami University). Fluorescent images of the 3D cellular interaction within the gel were taken with a Leica microscope (Leica DM-LB2).

For the cell migration study, a 3-mm-radius circular scratch was made on the bottom of each well of the 24-well plate, and 15 µL of the cell/gel mix was deposited onto the middle of the circle. The surface tension properties of the gel created a 3-mm-radius half dome. The gel was set to solidify for 30 min and then supplemented with 1 mL D10. The medium was changed every 3 days. The gel was monitored daily to observe the migration of the cells from the gel to the surrounding plate. The migration distance was measured in millimeters as the distance from the edge of the original gel to the cell front. The mean distance was derived as an average of four measurements at each quadrant of the dome. One set of gels was stained with Diff-Quik (Baxter Scientific Products, Los Angeles, CA) to visualize the cell migration, according to the manufacturer’s protocol. The gels were fixed for 2 min with Diff-Quik fixation solution, and stained for 5 min with Diff-Quik solution I and for 5 min with Diff-Quik solution II.

In vivo cell delivery with 3D gelatin–TGase

Four male 6-week-old Fisher 344 rats (weighing 190–210 g) were used for the experiment according to an approved

protocol by the Institutional Animal Care and Use Committee, University of Southern California. Prior to surgery, the rats were anesthetized with ketamine/xylazine (10:1, w/w). The area of surgery was shaved and disinfected with iodine and ethanol. A 1-cm incision was made above the shoulder. A portion of the deltoid was detached from the acromion to expose the infraspinatus tendon. Measuring about 5 mm from the humeral insertion of the tendon, a full-thickness defect across 50% of the total width of the infraspinatus tendon was made. About 200 μ L gelatin-TGase cocktail mixed with 10^6 cells/mL of GFP-labeled MSCs was injected through a 23-gauge needle onto the defect site. The injection site was identified with a small suture knot. For closure, deltoid muscle was sutured and the skin was subsequently sutured. No activity restrictions were imposed following surgery.

The injected gels and surrounding tissue were harvested at 2 and 7 days postsurgery and fixed in 10% formalin neutral buffer solution (pH 7.4), dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 5 μ m thick with a microtome (Reichert Jung HistoCut 820). Fluorescent micrographs were taken from deparaffinized sections with a fluorescent microscope (Leica DM-LB2). The serial sections from the same paraffin blocks were also stained with hematoxylin and eosin to visualize the cells and extracellular matrix.

Results

Determination of the gelation time

The optimum condition for proper cell delivery was established by assessing the gelation time. Depending on the amount of TGase or the concentration of gelatin, the onset of gelling was verified to be controllable. Decreasing either the concentration of TGase and/or gelatin had the effect of extending the gelling time. Although gelatin and TGase concentration both had effects of extension and onset time, one factor seemed to have a higher impact on the other. Low concentration of gelatin had a higher impact on extending the time window for injections, whereas low concentration of TGase shifted the onset of gelling to a later starting time. A tangible gel-like consistency required at least 10 min of reaction time (Table 1).

Determination of binding strength between gelatin-TGase and collagen

Localization of cell delivery required the adhesion of gelatin-TGase gel to the surrounding tissue. A burst pressure test

was performed to determine and quantify the strength of the adhesion between the gel and collagen substrate. A nonlinear increase in pressure resistance was observed as the concentration of TGase was increased (Fig. 1B). The gelatin sealant without TGase bursts immediately when water pressure increased beyond 2.9 mmHg. Each sealed defect broke at the interface border between the collagen and the solidified gel. Statistical significance of burst pressure was seen between 11.7 μ g/mL gelatin-TGase versus gelatin only ($p < 0.008$), 23.5 μ g/mL gelatin-TGase versus gelatin only ($p < 0.002$), 47 μ g/mL gelatin-TGase versus gelatin only ($p < 0.0001$), and 47 μ g/mL gelatin-TGase versus 11.7 μ g/mL gelatin-TGase ($p < 0.004$).

The breaking at the gel interface suggests that the TGase bonds between the collagen and gelatin were broken; thus calculating the bonding strength at the interface was possible.

The interface bonding strength was defined as the tensile stress at the interface between the gel and collagen matrix. The tensile stress was determined from the derived formula.³⁵

$$\text{Bonding strength} = 3P/4 \times (a/t)^2$$

where P is the pressure in N/m^2 , a is the radius, and t is the thickness of the collagen defect. Bonding strength was calculated from pressure data (Table 2). A high TGase concentration of 47 μ g/mL displayed high bond strength of 318 ± 10.13 kPa, whereas gelatin without TGase bonded with collagen showed a value of 14.67 ± 16.94 kPa.

Determination of cell attachment and viability

A direct stem cell overlay of gelatin and gelatin/TGase substrate was used to study cell behavior and performance. Through the 1, 3, and 5-day time course, distinct difference in MSC behavior was observed. The 10% gelatin/TGase appears to have the highest proliferation rate (Fig. 2A), whereas the 1% gelatin concentration had the lowest. Cell numbers were increased as the gelatin concentration increased. The same trends were observed at days 1 and 3 time points. Statistical differences at day 1 between 10% gelatin-TGase and noncrosslinked 10% gelatin ($p < 0.02$) and between 5% gelatin-TGase and 10% noncrosslinked gelatin ($p < 0.05$) were significant. There was no statistical significance between 1% gelatin concentration with TGase and noncrosslinked 10% gelatin at day 1, suggesting that a higher rate of crosslinking is required for cell attachment. At day 3,

TABLE 1. GELATION TIMES FOR DIFFERENT CONCENTRATIONS OF GELATIN AND TRANSGLUTAMINASE

Gelatin concentration (%)	Gelation time (min)		
	47.0 μ g/mL ^a	23.5 μ g/mL ^a	11.7 μ g/mL ^a
10	10–13	20–25	30–42
7.5	13–20	30–50	
5	75–100	95–120	

Values are represented in minutes from the time the reaction generates a gel-like consistency to the time the gel completely solidifies.

^aConcentration of transglutaminase.

TABLE 2. CALCULATED INTERFACE BONDING STRENGTH BETWEEN COLLAGEN AND GELATIN

10% gelatin mixed with TGase (μ g/mL)	Interface bonding strength (kPa)
47.0	318.08 ± 10.13
23.5	237.03 ± 88.13
11.7	150.92 ± 75.36
0	14.67 ± 16.94

Values show the mean \pm standard deviation of $n = 4$. These values were calculated from the values of the burst test.

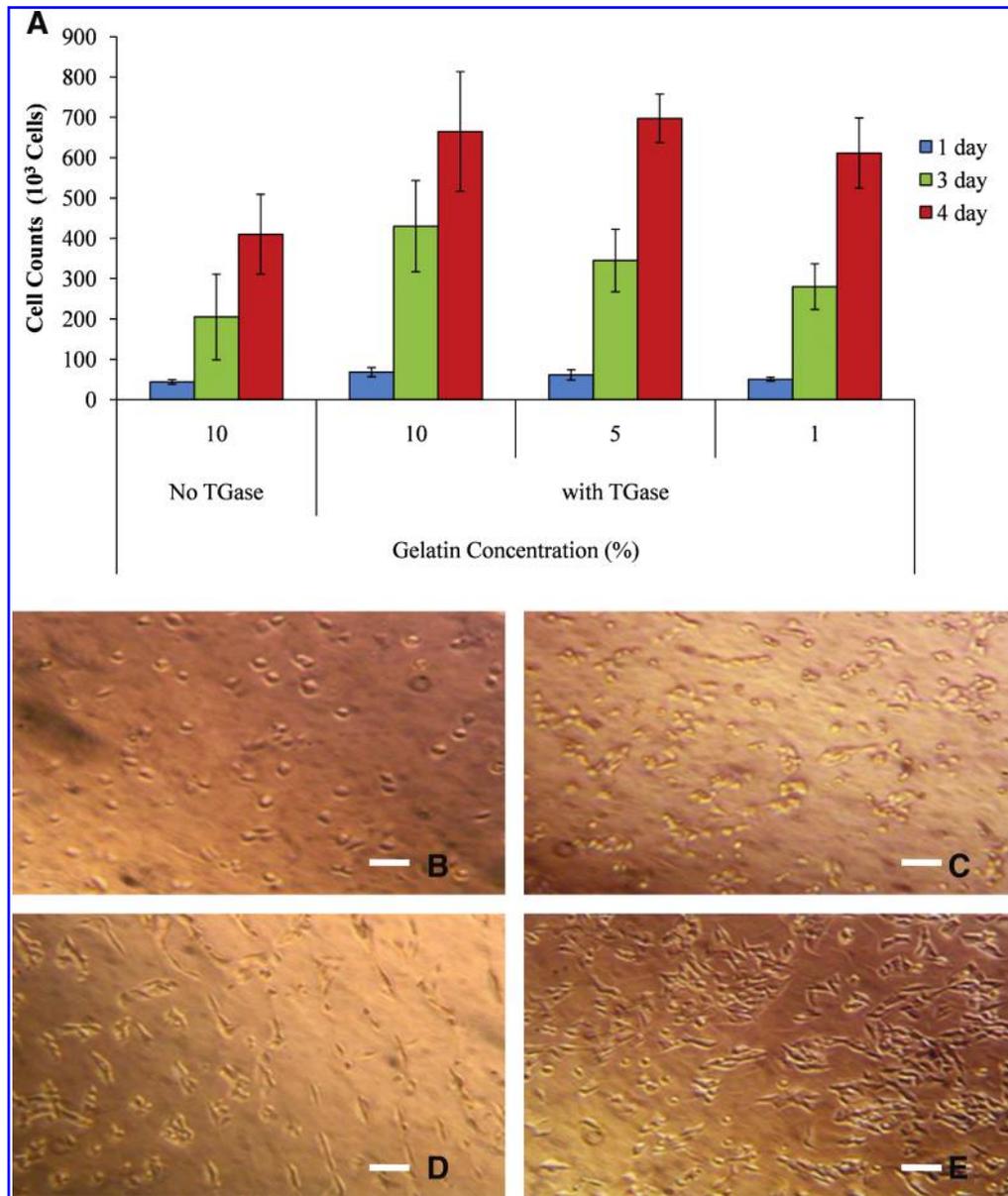


FIG. 2. (A) Cell counts of MSCs on two-dimensional gelatin–TGase gels after 1, 3, and 4 days. Different gelatin concentrations were used and all were treated with 23.5 $\mu\text{g}/\text{mL}$ of TGase. Ten percent + no TGase is the gelatin-only control. Error bars represent the standard deviation ($n = 4$). (B–E) Representative cell morphology images of the MSCs on 10% gelatin with no TGase (B). One percent gelatin with TGase (C), 5% gelatin with TGase (D), 10% gelatin with TGase (E) demonstrate the gelatin concentration effect on cell shape. High gelatin concentration without TGase shows cells still in their round form. Scale bars are 100 μm . MSC, mesenchymal stem cell. Color images available online at www.liebertonline.com/ten.

a statistical significance was observed between 10% gelatin–TGase and 10% noncrosslinked gelatin ($p < 0.04$). The high gelatin concentration with TGase was shown to support cell proliferation. Therefore, subsequent cell encapsulation studies were performed with 10% gelatin.

Cell morphology was dependent on the concentration of gelatin. The cell shape progressively assumed more of a spread-out elongated pattern as the percentage of gelatin was increased (Fig. 2B–E). A correlation was observed between the amount of gelatin and the cell morphology. MSCs were distinctly more elongated and less spherical in the gelatin with TGase in comparison to the gelatin alone.

Cell morphology and viability in 3D cell culture

The two-dimensional or monolayer cell culture showed that the crosslinked gelatin substrate can support cell proliferation; however, studies were necessary to determine if the MSCs could thrive in a 3D gelatin–TGase setting. When the MSCs were initially embedded, the cells assumed a round shape (Fig. 3A). On day 3, the cells began to aggregate, forming colony-like clusters (Fig. 3B). The cells exhibited elongated cell shape around day 7 and a network-like structure was observed (Fig. 3C). As the gels were monitored for 10 days, increases in cell density were observed within

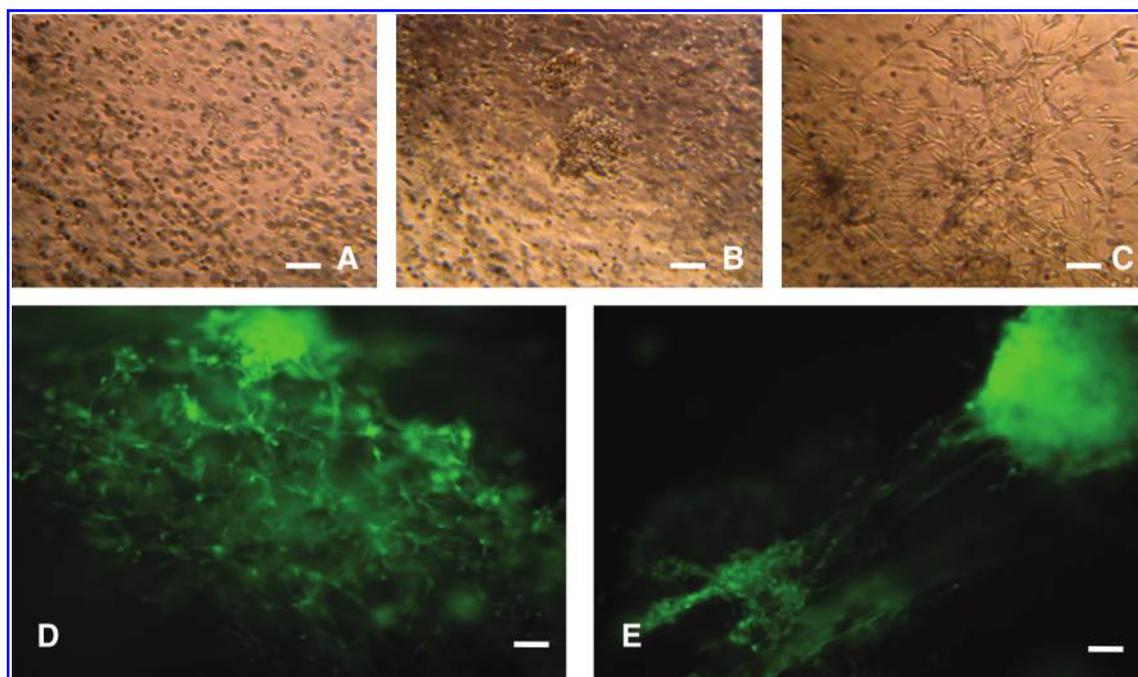


FIG. 3. (A–C) MSC morphology changes while embedded in three-dimensional cell culture after 1 (A), 4 (B), and 7 days (C). The gelatin concentration of gelatin–TGase is 10%. The cell density is at 1×10^6 /mL. Fluorescent pictures of the cell colonies embedded in the gel were taken. The scale bars for (A)–(C) is 100 μ m. (D) Colony with a network-like morphology, under higher magnification. Scale bar is 100 μ m. (E) Cell connection formation between colonies. Scale bar is 200 μ m. Color images available online at www.liebertonline.com/ten.

the gel. To further validate cell viability, trypan blue dye exclusion was performed at each stage. Staining showed that the embedded cells (>95%) were viable in all 3D 10% gelatin–TGase gels.

Fluorescent images of the GFP-labeled MSCs in the gel displayed a clear 3D interaction between the cells in the colonies. A mesh-like matrix between cells was observed within colonies (Fig. 3D) where the cells elongated and formed rod-like interconnections. Colonies of MSCs were also observed to stretch toward neighboring colonies (Fig. 3E).

Cell release rates from 3D gels

To demonstrate the potential for use in cell delivery, the ability to control the release of the MSCs from the gel was investigated. Cells migrated out from gelatin–TGase hydrogels, showing that the cells released were dependent on the concentration of TGase. At 11.7 μ g/mL concentration of TGase, the cells migrated out of the gel within 24 h (Fig. 4A). At 23.5 and 47 μ g/mL of TGase, the cells remained within the gel and were released after 96 h. Gelatin–TGase gels that had TGase concentrations lower than 5.8 μ g/mL were unable to retain the cells and dissolved at 37°C within 24 h. A possible correlation between the length of delayed release and increased crosslinks was observed. After their release, cells were able to migrate outward, and the migration rate was independent of TGase concentration. This suggested that the encapsulation had no negative impact on the cell's viability. Examples of cell migration from gelatin–TGase are shown in gel domes stained with Diff-Quik (Fig. 4B, C).

Feasibility of in vivo cell delivery

The feasibility of injecting gelatin–TGase hydrogel as a cell delivery vehicle to be used in tissue repair was studied in an animal model. A pilot study was conducted in which GFP-labeled MSCs in gelatin–TGase were injected into a rotator cuff of a rat. Using the gelation time table (Table 1), the gel was injected into the animal by using a 23-gauge needle with relative ease. The area of injection was located by the suture marker left at the time of initial surgery. From the histology, the gel was distinguished by the difference in density and appearance from the surrounding tissue. The histology showed that the gel was able to attach itself onto the surrounding muscle tissue (Fig. 5A, B). The identification of the MSCs, observed in Figure 5A and C, was confirmed by fluorescence imaging. In the 2-day explant, the cells were found concentrated in the gelatin–TGase (Fig. 5A, C). By the 7th day, the explants showed that the MSCs spread out uniformly into the surrounding tissue (Fig. 5B, D). The gel was also found to be smaller and held fewer cells.

Discussion

This study demonstrates that the gelatin–TGase hydrogel is a method that has promise for delivering viable cells into repair sites through injection. The hydrogel form of gelatin–TGase can be injected through fine 27-gauge needles. This can later gel *in situ* at the repair site, providing for ease in administration. The hydrogel further provides encapsulation of the cells, which allows for the injection of the cells without dissipation. The TGase and gelatin mixture and the process

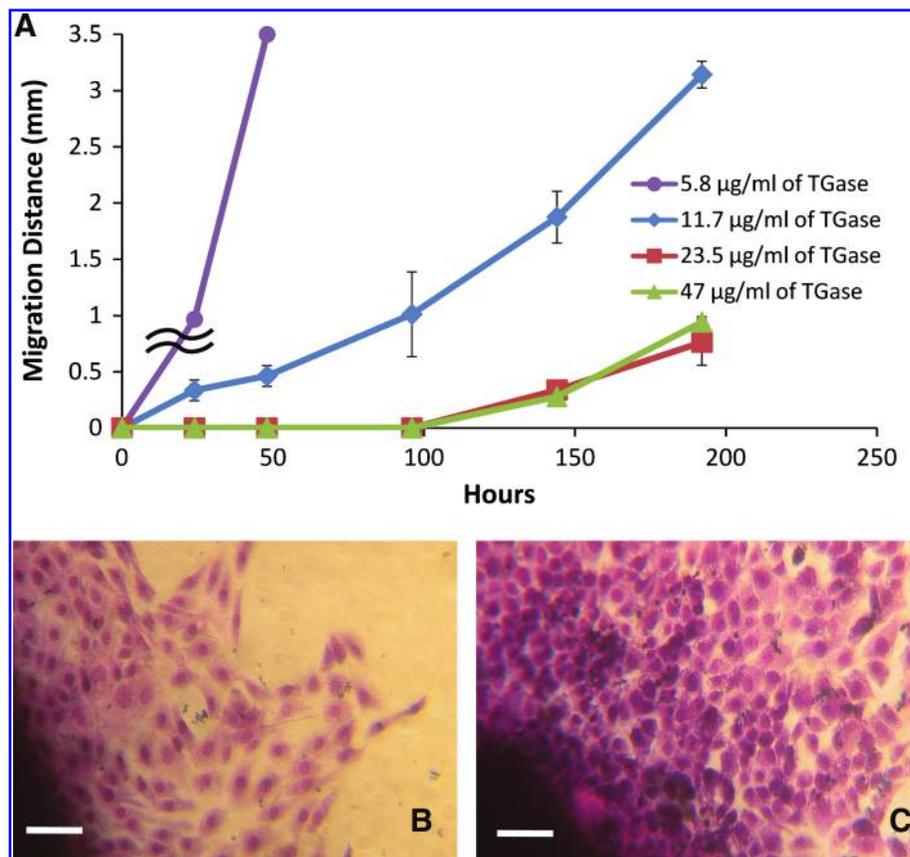


FIG. 4. (A) The graph shows the distance traveled after the encapsulated MSCs were released from the gelatin-TGase. The gelatin concentration of the gels were 10%. The double wavy line on the 5.8 $\mu\text{g/ml}$ of TGase represents gel dissolution and cell dispersion. The error bars represent the standard deviation ($n=3$). (B, C) Pictures show cells being released from the corners of the gelatin-TGase gel half dome after 4 (B) and 5 (C) days. The half dome along with the released cells were fixed and stained with Diff-Quik (magnification, 200 \times). Scale bars are 100 μm . Color images available online at www.liebertonline.com/ten.

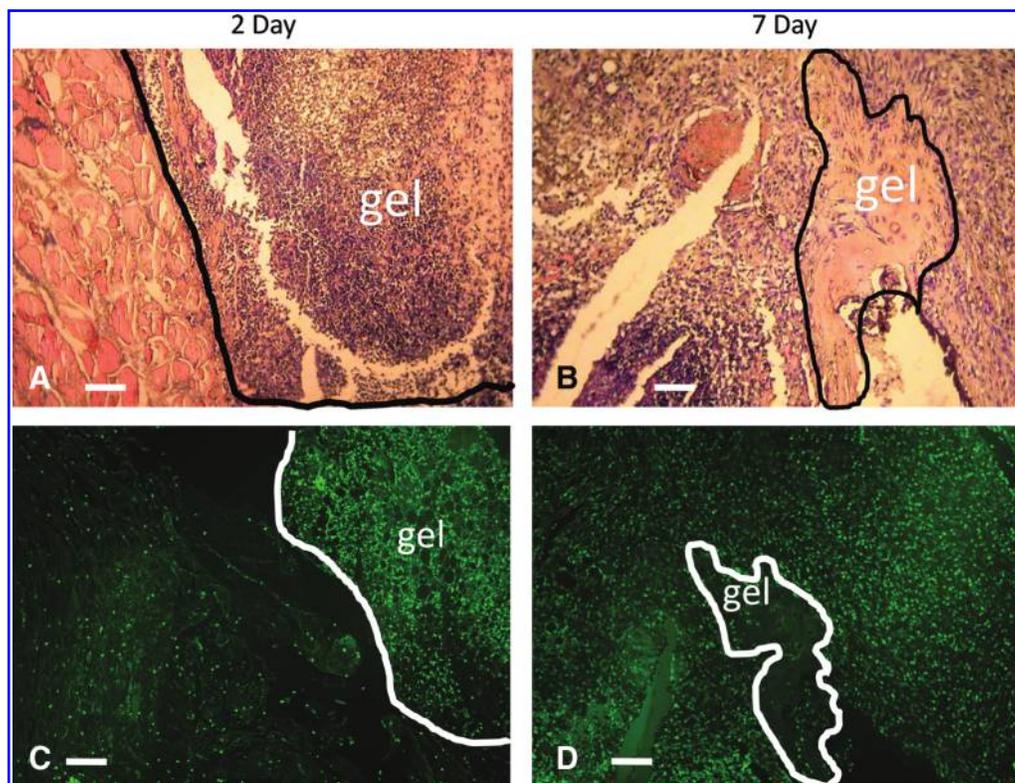


FIG. 5. Histological and fluorescent images of the green fluorescent protein-labeled MSC-encapsulated gelatin-TGase injected areas. (A) Histological hematoxylin and eosin staining of the site 2 days after injection. (B) Hematoxylin and eosin staining of the site 7 days after injection. (C) Fluorescent imaging of the injection site at 2 days. (D) Fluorescent imaging of the injection site at 7 days. Viewed at 40 \times magnification. All scale bars are 250 μm . Color images available online at www.liebertonline.com/ten.

of crosslinking were proven to be nondetrimental to cell viability, through both our studies and other studies.^{33,36–38} These studies demonstrated that the unique characteristics of this hydrogel, such as its ability to encapsulate cells, qualities of adhesion and localization, and its nontoxic nature, are of significant value. They aid in maintaining cell viability and overcome some of the challenges posed by other delivery mechanisms. Further, the results suggest that the level of adhesion and the release of cells may be controllable.

Encapsulation under high concentration of gelatin can be constrictive. Yung *et al.*³⁶ commented that a constrictive gel may pose a problem in the encapsulation of cells. For example, Hoshikawa *et al.*¹³ used a styrenated natural polymer gelatin to encapsulate chondrocytes. After initiating polymerization with light and subsequently injecting through a needle, the hydrogel gelled *in situ*. However, because of its tightly crosslinked structure, the cell viability was low. In our study, a high concentration of gelatin (10%) was used, and the encapsulated cells were not found to be constricted. The cells were able to form colonies around day 3 and form a network-like mesh around day 7.

The cells' changing morphology from round cells to network-like structures demonstrates the gel's ability to sustain cells and keep them viable. Studies showed that cells that remain round and unattached eventually lead to apoptosis or death.³⁹ The highly dense gelatin in the gelatin-TGase gel may have provided ample integrin sites for the cells to change their morphology and spread. PEG-based and polysaccharide-based hydrogels have no integrin sites and allowed the cells to maintain their round morphology. For example, an *in situ* gelable hydrogel composed of chitosan and oxidized dextran shows that the encapsulated cells take a week to adhere and 2 weeks to form colonies,¹⁰ a considerably longer time compared with gelatin-TGase. Network-like structures were never mentioned or shown in the study.

Cell proliferation increased as the percentage amount of gelatin in the gelatin-TGase mixture is increased. Chau *et al.*³³ has shown that adding TGase on to collagen can increase the proliferation of HFDF and HOB cells in comparison to a nontreated collagen matrix by altering the collagen configuration and exposing a larger number of cells binding sites. The higher amount of gelatin may have increased the number of integrin binding sites. However, in our subsequent study, where the gelatin percentage was kept constant and the TGase concentration modified (data not shown), the gel with a higher concentration of TGase had more cell attachment. Therefore, proliferation and attachment may be related to the rigidity of the gel rather than to the number of attachment sites. Lo *et al.*⁴⁰ has shown that cells have an affinity toward the rigid substrates and will migrate toward it. Matrix rigidity in tumors enhances cell growth and even upregulate fibronectin expression.⁴¹ It is possible that rigidity may be a factor in the cell proliferation and attachment, and this line of study is under investigation.

The gelatin-TGase adherence to the surrounding tissue enables localization of the injected cells at the repair site. Gelatin by itself has adhesive properties and has been considered a nontoxic alternative for solvent-based glue.⁴² But with crosslinked gelatin, adhesiveness can be increased depending on the substrate bonded to the gelatin. Chen *et al.*⁴³ demonstrated the potential of gelatin mixed with TGase as an adhesive to retinal tissue. The study found that lap shear

stress of the gelatin-TGase was comparable to other adhesives such as fibrin sealants,⁴⁴ chitosan,⁴⁵ and other various crosslinked gelatin.⁴⁶ The method used was based on ASTM F2255-03 (Test Method for Strength Properties of Tissue Adhesives in Lap-Shear by Tension Loading). In this study, we calculated the adhesion strength by measuring the stress required to break at the collagen and gelatin interface. Gelatin side chains were able to adhere with the surrounding pericardial collagen with the aid of TGase. As the concentration of gelatin was kept constant, the increase in stress was assumed to be from the TGase's construction of covalent crosslinks between gelatin and collagen. This reflects the value of controlling the strength of the adhesion of the gelatin-TGase to the surrounding tissue.

Finally, the ability to control the release of cells was dependent on the amount and concentration of the TGase enzyme. Yung *et al.*³⁶ showed that gelatin crosslinked by microbial TGase did not inhibit proliferation, and cells continued to proliferate once released from the gel. In their study, the cells in the hydrogel were released through a simulated proteolytic attack by trypsin. *In vivo*, proteolytic enzymes can originate from native cells in the surrounding tissue, dissolving the gel, and may influence an earlier release of cells. Nevertheless, the amount of enzymes released is hard to estimate and calculate, making the actual timing of *in vivo* release difficult to simulate.

In our studies, the cells were not exposed to a proteolytic attack but were left alone to migrate out (Fig. 4A–C). When the cells were left to incubate on top of gelatin-TGase gels, digestive rings surrounding the cells were observed. This may indicate that cells were releasing proteolytic matrix metalloproteinase enzymes which are known to play a role in cell migration and tissue regeneration.⁴⁷ Further studies may need to be done to correlate matrix metalloproteinase production with the crosslinking degree of gelatin-TGase.

In identifying the gels *in vivo*, it was difficult to locate the gels after the implant. Because of the gelatin-TGase adherence to the surrounding tissue, there were no clear border between the gel and tissue. The implant sites were located by a suture marker left after wound closure. On the slides, the gels were distinguished from the surrounding tissue by the differences in stain density by hematoxylin and eosin. The gel borders marked in Figure 5, especially the fluorescent images, were inferred from viewing numerous slides of serial sections.

This *in vivo* study reflects the clinical applications of gelatin-TGase. The use of GFP-labeled cells enabled in distinguishing the MSCs from other cells in the tissue. The fluorescent images show the gelatin-TGase cocktail held in the GFP-labeled MSCs for at least 2 days. Images from the 7th day showed cells scattered around the gels, indicating that the cells have dispersed and migrated from the gel. Such encapsulation of the cells may provide a barrier for the encapsulated cells against an inflammation response. Acute inflammatory response can create an inhospitable microenvironment for stem cells.⁴⁸ Molcanyi *et al.* reported that the injected stem cells are vulnerable to macrophages released during the inflammatory response.⁴⁹ Inflammatory responses are time dependent and, in the case of neural tissue, last for about 3 days.⁴⁸ Through gelatin-TGase's controlled release shown *in vivo*, the gel may be able to harbor the cells until after the initial inflammation phase. Further studies may

need to be done to see whether gelatin–TGase can block inflammatory effects on the encapsulated cells. The gelatin–TGase's ability to adhere and locally deliver cells can also be applied to wound healing, and soft and hard tissue repair. As MSCs can repair damaged tissues, a more in-depth rotator cuff study, where the healing of the defect is monitored, is currently under investigation.

Disclosure Statement

No competing financial interests exist.

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