Transglutaminase crosslinked gelatin as a tissue engineering scaffold

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Received 1 October 2006; revised 9 February 2007; accepted 12 March 2007
Published online 21 June 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31431

Abstract: Gelatin is one of the most commonly used biomaterials for creating cellular scaffolds due to its innocuous nature. In order to create stable gelatin hydrogels at physiological temperatures (37°C), chemical crosslinking agents such as glutaraldehyde are typically used. To circumvent potential problems with residual amounts of these crosslinkers *in vivo* and create scaffolds that are both physiologically robust and biocompatible, a microbial transglutaminase (mTG) was used in this study to enzymatically crosslink gelatin solutions. HEK293 cells encapsulated in mTG-crosslinked gelatin proliferated at a rate of 0.03 day^{-1}. When released via proteolytic degradation with trypsin, the cells were able to recolonize tissue culture flasks, suggesting that cells for therapeutic purposes could be delivered *in vivo* using an mTG-crosslinked gelatin construct. Upon submersion in a saline solution at 37°C, the mTG-crosslinked gelatin exhibited no mass loss, within experimental error, indicating that the material is thermally stable. The proteolytic degradation rate of mTG-crosslinked gelatin at RT was slightly faster than that of thermally-cooled (physically-crosslinked) gelatin. Thermally-cooled gelatin that was subsequently crosslinked with mTG resulted in hydrogels that were more resistant to proteolysis. Degradation rates were found to be tunable with gelatin content, an attribute that may be useful for either long-time cell encapsulation or time-released regenerative cell delivery. Further investigation showed that proteolytic degradation was controlled by surface erosion. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 83A: 1039–1046, 2007

Key words: transglutaminase; tissue engineering; gelatin; hydrogel; cell encapsulation; scaffold

INTRODUCTION

In the field of tissue engineering, there is great interest in designing cellular scaffolds that are not only biocompatible and physically robust, but also biodegradable at a controlled rate. Hydrogels made from thermally-cooled gelatin are one of the most commonly used support matrices for mammalian cell growth.[1] The prevalence of gelatin hydrogels is mainly due to their ability to closely simulate the native environment of mammalian cells since gelatin is the basic building block of collagen, a major component of the extracellular matrix (ECM).[1–3] The employment of these natural biopolymers thus offers a growth medium that is highly biocompatible with both encapsulated cells and host tissues. However, these physically-crosslinked gelatin hydrogels melt at physiological temperature and are prone to premature degradation by proteolytic enzymes such as gelatinase and collagenases.[1] Many researchers have sought to create more stable hydrogels by using either UV-light or chemical crosslinkers (e.g. glutaraldehyde, carbodiimide, and diphenylphosphoryl azide).[2,4–6] Despite the improved mechanical strength and proteolytic stability of synthetically crosslinked hydrogels, the crosslinkers often elicit either cytotoxic side-effects or immunological responses from the host.[5–10] Photocrosslinked hydrogels may also encounter a limitation in applications of deep tissue implants, where light is unable to penetrate the host tissue. These effects, in turn, diminish their overall applicability as crosslinking agents. Thus, there is a continuing need to develop cellular scaffolds that do not make concessions for either the biocompatibility or the physical stability of the hydrogel.

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Contract grant sponsor: National Science Foundation Research; contract grant number: EEC-0139595
Contract grant sponsor: University of Maryland, College Park

To overcome the need to compromise either compatibility or stability, a naturally occurring protein crosslinking enzyme, transglutaminase, was used in this work to form a thermally stable hydrogel from gelatin. Transglutaminase functions by catalyzing the formation of covalent N ε-(γ-glutamyl) lysine amide bonds between individual gelatin strands to form a permanent network of polypeptides.[11,12] This enzyme is ubiquitous in nature, being found in many species of the plant and animal kingdoms (e.g. peas, oysters, shrimp, tuna, chickens, cows, and humans).[13,14] Microbial transglutaminase (mTG) is a native protein that is innocuous[15] and commonly used in food manufacturing processes approved for human consumption by the U.S. Food and Drug Administration.[5,6] The functional independence of mTG with respect to proenzymes or calcium ions and its high level of activity over a wide range of temperatures (~50% at 37°C, max. at 50°C) and pH values (~90% between 5 and 8) make the enzyme amenable to a wide variety of gel formation and cell encapsulation techniques and conditions.[5,6,16,17] The improved mechanical strength and the short process time (<30 min for 4% gelatin at 37°C) for mTG to form a permanent gelatin network make this type of hydrogel appropriate for live surgery procedures, as well as injectable tissue engineering applications.[18–20] To advance the usage of mTG towards these applications, this work seeks to (1) demonstrate the biocompatibility of mTG-crosslinked gelatin hydrogels as 3D cellular scaffolding media, and (2) characterize the thermal stability and proteolytic degradability of these hydrogels. Biocompatible cellular scaffolds with tunable degradation rates can potentially be utilized in a wide variety of applications, ranging from the immuno-isolative encapsulation of cells to time-released delivery of cells for regenerative medicine.

MATERIALS AND METHODS

Maintenance of cell cultures

The attachment-dependent, human embryonic kidney cell line, HEK293 (Flp-In™-293, Invitrogen) was cultured in Dulbecco's Modified Eagle's Media containing high glucose (4.5 g/L), GlutMAX™ I (3.97 m*M*) (DMEM, Invitrogen), with added fetal bovine serum (10%, FBS, Sigma) and zeocin (100 m*M*, Invitrogen) (complete media). Cells were cultured in 75 cm^2-tissue culture flasks (Costar) and subcultured every 2–3 days as the population approached 80–90% confluency. Cells were passaged by first aspirating the spent media and then rinsing with Dulbecco's Phosphate Buffer Solution without Ca^{2+} or Mg^{2+} (DPBS-cm, Invitrogen). Rinsed cells were then incubated in 2 mL of a 0.25% trypsin with 0.03% EDTA solution (Sigma) for 5 min in their normal growth environment (37°C, 8% CO_2 incubator). Cells were passaged with a 1:10 split ratio and supplemented with fresh complete media up to 12 mL for further growth.

Thermally- and transglutaminase-crosslinked gelatin hydrogels

Three different methods were employed to generate gelatin hydrogels for this study: (1) physical crosslinking via thermal cooling (T-Gels), (2) covalent crosslinking via the enzyme mTG (mTG-Gels), and (3) a combined approach, thermal cooling followed by mTG crosslinking (Hybrid-Gels). T-Gels were formed by first dissolving 300-bloom gelatin (Type A, Sigma) in DMEM + FBS at 70°C for 10 min at a gelatin concentration of 0.04 g/mL (4%), followed by sterile filtration through 0.22 μm SteriFlip (Millipore) media filters before thermally setting at 4°C for 24 h. These physically-crosslinked hydrogels are not permanent and are thermally reversible. mTG-Gels (4%) were made by mixing 4 mL of a 10% gelatin solution in DMEM + FBS with 0.8 mL of a sterile filtered stock solution of mTG (10%, Ajinomoto) and 5.2 mL of DMEM + FBS. Stock mTG solution was made by first dissolving mTG powder in DMEM + FBS at 50°C for 10 min and then sterile filtering through 0.22 μm Millex (Millipore) syringe filters. The crosslinking reaction took place in a cell culture incubator set at 37°C for 24 h. Hybrid-Gels were formed by first cooling gelatin in DMEM + FBS (4, 10, 15, or 25%) at 4°C for 12 h without mTG. Then, an equal volume of the 10% mTG stock solution was overlaid on top of the thermally-cooled hydrogel to enzymatically crosslink the physical hydrogel. The reaction phase took place in an incubator at 37°C for an additional 12 h. High concentration gelatin solutions (15 and 25%) could not be obtained by simple mixing owing to the high viscosity of gelatin solutions as dissolution proceeds. Instead, gelatin was initially dissolved at a lower concentration and water evaporated at 70°C to arrive at the desired concentration. The final concentration was calculated from the difference in initial and evaporated weights.

Cell encapsulation and culture

Three-dimensional cell encapsulates were formed in an mTG-Gel with a gelatin concentration of 0.04 g/mL (4%). The gelatin and mTG solutions were sterile filtered and equilibrated in a 37°C water bath for 20 min prior to mixing with the HEK293 cells in order to prevent heat shock. Encapsulates were formed in white-wall, clear-bottom, 96-well tissue culture plates (Costar) with 60 μL/well. HEK293 cells were used at a density of 2000 cells per 60 μL. Mixtures of cells and the hydrogel precursor solution were immediately aliquoted into well plates in order to avoid thermal cooling. Once the hydrogel was formed, the cell encapsulates were incubated under normal cell culture conditions (37°C, 8% CO_2) with an overlay of 100 μL of complete media.

In situ cell proliferation assay

The proliferation of encapsulated HEK293 cells was monitored with two membrane-permeable nucleic acid

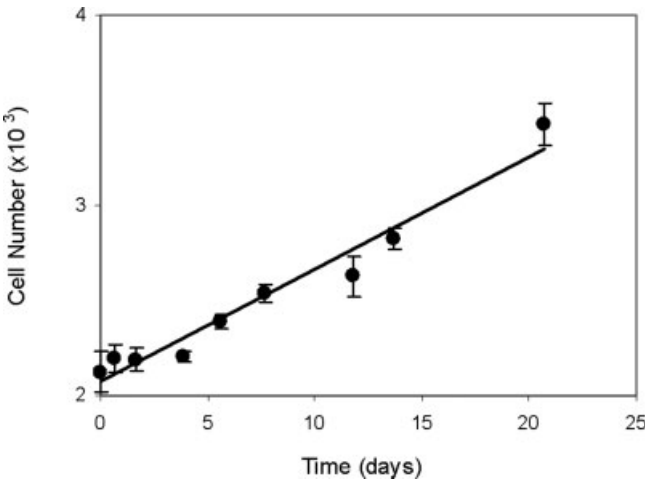

Figure 1. HEK293 cells (2,000 cells/well) were encapsulated in 4% mTG-Gels (60 μL/well) and initially seeded into 96-well plates with white walls and clear bottoms. Hoechst 33342 staining (4–h incubation) showed that encapsulated cells were proliferating at a linear rate of $\sim 0.03\ \text{day}^{-1}$ ($r^2 = 0.98$, $p = 4.4 \times 10^{-6}$).

dyes, SYTO-16 and Hoechst 33342 (Molecular Probes; Invitrogen). Although both dyes can bind DNA, SYTO-16 can also stain RNA, cytoplasm, and mitochondria. Both dyes have the property of fluorescing only when bound to nucleic material, but not while they are free or simply interacting with proteins such as gelatin. Cell encapsulates were stained within the 96-well plates by first aspirating the media overlays and then incubating each well with 60 μL of either dye at 2× concentrations (SYTO-16 = 2 μ*M*, max E_x/E_m = 489/520 nm; Hoechst 33342 = 20 μg/mL, max E_x/E_m = 350/460 nm). Stock reagents of both dyes were made with Dulbecco's Phosphate Buffer Solution (DPBS, Invitrogen) and stored in the dark at 4°C until needed. The fluorescence of stained cells was measured with an LS55 fluorescence microplate reader (Perkin–Elmer). Since fluorescence intensity measurements were found to vary with incubation time (data not shown), an arbitrary incubation period of 4 h was fixed for all *in situ* assays.

Thermal stability and proteolytic degradability

The three types of gelatin hydrogel were tested for their ability to resist thermal and proteolytic degradation using mass loss experiments. Two milliliter volumes of each hydrogel precursor solution were cast in separate 35-mm Petri dishes (9.6 cm^2) and allowed to set overnight before thermally or proteolytically challenged. Thermal stability was examined by incubating the hydrogels in a DPBS solution at 37°C. Proteolytic degradability was examined by incubating the hydrogels in a solution of 0.25% trypsin with 0.03% EDTA at room temperature (RT). All treatments were performed on an orbital shaker (75 rpm). Petri dishes were weighed on an analytical balance (Mettler Toledo AE50) immediately prior to immersion in a treatment solution and at various time points until the gel had fully degraded. Special care was taken to remove excess treatment solution by using blotting paper on the gel surface as well as the exposed surfaces of the dish. To determine whether or not surface or bulk erosion controlled proteolytic degradation, hydrogels were cast either in Petri dishes of variable surface area (9.6, 28.3, and 78.5 cm^2) at a fixed height (0.21 cm) or in Petri dishes with a fixed sur-

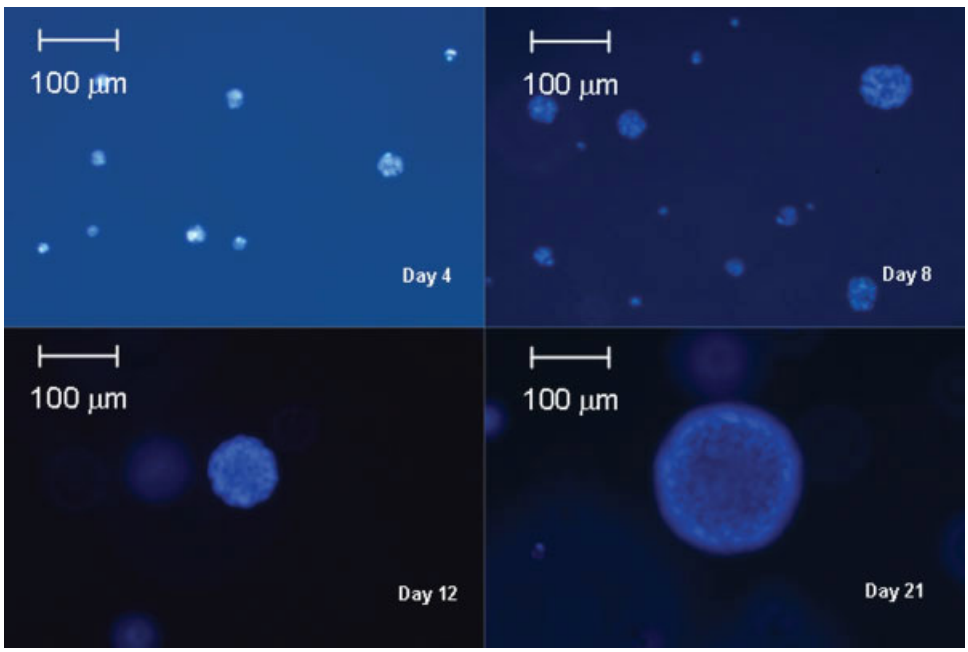

Figure 2. HEK293 cells stained with Hoechst 33342 can be seen proliferating while they are encapsulated in the 4% mTG-Gel. Cells formed spherical clusters that increased in size with time. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

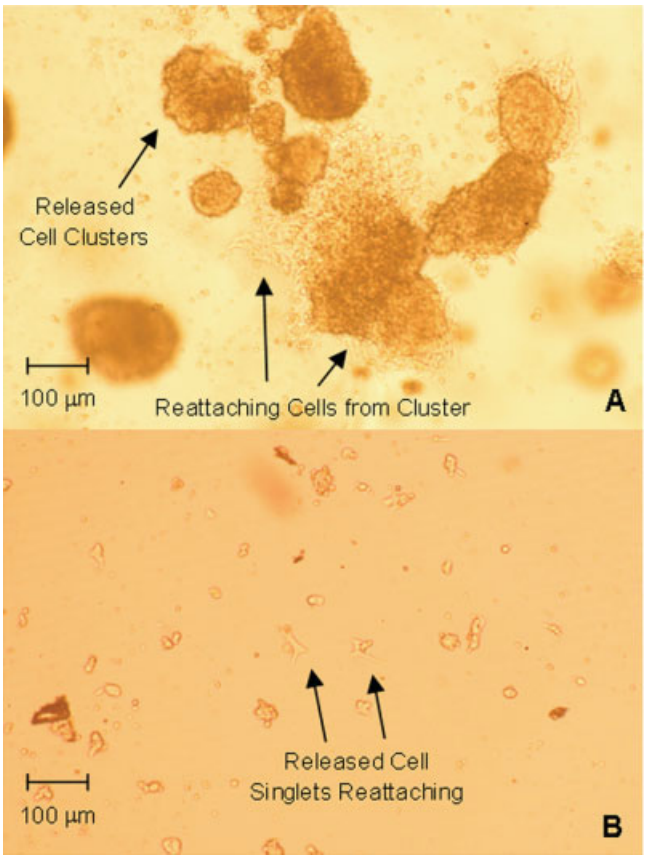

Figure 3. Encapsulated HEK293 cells were released by masticating the mTG-Gels followed by a 20-min incubation with a 0.25% trypsin + 0.03% EDTA solution at 37°C. Released cells appeared as clusters (A), as well as singlets (B). Both forms of released cells were able to re-attach to tissue culture plates under normal incubation conditions (37°C, 8% CO_2). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

face area (9.6 cm^2) at variable heights (0.10, 0.21, 0.31, and 0.42 cm).

Statistical analysis

All data points shown in this work represent averaged values from experiments done in triplicate, at a minimum. Error bars represent standard deviations. Single-factor ANOVA calculations, using Mathematica v 5.1 and MS Excel 2003, are used to determine the coefficient of determination (r^2) and probability of statistical significance (p) for linear regressions.

RESULTS AND DISCUSSION

Biocompatibility and cell proliferation

To determine the suitability of nucleic acid staining for measuring cell proliferation, calibration curves between cell number and fluorescence intensity within each well were developed for both the SYTO-16 and Hoechst 33342 dyes. SYTO-16 staining produced a Langmuir-like relationship between cell number and fluorescence, presumably due to saturation. This was less suitable as a calibration curve as compared to Hoechst 33342 staining, which yielded a reasonable linear fit of 5.05×10^5 cells/RFU between 0 and 40,000 cells/well ($r^2 = 0.99$, $p = 6.3 \times 10^{-10}$).

The proliferation of HEK293 cells encapsulated in 4% mTG-Gels, with an initial concentration of 2000 cells/well, was monitored with Hoechst 33342 staining for a 3-week period. Negative controls using stained gelatin hydrogels without any cells were made for each sampling time point. Cell numbers were calculated by multiplying the normalized ratio of fluorescence intensity ($RFU_{sample}/RFU_{negative\ control}$) by the initial cell concentration. As shown in Figure 1, the HEK293 cells proliferated within the hydrogel at a linear growth rate of 0.03 day^{-1} ($r^2 = 0.98$, $p = 4.4 \times 10^{-6}$).

Images of the fluorescently stained cells (Fig. 2) showed that singly encapsulated cells were replicating in place and forming spherical clusters within the hydrogels. Similarly, encapsulated HEK293 cells that were cast in 6-well tissue culture plates and grown for over 1 month were observed to form clusters 1–2 mm in diameter. As a direct and qualitative demonstration of cell viability, these hydrogels were physically broken into smaller pieces (length = 4 mm) and incubated in a 0.25% trypsin + 0.03% EDTA solution at 37°C for 20 min, in order to release the proliferating cells. The majority of these clusters (>90%), when incubated under normal cell culturing conditions, were able to spread out and re-colonize plate surfaces [Fig. 3(A)]. Released cells that were of smaller cluster sizes or even singlets were also able to re-attach and propagate [Fig. 3(B)]. Together, the

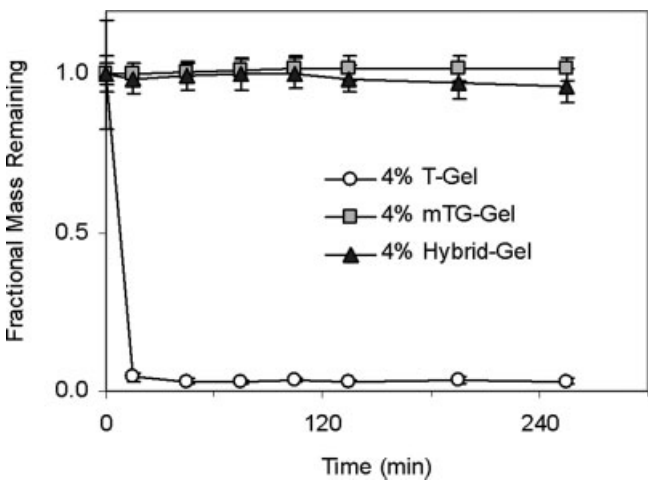

Figure 4. Exposure of the three 4% hydrogels (T-Gel, mTG-Gel, and Hybrid-Gel) to 37°C (in DPBS) showed that only the T-Gel was susceptible to thermal degradation.

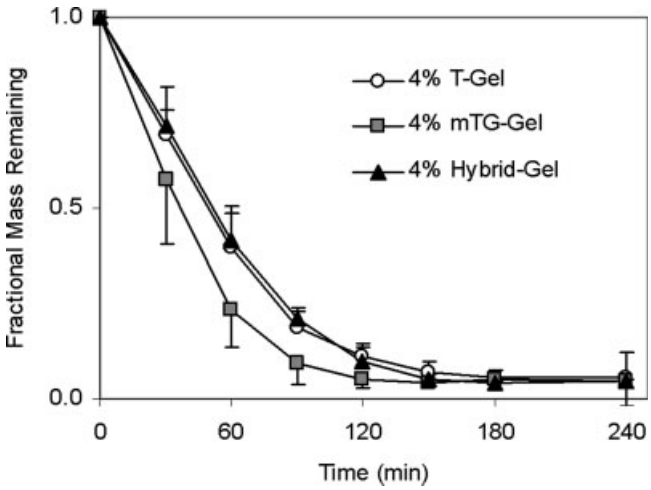

Figure 5. Proteolytic degradation by trypsin at RT revealed that mTG-Gels were slightly less resistant to proteolytic degradation than T-Gels, but that Hybrid-Gels, formed by thermal cooling prior to mTG exposure, matched the T-Gels.

results in Figures 1–3 demonstrate that HEK293 cells encapsulated in mTG-crosslinked gelatin hydrogels were able to maintain high viability for at least 3 weeks.

Thermal stability of mTG-gels

Since mTG crosslinks gelatin covalently, the resulting hydrogel should be stable at physiological temperatures. To simulate thermal conditions *in vivo*, 4% mTG-Gels and 4% T-Gels were placed in a 37°C DPBS bath and removed for weighing at various time points. As shown in Figure 4, physically crosslinked T-Gels were highly susceptible to melting, as expected, at physiological temperature (initial degradation rate, $-R_i = 63.8\ \text{min}^{-1}$; $p = 1.6 \times 10^{-8}$). Most of the original mass of the hydrogel disappeared within the first 30 min. mTG-Gels, however, were resistant to thermal degradation, within experimental error, and remained intact for the 4 h of observation (Fig. 4).

Proteolytic degradation

To simulate enzymatic degradation *in vivo*, 4% mTG-Gels and 4% T-Gels were placed in the trypsin/EDTA solution described above at RT and again removed at various time points for weighing (Fig. 5). The mTG-Gels degraded at a slightly faster rate (10.1 min^{-1}; $p = 1.4 \times 10^{-19}$) when exposed to trypsin than the T-Gels (9.0 min^{-1}; $p = 7.0 \times 10^{-23}$). It was

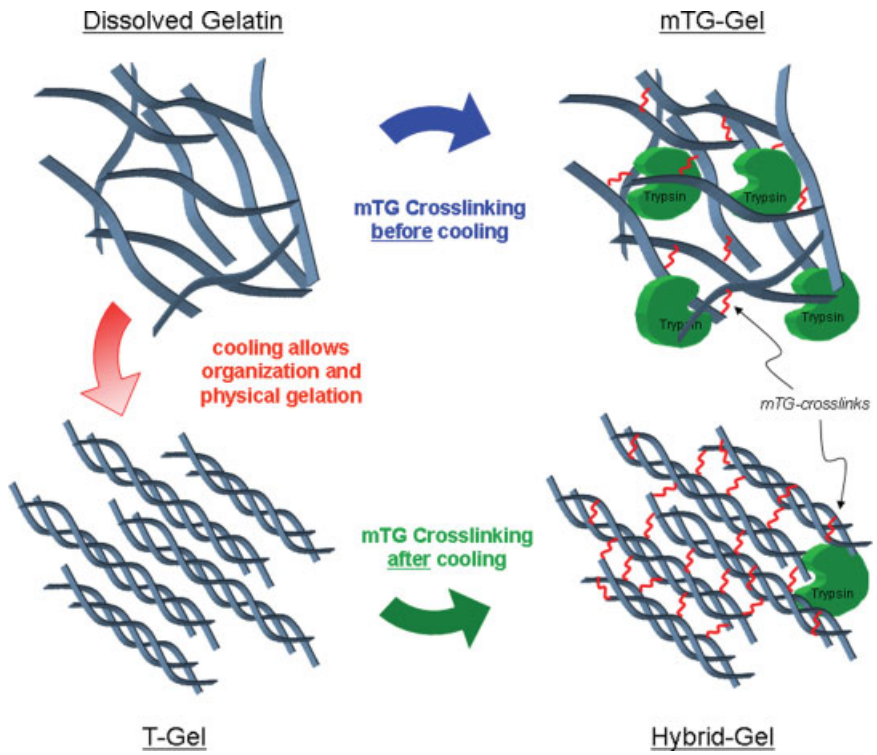

Figure 6. Proposed scheme of hydrogel formation: Hybrid-Gels were found to be more resistant to proteolysis compared mTG-Gels. An explanation may be that thermal cooling allows gelatin to first self-organize into a tight network of polypeptides through hydrogen bonding. Once this network is formed, there are more potential junction points where mTG crosslinking can occur, creating a denser hydrogel. mTG-Gels, however, are unable to form this tight network since mTG is added directly to the gelatin solution. The result is a less tightly packed hydrogel with a larger mesh size, which may be more susceptible to proteolytic attack. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

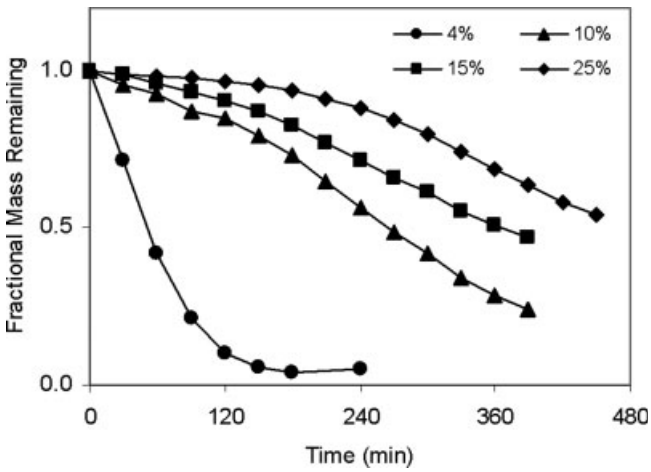

Figure 7. Proteolytic degradation by trypsin at RT for four Hybrid-Gels (4, 10, 15, and 25%). Disproportionate drop in initial rate between 4 and 10% may indicate a critical mesh size for trypsin permeation over the time scale of measurement.

hypothesized that mTG-Gels may be more susceptible to degradation because mTG creates a looser network than that formed by thermal cooling (Fig. 6). As a result, mTG-Gels have a larger mesh size than T-Gels, allowing them to be more susceptible to proteolytic attack despite being thermally stable. From these results, it was hypothesized that a 4% Hybrid-Gel would have the same mesh size as a T-Gel with the added thermal stability of an mTG-Gel with its covalent crosslinks. When 4% Hybrid-Gels were subjected to the same trypsin bath as before, they were found to be more resistant to proteolytic degradation than 4% mTG-Gels. The rate of degradation (8.9 min^{-1}) was, in fact, similar to the 4% T-Gels (Fig. 5). The Hybrid-Gels also proved to be resistant to thermal degradation, as no mass was lost over a 4-h period, when exposed to a 37°C DPBS solution (Fig. 4).

Tunable degradability of Hybrid-Gels

Since degradation rates of hydrogels can typically be influenced by polymer and crosslinking densities (mesh size), increasing gelatin concentration may be a simple method of retarding the proteolytic degradation rate. Hybrid-Gels of four concentrations (4, 10, 15, and 25%) were tested for their resistance to proteolytic degradation by the trypsin solution. Measurements of fractional mass remaining in the hydrogels showed that initial degradation rates decreased as a function of increasing gelatin concentration, as expected (Fig. 7 and Table I). The reduction in initial degradation rate, however, was not linear with respect to gelatin concentration. A sharp decrease in the rate of proteolytic degradation was observed between concentrations of 4 and 10% (Fig. 7). This disproportionate decrease in the degradation rate may reflect a critical mesh size necessary for trypsin to penetrate the hydrogel beyond the surface, over the time scale of the degradation process. In other words, below this critical mesh size, the rate at which trypsin permeates the hydrogel is significantly less than the rate of degradation.

Visually, the hydrogels appear to degrade by a surface erosion mechanism. The surfaces remain smooth with no significant defects and only the height changed as mass was lost. To investigate whether or not proteolytic degradation was controlled by surface erosion, 10% Hybrid-Gels were set in Petri dishes of either variable surface area at a fixed height or fixed surface area at different heights. Since degradation rate is calculated based on normalized mass loss ($[g_f/g_i]$/min), if degradation is controlled by surface erosion, the total mass lost per unit time (g/min) would scale linearly with surface area resulting in a normalized degradation rate (min^{-1}) that is independent of surface area at a fixed height. Similarly, normalized degradation rates that scale with $(height)^{-1}$ at a fixed surface area would also be indicative of surface controlled erosion. As shown in Figure 8, both conditions are met and surface erosion controls the rate of proteolytic degradation. This phenomenon would likely apply to Hybrid-Gels of a higher gelatin concentration, since increasing the gelatin content decreases the mesh size, further limiting proteolytic degradation to the surface of the hydrogel.

TABLE I
Thermal and Enzymatic Degradation Rates for Various Forms of Hydrogels ($\times 10^{-3}$ min^{-1})

	Initial Degradation Rate ($\times 10^{-3}$ min^{-1})				
Hydrogel Form	37°C DPBS	0.25% Trypsin + 0.03% EDTA at RT			
% Gelatin	4	4	10	15	25
T-Gel	63.8 ± 0.0	9.0 ± 0.8	–	–	–
mTG-Gel	−0.1 ± 0.2	10.1 ± 1.2	–	–	–
Hybrid-Gel	0.1 ± 0.2	8.9 ± 0.7	1.3 ± 0.3	0.9 ± 0.2	0.3 ± 0.2
Fully hydrated Hybrid-Gel	–	–	2.7 ± 0.3	1.5 ± 0.2	–

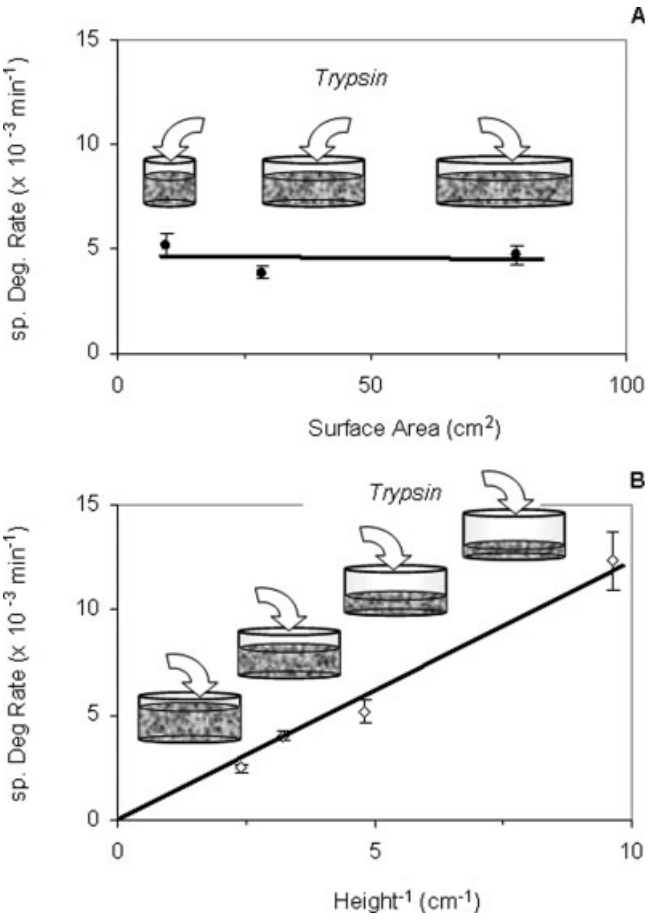

Figure 8. Proteolytic degradation rate at RT for 10% Hybrid-Gels as a function of (A) surface area at fixed height (0.21 cm) and (B) $(\text{height})^{-1}$ at fixed surface area (9.69 cm^2). Both results suggest proteolysis is controlled by surface erosion.

To further test the applicability of using mTG crosslinked hydrogels as cellular scaffolds *in vivo*, the degradation rate was determined for hydrogels in a fully hydrated state. Ten and 15 % Hybrid-Gels were formed as before and soaked in DPBS for 24 h before performing degradation experiments with trypsin. As Figure 9 shows, swelling the hydrogels to their equilibrium water content made them more susceptible to proteolytic degradation at both gelatin concentrations. This result suggests that Hybrid-Gels may have slightly faster degradation rates once implanted as a result of scaffold hydration by the host.

CONCLUSIONS

The objective of this study was to demonstrate that gelatin hydrogels crosslinked by mTG were biocompatible with encapsulated cells, such that they may be used as a 3D cellular scaffold. The ability of HEK293 cells to proliferate within the hydrogel and then re-colonize cell culture surfaces once released from encapsulation demonstrated that mTG-Gels were indeed biocompatible. mTG-crosslinked hydrogels were also shown to be resistant to thermal degradation, in contrast to physically-crosslinked (thermally-cooled) hydrogels, but were proteolytically degraded at a slightly faster rate. Thermally setting a gelatin solution followed by enzymatic crosslinking with mTG (Hybrid-Gels), resulted in a hydrogel that was more resistant to proteolytic attack than mTG-Gels, yet maintained thermal stability. Measurement of the degradation rate, under conditions of either variable surface area or variable height, supported the hypothesis that degradation occurred through a mechanism of surface erosion. Degradation rates of Hybrid-Gels were found to be tunable with gelatin concentration. Fully hydrated Hybrid-Gels, which were designed to more closely simulate physiological conditions, resulted in slightly higher degradation rates for both 10 and 15% Hybrid-Gels. This observation, together with the sudden increase in degradation rate seen in Figure 7 between 10 and 4%, suggests that the degradation mechanism *in vivo* could shift from surface to bulk erosion, or at a minimum, a mixed-control mechanism including both.

Gelatin concentrations above 10% were found to be much more resistant to proteolytic degradation compared to the 4% hydrogel used for cell encapsulation. Higher gelatin concentrations could be used for cellular scaffolding, but are not preferable since denser structures may be too confining for most cell types, which do not proliferate well in a rounded morphology.[21–24] Although the ability of cells to survive direct encapsulation at higher gelatin concentrations was not examined, these hydrogels could serve

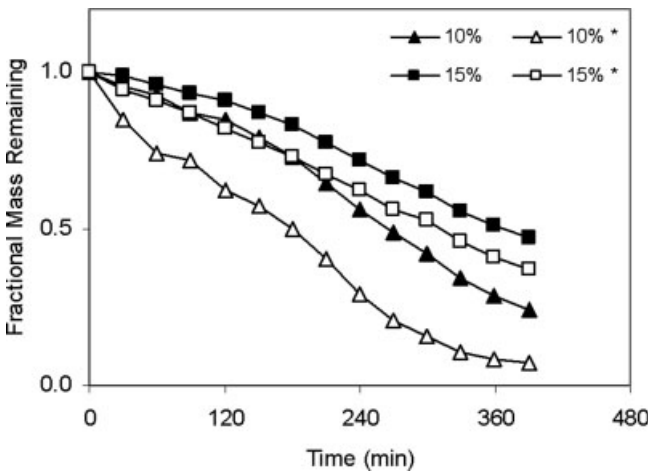

Figure 9. Effect of water content on proteolytic degradation at RT for 10 and 15% Hybrid-Gels. Those allowed to swell to equilibrium in DPBS (*, open symbols) were found to be more susceptible than the as-prepared hydrogels.

as an outer protective coating for cells that are encapsulated in a 4% mTG-Gel. Encapsulated cell cores surrounded by high-density gelatin shells, both utilizing mTG-Gels with customizable degradation rates, may be one means of delivering regenerative cells in a controlled manner.

Facilities and equipment were provided by the University of Maryland Biotechnology Institute (UMBI), Center for Biosystems Research (CBR).

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