Published in final edited form as:

Biomaterials. 2014 March ; 35(8): 2477–2487. doi:10.1016/j.biomaterials.2013.11.044.

Injectable, porous, and cell-responsive gelatin cryogels

Sandeep T. Koshy^{1,2,3}, Thomas C. Ferrante², Sarah A. Lewin², and David J. Mooney^{1,2,*}

¹School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA

²Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02115, USA

³Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA 02139, USA

Abstract

The performance of biomaterials-based therapies can be hindered by complications associated with surgical implant, motivating the development of materials systems that allow minimally invasive introduction into the host. In this study, we created cell-adhesive and degradable gelatin scaffolds that could be injected through a conventional needle while maintaining a predefined geometry and architecture. These scaffolds supported attachment, proliferation, and survival of cells *in vitro* and could be degraded by recombinant matrix metalloproteinase-2 and -9. Prefabricated gelatin cryogels rapidly reassumed their original shape when injected subcutaneously into mice and elicited only a minor host response following injection. Controlled release of granulocyte-macrophage colony-stimulating factor from gelatin cryogels resulted in complete infiltration of the scaffold by immune cells and promoted matrix metalloproteinase production leading to cell-mediated degradation of the cryogel matrix. These findings suggest that gelatin cryogels could serve as a cell-responsive platform for biomaterial-based therapy.

Keywords

injectable; gelatin; cryogel; degradable; matrix metalloproteinase

Introduction

Implantable biomaterials have been proposed to locally deliver or recruit cells, or provide sustained release of therapeutic molecules for applications such as tissue engineering, drug delivery, gene therapy, and vaccines [1–4]. The clinical implantation of prefabricated biomaterials for these purposes typically requires trained physicians, causes patient distress, creates potential scarring, poses a risk of infection, and often causes inflammation at the surgical site that may inhibit the performance of the implant. Biomaterials that can be introduced in a minimally invasive manner are thus of interest in many therapeutic applications.

© 2013 Elsevier Ltd. All rights reserved.

*To whom correspondence should be addressed. mooneyd@seas.harvard.edu. Fax: (617) 495–9837.

Author Contributions: STK and DJM designed the studies. STK conducted the studies. TCF and STK recorded high-speed movies, performed 2-photon microscopy, and image processing. SAL and STK performed longitudinal IVIS imaging of cryogel degradation. STK analyzed the data. STK and DJM interpreted the data, and wrote the paper.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Injectable hydrogels have been used as biomaterial implants without the need for surgery [5,6]. Many of these materials systems involve the injection of a polymer solution and subsequent crosslinking of the polymer chains by chemical or physical means to form a solid [7,8]. The use of liquid precursors may result in leakage from the implant site to unwanted tissues and poses difficulties in generating the desired implant geometry. We have recently created preformed hydrogel scaffolds with a defined geometry and microstructure that can be introduced to the body in a minimally invasive manner through a conventional needle [9]. These hydrogels are formed by cryopolymerization of methacrylated alginate using radical polymerization at sub-zero temperatures. Although these scaffolds are capable of delivering cells and biomolecules in a non-invasive manner, their ability to be remodeled and degraded locally by cells is limited, which could reduce their ability to integrate with host tissue in certain applications. Additionally, these hydrogels require modification of the alginate polymer with cell adhesive peptides to allow cell attachment, which requires additional synthesis steps.

We hypothesized that using a material with inherent cell-responsive (e.g. cell binding and enzymatically degradable) elements may improve the performance of cryogel implants by allowing direct cell attachment and local remodeling. Gelatin, derived from collagen, contains inherent peptide sequences that facilitate cell adhesion and enzymatic degradation. Additionally, its low cost, lack of immunogenicity, and safety record in medicine (hemostatic agent, blood volume expander etc.) makes gelatin an attractive implantable biomaterial. Modification of gelatin with pendant methacrylate groups (GelMA) allows crosslinked hydrogels to be formed using radical polymerization, which have been used extensively in cell culture and tissue engineering studies [10–15]. In this report, we fabricated and characterized scaffolds formed by cryopolymerization of GelMA (cryoGelMA). We studied the bulk mechanical behavior, structure, and degradation of the cryoGelMA gels, as well as the ability of these scaffolds to facilitate cell attachment, proliferation, and survival. Finally, the ability of gelatin cryogels to locally deliver a chemoattractant protein, recruit host cells, and undergo cell-mediated degradation *in vivo* was explored.

Materials and Methods

Mice

All work with C57BL/6J and C57BL/6J-Tyr^{c-2J} mice (female, aged 6–8 weeks; Jackson Laboratories) was performed in compliance with National Institutes of Health and institutional guidelines.

Methacrylated gelatin synthesis

Methacrylated gelatin (GelMA) was synthesized (Fig. 1-A) by allowing Type A porcine skin gelatin (Sigma) at 10% (w/v) to dissolve in stirred Dulbecco's phosphate buffered saline (DPBS; GIBCO) at 50 °C for 1 h [10,12]. Methacrylic anhydride (Sigma) was added dropwise to a final volume ratio of 1:4 methacrylic anhydride:gelatin solution. This resulted in GelMA with a degree of substitution of 79% (Fig. S1). The solution was stirred at 50 °C for 1 h, and then diluted 5x with DPBS. The resulting mixture was dialyzed in 12–14 kDa molecular weight cutoff tubing (Spectrum Labs) for 4 d against distilled water (dH₂O) with frequent water replacement. The dialyzed solution was lyophilized, and the resulting GelMA was stored at -20 °C until use. Rhodamine-labeled GelMA, created from the reaction of GelMA with NHS-rhodamine (Thermo Scientific), was purified using an identical dialysis and lyophilization process.

Gelatin cryogel preparation

Cryogels were formed by dissolving GelMA in dH₂O to the final desired concentration in the presence of 0.5% (w/v) ammonium persulfate (APS; Bio-Rad) and 0.1% (w/v) tetramethylethylenediamine (TEMED; Bio-Rad). This prepolymer solution was pipetted into cylindrical (5 mm diameter, 2 mm thickness) polystyrene molds and placed in a freezer set to -12 °C (Fig. 1-B). Cryopolymerization was allowed to proceed for 18 h, and the resulting cryogels were thawed and hydrated in dH₂O prior to use.

Interconnected porosity

To test for cryogels for interconnected porosity, scaffolds were first thawed and hydrated for 1 d. Hydrated scaffolds were weighed on a scale, and a Kimwipe was lightly applied to the scaffold surface for 30 s to wick away loosely held water, and the mass was again recorded. The interconnected volume was calculated as the mass of water wicked away divided by the total hydrated mass.

Scanning electron microscopy

For scanning electron microscopy, cryogels were serially transitioned from dH₂O into absolute ethanol with 20 min incubations in 30, 50, 70, 90, and 100% ethanol solutions. Samples were incubated in hexamethyldisilazane (Electron Microscopy Sciences) for 10 min and dried in a desiccator for 1 h. Dried cryogels were adhered onto sample stubs using carbon tape and coated with a platinum/palladium in a sputter coater. Samples were imaged using secondary electron detection on a Carl Zeiss Supra 55 VP field emission scanning electron microscope (SEM). Cell-laden cryogels were fixed in 4% paraformaldehyde (PFA) and prepared for SEM as described above. Images were false-colored in Adobe Photoshop CS6 to highlight cells.

2-photon microscopy

To characterize the hydrated cryogel structure, rhodamine-GelMA cryogels were placed in dH₂O in a 35mm glass-bottom culture plate (MatTek), and imaged on a Leica SP5 inverted laser scanning confocal microscope. 2-photon excitation was achieved using a Chameleon Vision 2 pulsed infrared (IR) laser (Coherent) at 820 nm, and fluorescence emission was collected through a 565–605 nm bandpass filter by a non-descanned detector. For imaging of cell-laden cryogels, cells were first labeled with 5-chloromethylfluorescein diacetate (CMFDA) according to the manufacturer's instructions (Molecular Probes) prior to seeding on scaffolds. After cell attachment, cells were fixed with 4% PFA in DPBS, and cell nuclei were stained with Hoechst 3342 (Molecular Probes). For 3-color imaging of cell-laden rhodamine-cryoGelMA scaffolds, the IR laser was tuned to 800 nm and Hoechst 3342, CMFDA, and rhodamine-GelMA were detected through 430–480 nm, 500–550 nm, and 565–605 nm bandpass filters, respectively.

Bulk cryogel imaging

Bright field and fluorescence images of bulk cryogels were acquired using a Zeiss Axio Zoom V16 stereomicroscope. To capture high-speed cryogel injection videos, a Hamamatsu Orca-Flash 4.0 sCMOS camera was used at 200 frames per second.

Cells and cryogel seeding

NIH 3T3 cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% (v/v) fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37 °C in a 5% CO₂ atmosphere. Prior to seeding into cryogels, cells were harvested using a non-enzymatic cell dissociation solution (Sigma) and resuspended at 10⁷ cells/ml in complete medium. Cryogels were dehydrated by wicking with a Kimwipe, and rehydrated in 100 µl of

cell suspension in a 2 ml round-bottom centrifuge tube. Tubes were incubated at 37 °C for 45 min to allow for cell attachment. Gels were placed directly in complete medium following incubation, and cultured on an orbital shaker at 80 rpm.

In vitro cell viability

To assess viability, cells were retrieved after culture on cryogels using 0.05% trypsin/EDTA at 37 °C for 45 min. Scaffolds were mechanically disrupted with a pipette, and filtered through a 70 µm cell strainer. Cells were stained using the Muse Count and Viability Assay Kit and analyzed using a Muse Cell Analyzer (EMD Millipore). To assess metabolic activity, cryogels were transferred to new wells with 10% AlamarBlue (AbD Serotec) in cell culture medium and incubated for 6 h. The reduction of the substrate was assessed as described in the manufacturer's instructions.

Cryosection preparation and staining

Prior to freezing and sectioning, samples were fixed in 4% PFA in DPBS at room temperature for 20 min. The gels were washed three times in DPBS and placed in a 30% (w/v) sucrose in DPBS solution overnight at 4 °C. Cryogels were then incubated at room temperature in a 15% (w/v) sucrose + 50% OCT compound (Tissue-Tek) for 4 h, and then neat OCT for 4 h. Gels were frozen in OCT on dry ice. The frozen blocks were sectioned at -20 °C into 20 µm sections on a Leica CM1950 cryostat. F-actin was stained using Alexa Fluor 488 phalloidin (Molecular Probes). Samples were mounted in Prolong Gold Antifade Reagent with DAPI (Molecular Probes), coverslipped, and imaged on a Zeiss LSM 710 upright confocal microscope. To visualize proliferation within the cryogels, cells were pulsed with 5-ethynyl-2'-deoxyuridine (EdU; Molecular Probes) for 6 h, fixed, cryosectioned, and stained according to the manufacturer's instructions.

Gelatin zymography

Zymography was performed using a 10% polyacrylamide gel, containing 0.1% (w/v) gelatin or GelMA using the standard protocol [16]. Recombinant human (Chemicon) and murine (R&D Systems) pro-MMP-2 and -9 were used to assess GelMA zymogram degradability. For zymography of *in vivo* implant sites, scaffolds were mechanically disrupted using a 20 G needle in 300 µl of lysis buffer composed of 0.025 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1% v/v IGEPAL CA-630, 10 µg/ml aprotinin, 2 µg/ml leupeptin, and 4 mM benzamidine (Sigma). Samples were vortexed briefly, incubated for 30 min on ice, and centrifuged at 16 000xg to remove debris. The protein content of the resulting lysate was quantified using a bicinchoninic acid assay (Thermo Scientific), and samples were diluted to an equal protein concentration prior to loading for zymography.

In vitro cryogel degradation

Cryogels created using rhodamine-GelMA were incubated with 25 U/ml collagenase type II (Worthington) in DPBS and were placed on an orbital shaker at 80 rpm and 37 °C. The collagenase solution was collected periodically and completely replaced with fresh enzyme solution. Fluorescence of the supernatants with excitation at 550 nm and emission at 580 nm were compared to a standard curve of rhodamine-GelMA also prepared in collagenase. To test the degradability of cryogels by mammalian enzymes, rhodamine-cryoGelMA gels were treated with recombinant murine and human MMP-2 and -9. Recombinant proenzymes were activated with 2.5 mM *p*-aminophenylmercuric acetate (Calbiochem) in zymogram development buffer for 3 h at 37 °C, and each gel was incubated in 100 µl of buffer containing 1 µg of enzyme for a subsequent 18 h. Fluorescence signal from the supernatants was measured on a plate reader, and normalized to fluorescence signal from gels incubated in zymogram development buffer only.

In vivo imaging

In vivo imaging studies were performed using an IVIS Spectrum system (PerkinElmer). Living Image 4.0 (PerkinElmer) software was used for quantitative analysis of all images. Cryogels were injected subcutaneously into mice using a 1 ml syringe equipped with a 16 G needle in 200 μ l DPBS. To monitor gel degradation *in vivo*, rhodamine-GelMA was injected subcutaneously in the flank of C57BL/6J-Tyr^{c-2J} mice and fluorescence signal was monitored longitudinally with excitation and emission measured at 570 nm and 620 nm, respectively. The opposite flank was injected with an unlabeled cryogel, and the fluorescence signal from this site was subtracted to correct for baseline.

To visualize local MMP activity at the cryogel site, mice were maintained for two weeks prior to imaging on an alfalfa-free purified rodent diet (Harlan) in order to reduce food autofluorescence. Next, mice were injected with rhodamine-labeled cryogels, and injected intravenously with MMPsense 750 FAST 1 w later. 6 h post-injection, fluorescence signal was measured at 745 nm excitation and 800 nm emission. Baseline fluorescence signal at the scaffold site prior to MMPsense 750 FAST injection was subtracted for quantitative analysis.

Histology of injected cryogels

Cryogels injected in C57BL/6J mice were retrieved along with the surrounding 1x1 cm skin samples and fixed overnight in 10% neutral buffered formalin. Samples were embedded in paraffin and sectioned at 7 μ m thickness by the Harvard Rodent Histopathology Core. Hematoxylin and eosin (H&E) staining was performed to assess inflammation and cell recruitment at the implant site.

GM-CSF release

5 μ g of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) (PeproTech) was incorporated per cryogel by direct encapsulation prior to polymerization. Gels were retrieved while frozen and placed in 1% BSA fraction V (Roche) in DPBS and placed on an orbital shaker at 80 rpm. The complete supernatant was recovered periodically and frozen until it was analyzed using a murine GM-CSF ELISA (R&D Systems). The amount of GM-CSF in the supernatant of the thawing solution after 1 h was considered unencapsulated and was used to calculate the encapsulation efficiency.

Quantification of in vivo cell recruitment

Blank and GM-CSF-releasing gelatin cryogels were thawed and hydrated for 1 h prior to injection. After the desired implantation period, scaffolds were retrieved and digested in 250 U/ml type II collagenase in DPBS for 45 min at 37 °C, and counted using a Muse Cell Analyzer.

Results

Bulk cryogel behavior

The bulk behavior of cryogels formed with GelMA macromonomer concentrations between 1 and 2% (w/v) was studied to assess their suitability as injectable preformed hydrogels. We found that 1.0% cryoGelMA showed a superior interconnected porosity of $91 \pm 2\%$, relative to 1.5 and 2.0% gels which had mean interconnected porosities of less than 5% (Fig. 1-C). Mechanical testing revealed that cryogels made with 1.5% and 2.0% GelMA led to brittle gels that could not undergo large strains (Fig. S2). However, 1.0% cryoGelMA gels demonstrated complete shape recovery when released after deformation to high strains (Fig. 2-A, Movie S1). 1.0% cryoGelMA gels collapsed dramatically when water was wicked

away from the interconnected pores, and rapidly reassumed their previous shape when rehydrated (Fig. 2-B, Movie S2). Due to their ability to undergo large deformation and allow rapid influx/efflux of water, the potential of 1.0% cryoGelMA gels to be injected through a conventional needle was next analyzed. We found that disc-shaped cryoGelMA gels (5 mm diameter, 2 mm thickness) could collapse, travel through a 16 G needle (1.65 mm inner diameter), and quickly (260 ± 80 ms, $n=10$) return to their original geometry after exiting the needle (Fig. 2-C, Movie S3). Based on these findings, 1.0% cryoGelMA was used in all subsequent studies.

Scaffold architecture

Since cell trafficking within scaffolds is subject to sufficient pore size ($>10 \mu\text{m}$) and interconnectivity, the microarchitecture of cryoGelMA gels was next assessed. SEM revealed a highly porous surface and scaffold interior (Fig. 3-A). Since the processing used to prepare the hydrogels for SEM can cause shrinkage, 2-photon fluorescence imaging of rhodamine-cryoGelMA was used to study the structure of these scaffolds in their hydrated state (Fig. 3-B). The gels demonstrated a highly interconnected pore structure, with the pore diameter increasing with depth into the scaffold from the surface to the interior (Movie S4), likely due to a temperature gradient within the scaffold during the freezing process. The hydrated pore size from the scaffold surface to a depth of $350 \mu\text{m}$ varied between $20\text{--}300 \mu\text{m}$. Together these data indicate that cryoGelMA has an interconnected porous structure with pore sizes compatible with cell trafficking.

Cell compatibility

The suitability of cryoGelMA gels as a substrate for cell attachment and growth was then tested. Free fluid was first wicked from cryoGelMA gels, and a cell suspension of NIH 3T3 fibroblasts, a cell line commonly used for cell compatibility testing, was then placed on the gels. 3T3 cells were found distributed throughout the scaffold volume, as seen with 2-photon microscopy (Fig. 4-A). To study the interaction of cells with cryoGelMA, SEM was used to assess 3T3 cell morphology at the scaffold surface (Fig. 4-B). 3T3 cells attached to the cryoGelMA surface and assumed their characteristic spindle-like morphology after 1 d of culture. Subsequent culture for 2 d led to the formation of a monolayer of cells on the surface of the cryogel, and deposition of extracellular matrix by the fibroblasts on the cryogel surface (Fig. S3). Cell coverage of the cryogel surface was also seen to increase over the culture period using fluorescence microscopy, and high cell viability was maintained on the scaffold surface (Fig. S4). Cryogels were also seen to contract slightly over the 3 d culture period, indicating 3T3 cells were exerting traction forces on the cryogel. Consistent with this observation, F-actin staining of histological sections showed actin stress fiber formation within cells in the scaffold interior (Fig. 4-C). Retrieval and analysis of cells from cryoGelMA scaffolds over the culture period showed maintenance of $>90\%$ cell viability (Fig. 4-D). Cell number and metabolic activity increased over the culture period (Fig. 4-D). Cell proliferation on cryoGelMA scaffolds was further studied by characterizing new DNA synthesis. BrdU incorporation after 1 d of culture showed cells were proliferating on cryoGelMA gels, albeit at a lower rate than cells cultured on tissue culture polystyrene (Fig. S5), as is expected when a high density of cells is cultured in a 3D environment relative to a sparse 2D culture. To assess whether proliferation occurred uniformly on cryoGelMA scaffolds, histological sections were stained after pulsing cell-laden scaffolds with EdU (Fig. 4-E). EdU-positive nuclei could be seen throughout the scaffold thickness. Collectively, these results indicate cryoGelMA is capable of providing a substrate for cell attachment, sustaining cell viability, and allowing cell proliferation.

Enzymatic degradation *in vitro*

Next we tested whether modification of gelatin with methacrylate groups and subsequent cryopolymerization preserved the inherent enzymatic degradability of gelatin. Fluorescent cryogels were first incubated in the presence of 25 U/ml collagenase type II, and fluorescence in the supernatant was monitored as a proxy for gel degradation (Fig. 5-A). This analysis revealed cryoGelMA gels could be degraded completely in the presence of collagenase over a period of 10 d. The ability of the mammalian gelatinases, MMP-2 and -9, to degrade gelatin with a high degree of methacrylation was next assessed. GelMA was incorporated as a substrate into a polyacrylamide gel and zymography was performed with recombinant mouse and human gelatinases (Fig. 5-B). After Coomassie staining of the gel, bands of GelMA degradation were observed with all of the enzymes tested, indicating preservation of enzymatic degradability following methacrylation of gelatin. Short-term degradation studies of rhodamine-cryoGelMA gels by activated mammalian gelatinases were also conducted (Fig. 5-C). All of the enzymes tested were seen to accelerate gel degradation compared to buffer alone. Together these results demonstrate that cryoGelMA can be enzymatically degraded by collagenase and mammalian gelatinases.

In vivo injection

The *in vivo* response to cryoGelMA scaffolds was explored next. CryoGelMA gels injected subcutaneously through a conventional needle regained their original shape under the skin (Fig. 6-A,B). Implanted scaffolds were covered by a thin fibrous capsule after 2 m and underwent a reduction in bulk size. H&E staining revealed mild inflammation at the scaffold border after 1 w with the presence of mononuclear phagocytes and lymphocytes and sparse infiltration of the scaffold by mononuclear cells (Fig. 6-C). At 2 m post-implant, a foreign body reaction was present at the scaffold border with the presence of macrophages, multinucleated giant cells, fibroblasts, and collagen deposition (Fig. 6-D). The interior of the scaffold was acellular at 2 m and no adverse reaction was seen in the skin. These results show that cryoGelMA gels induce only mild acute inflammation after injection followed by a foreign body reaction.

In vivo cell recruitment

As a demonstration of the potential utility of cryoGelMA *in vivo*, the ability of this material to release a chemotactic protein and allow host cell infiltration into the scaffold was analyzed. GM-CSF, a cytokine involved in immune cell development that is being widely explored in cancer vaccines [17], was used as a recruitment signal for immune cells (Fig. 7-A). Direct incorporation of GM-CSF in the prepolymer solution led to $84 \pm 2\%$ encapsulation efficiency after cryopolymerization. GM-CSF is likely to be released by both diffusion out of the cryogel walls and by degradation of the walls *in vivo*. To study the diffusive element of this process, *in vitro* GM-CSF release over a period of 14 d in DPBS containing 1% BSA was analyzed (Fig. 7-B,C). Sustained release of GM-CSF was seen over this period, with an initial burst release followed by a more constant release rate. The total amount of GM-CSF released over this period accounted for only $\sim 2\%$ of the amount encapsulated, indicating that gel degradation is required for significant payload delivery.

Next, GM-CSF-releasing cryoGelMA was injected subcutaneously to assess cell recruitment. Implantation of GM-CSF-releasing scaffolds into C57/Bl6J mice led to recruitment of $\sim 20\times$ more live cells at 14 d post-implant relative to blank cryoGelMA (Fig. 7-D). H&E staining showed thin fibrous capsule formation, and minimal cellular infiltration of blank scaffolds, whereas GM-CSF-releasing gels were surrounded by a thick fibrous capsule and contained a large primarily granulocytic cellular infiltrate (Fig. 7-E). These results show that cryoGelMA can deliver a chemotactic protein and allow cell infiltration and recruitment *in vivo*.

In vivo cryogel degradation

Next, the relative degradation of blank- and GM-CSF-cryoGelMA was monitored by *in vivo* fluorescence imaging of rhodamine-GelMA cryogels. GM-CSF-releasing cryogels degraded much more rapidly than blank cryogels over the course of 18 w (Fig. 8-A,B). Since MMPs are thought to be key players in gelatin degradation, 7 d implanted scaffolds were assessed for the presence of MMPs using gelatin zymography (Fig. 8-C). Degradation bands corresponding to the various molecular weight forms of MMP-2 and -9 [18], were greatly enhanced in GM-CSF-cryoGelMA relative to blank-cryoGelMA. *In vivo* imaging using an MMP-sensitive fluorescent dye revealed MMP activity was concentrated at the implant site of both blank and GM-CSF-releasing cryogels (Fig. 8-D). The magnitude of fluorescent signal from the MMP-sensitive dye at the scaffold site was significantly enhanced in GM-CSF-releasing scaffolds (Fig. 8-E). Collectively, these results suggest that cryoGelMA can be degraded by recruited cells, likely via MMP expression.

Discussion

Our results show that porous cryoGelMA gels of a defined shape can be injected through a conventional needle and regain their geometry and architecture after ejection from the needle bore. This is a result of the thin-walled and highly porous structure of these gels imparted through cryopolymerization [19]. Application of a mechanical stress during injection causes rapid efflux of water from the interconnected pores and collapse of the scaffold structure. Removal of stress after injection releases stored elastic energy and causes water influx into the hydrophilic gel, resulting in recovery of the cryogel shape. Previously reported injectable gelatin-based gels have relied on liquid prepolymers that require crosslinking agents to allow *in situ* gelation [20,21]. The use of a liquid precursor generally does not allow a defined geometry or microarchitecture to be created in the resulting gel, and prepolymer leakage to unwanted tissues may occur prior to gel formation. CryoGelMA gels of predefined shape and porosity can be implanted in a highly localized manner at any site that can be safely accessed with a needle.

In vitro and *in vivo* testing showed that cryoGelMA gels are cell and tissue compatible. CryoGelMA gels allowed cell attachment, spreading, proliferation, and sustained viability of fibroblasts *in vitro*. *In vivo* implanted gels produced mild acute inflammation followed by a foreign body response at the scaffold periphery, which is typical of many biomaterials [22]. Gelatin has been used a substrate for culture of many cell types, motivating our use of this material for the creation of injectable scaffolds. Recently, nanoporous GelMA hydrogels have been shown to be compatible in cell culture applications for a variety of mouse and human cell types in 2D and 3D [10,14,15,23,24]. Cell-laden nanoporous GelMA hydrogels introduced into athymic nude mice were also shown to elicit minimal inflammation and cell infiltration [11,13]. The cell and tissue compatible nature of porous cryoGelMA gels are favorable for applications where cell trafficking within the scaffold is important, such as cell delivery and recruitment.

The results of both *in vitro* and *in vivo* degradation studies show that cryoGelMA gels are enzymatically degradable. Collagenase preparations, and mammalian MMP-2 and -9 were capable of degrading cryoGelMA gels *in vitro*, indicating that the enzymatic degradability of gelatin was preserved after modification with methacrylate pendant groups and cryopolymerization. Controlled release of GM-CSF from the scaffold resulted in complete scaffold infiltration by immune cells, increased MMP activity, and accelerated scaffold degradation. These results are consistent with the known role of GM-CSF as a mediator of immune cell recruitment [25], and as an inducer of MMP production [26,27]. CryoGelMA degraded at a slower rate in the presence of collagenase type II *in vitro* than has been previously reported for nanoporous GelMA hydrogels [28]. CryoGelMA degradation *in vivo*

was also substantially slower than reported for other implantable gelatin formulations [11,29,30]. This enhanced stability of cryoGelMA may be due to an overall higher degree of crosslinking and entangled polymer network density achieved with cryopolymerization relative to conventional bulk hydrogel formation. CryoGelMA gels can undergo cell-mediated remodeling at the injection site, alleviating the need for surgical explant in certain applications.

Our data show that cryoGelMA can provide localized controlled release of proteins. We found that cryoGelMA could release GM-CSF in a sustained manner *in vitro*, and that subcutaneously injected GM-CSF-cryoGelMA released bioactive GM-CSF that caused immune cell recruitment to the scaffold. CryoGelMA allows the encapsulation of proteins in a single step that occurs concurrently with gel formation. This poses an advantage over commonly used protein-loaded gelatin hydrogel fabrication schemes that use crosslinking agents that cause protein deactivation during direct encapsulation (e.g. glutaraldehyde) [31]. Potential limitations of the cryoGelMA system for protein encapsulation may include free radical damage to proteins during polymerization and reaction of free thiol and amine groups on proteins with methacrylate groups on GelMA via Michael addition. Using gelatin as the scaffold material allows release of molecules of various net charges based on the processing used for gelatin fabrication and/or through chemical modifications to alter the electrostatic properties of gelatin [31]. Additionally, the tightly packed, entangled, and highly crosslinked polymer network formed by phase separation during cryopolymerization provides an increased physical barrier to protein release relative to conventionally formed hydrogels. Enzymatic degradation of the cryogel walls by recruited host cells likely facilitates release of entrapped proteins. These properties could allow cryoGelMA to provide cell-triggered release of therapeutic proteins at sites of disease.

Conclusions

The results of this study show that cryoGelMA is a cell and tissue compatible biomaterial that can be injected in a minimally invasive manner through a conventional needle. CryoGelMA can be degraded by MMPs, is capable of controlled release of proteins, and allows cell trafficking within its interconnected pores. These attractive properties position cryoGelMA as a platform for cell-triggered scaffold remodeling and protein release for applications in biomaterials-based therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grant R01 EB015498 to DJM. SK was supported by a NSERC PGS-D and a HHMI ISRF. This work was performed in part at the Center for Nanoscale Systems (CNS) at Harvard University, a member of the National Nanotechnology Infrastructure Network (NNIN), which is supported by the National Science Foundation under NSF award no. ECS-0335765. The authors would like to thank Alexander Cheung, Dr. Manav Mehta, Rajiv Desai, Max Darnell, Anup Tuladhar, Dr. Cathal Kearney and Dr. Yevgeny Brudno for scientific discussions. We especially thank Dr. Roderick Bronson of the Harvard Rodent Histopathology Core for his pathological assessment of H&E sections, and Charles Sheahan of the Harvard Medical School East Quad NMR facility for assistance with NMR.

References

1. Langer R, Vacanti JP. Tissue engineering. *Science*. 1993; 260:920–41. [PubMed: 8493529]
2. Langer R. New methods of drug delivery. *Science*. 1990; 249:1527–33. [PubMed: 2218494]

3. Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. *Nat Biotechnol.* 1999; 17:551–4. [PubMed: 10385318]
4. Ali OA, Huebsch N, Cao L, Dranoff G, Mooney DJ. Infection-mimicking materials to program dendritic cells in situ. *Nat Mater.* 2009; 8:151–8. [PubMed: 19136947]
5. Bae KH, Wang L-S, Kurisawa M. Injectable biodegradable hydrogels: progress and challenges. *J Mater Chem B.* 2013; 1:5371.
6. Yu L, Ding J. Injectable hydrogels as unique biomedical materials. *Chem Soc Rev.* 2008; 37:1473–81. [PubMed: 18648673]
7. Elisseeff J. Injectable cartilage tissue engineering. *Expert Opin Biol Ther.* 2004; 4:1849–59. [PubMed: 15571448]
8. Silva EA, Mooney DJ. Effects of VEGF temporal and spatial presentation on angiogenesis. *Biomaterials.* 2010; 31:1235–41. [PubMed: 19906422]
9. Bencherif SA, Sands RW, Bhatta D, Arany P, Verbeke CS, Edwards DA, et al. Injectable preformed scaffolds with shape-memory properties. *Proc Natl Acad Sci USA.* 2012; 109:19590–5. [PubMed: 23150549]
10. Nichol JW, Koshy ST, Bae H, Hwang CM, Yamanlar S, Khademhosseini A. Cell-laden microengineered gelatin methacrylate hydrogels. *Biomaterials.* 2010; 31:5536–44. [PubMed: 20417964]
11. Chen Y-C, Lin R-Z, Qi H, Yang Y, Bae H, Melero-Martin JM, et al. Functional human vascular network generated in photocrosslinkable gelatin methacrylate Hydrogels. *Adv Funct Mater.* 2012; 22:2027–39. [PubMed: 22907987]
12. Van Den Bulcke AI, Bogdanov B, De Rooze N, Schacht EH, Cornelissen M, Berghmans H. Structural and rheological properties of methacrylamide modified gelatin hydrogels. *Biomacromolecules.* 2000; 1:31–8. [PubMed: 11709840]
13. Lin R-Z, Chen Y-C, Moreno-Luna R, Khademhosseini A, Melero-Martin JM. Transdermal regulation of vascular network bioengineering using a photopolymerizable methacrylated gelatin hydrogel. *Biomaterials.* 2013; 34:6785–96. [PubMed: 23773819]
14. Benton JA, DeForest CA, Vivekanandan V, Anseth KS. Photocrosslinking of gelatin macromers to synthesize porous hydrogels that promote valvular interstitial cell function. *Tissue Eng Part A.* 2009; 15:3221–30. [PubMed: 19374488]
15. Qi H, Du Y, Wang L, Kaji H, Bae H, Khademhosseini A. Patterned differentiation of individual embryoid bodies in spatially organized 3D hybrid microgels. *Adv Mater.* 2010; 22:5276–81. [PubMed: 20941801]
16. Toth, M.; Sohail, A.; Fridman, R. *Methods in Molecular Biology.* Vol. 878. Totowa, NJ: Humana Press; 2012. Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography; p. 121–35.
17. Dranoff G. GM-CSF-based cancer vaccines. *Immunol Rev.* 2002; 188:147–54. [PubMed: 12445288]
18. Snoek-van Beurden PAM, den Hoff Von JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *BioTechniques.* 2005; 38:73–83. [PubMed: 15679089]
19. Henderson TMA, Ladewig K, Haylock DN, McLean KM, O'Connor AJ. Cryogels for biomedical applications. *J Mater Chem B.* 2013; 1:2682–95.
20. Kuwahara K, Yang Z, Slack GC, Nimni ME, Han B. Cell delivery using an injectable and adhesive transglutaminase-gelatin gel. *Tissue Eng Part C Methods.* 2010; 16:609–18. [PubMed: 19757996]
21. Sakai S, Hirose K, Taguchi K, Ogushi Y, Kawakami K. An injectable, in situ enzymatically gellable, gelatin derivative for drug delivery and tissue engineering. *Biomaterials.* 2009; 30:3371–7. [PubMed: 19345991]
22. Mikos A, McIntire L, Anderson J, Babensee J. Host response to tissue engineered devices. *Adv Drug Deliv Rev.* 1998; 33:111–39. [PubMed: 10837656]
23. Ramón-Azcón J, Ahadian S, Obregón R, Camci-Unal G, Ostrovidov S, Hosseini V, et al. Gelatin methacrylate as a promising hydrogel for 3D microscale organization and proliferation of dielectrophoretically patterned cells. *Lab Chip.* 2012; 12:2959–69. [PubMed: 22773042]
24. Aubin H, Nichol JW, Hutson CB, Bae H, Sieminski AL, Cropek DM, et al. Directed 3D cell alignment and elongation in microengineered hydrogels. *Biomaterials.* 2010; 31:6941–51. [PubMed: 20638973]

25. Khajah M, Millen B, Cara DC, Waterhouse C, McCafferty DM. Granulocyte-macrophage colony-stimulating factor (GM-CSF): a chemoattractive agent for murine leukocytes in vivo. *J Leukocyte Biol.* 2011; 89:945–53. [PubMed: 21393420]
26. Mollinedo F, Nakajima M, Llorens A, Barbosa E, Callejo S, Gajate C, et al. Major co-localization of the extracellular-matrix degradative enzymes heparanase and gelatinase in tertiary granules of human neutrophils. *Biochem J.* 1997; 327 (Pt 3):917–23. [PubMed: 9581574]
27. Zhang Y, McCluskey K, Fujii K, Wahl LM. Differential regulation of monocyte matrix metalloproteinase and TIMP-1 production by TNF-alpha, granulocyte-macrophage CSF, and IL-1 beta through prostaglandin-dependent and -independent mechanisms. *J Immunol.* 1998; 161:3071–6. [PubMed: 9743373]
28. Hutson CB, Nichol JW, Aubin H, Bae H, Yamanlar S, Al-Haque S, et al. Synthesis and characterization of tunable poly(ethylene glycol): gelatin methacrylate composite hydrogels. *Tissue Eng Part A.* 2011; 17:1713–23. [PubMed: 21306293]
29. Kang H-W, Tabata Y, Ikada Y. Effect of porous structure on the degradation of freeze-dried gelatin hydrogels. *J Bioact Compat Pol.* 1999; 14:331–43.
30. Tabata Y, Hijikata S, Ikada Y. Enhanced vascularization and tissue granulation by basic fibroblast growth factor impregnated in gelatin hydrogels. *J Control Release.* 1994; 31:189–99.
31. Young S, Wong M, Tabata Y, Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *J Control Release.* 2005; 109:256–74. [PubMed: 16266768]

Figure 1.

Fabrication of gelatin cryogels with highly interconnected pores. (A) Schematic of GelMA synthesis and crosslinking. Pendant methacrylate groups are added primarily to the free amines of gelatin by reaction with methacrylic anhydride. Free radical polymerization results in crosslink formation between methacrylate groups. (B) Cryopolymerization of methacrylated gelatin. Freezing of methacrylated gelatin in the presence of radical initiators (APS and TEMED) allows polymerization to occur in the partially frozen state (cryopolymerization). Ice crystals formed during the freezing process and thawing after cryopolymerization results in the formation of a hydrogel with micron-scale pores. (C) Volume of interconnected pores in gelatin cryogels (normalized to total gel volume). Values

represent mean and standard deviation (n=10). Data were compared using ANOVA with Bonferroni's post-hoc test (**p<0.01, ***p<0.001).

Figure 2.

Bulk mechanical behavior and injectability of gelatin cryogels. (A) Images demonstrating ability of an individual rhodamine-gelatin cryogel to be compressed between forceps (dashed white line) to large strain, followed by release and resumption of its original shape. (B) Rhodamine-gelatin cryogel following collapse due to wicking of free water (0 ms), and rapid rehydration following exposure to excess DPBS. (C) Rhodamine-gelatin cryogel exiting the bore of a 16 G needle (0 ms, outer needle wall outlined with dashed line), and its expansion to original size and shape after injection. All scale bars = 2 mm.

Figure 3. Microarchitecture of gelatin cryogels. (A) Surface and cross-sectional SEM micrographs of highly porous 1.0% (w/v) gelatin cryogels (scale bar = 50 μm). (B) 2-photon imaging at a depth of 150 μm below the surface of a rhodamine-gelatin cryogel (scale bar = 1 mm). The inset shows a magnified view at the center of the scaffold diameter (scale bar = 100 μm). Images are representative of at least 5 gels imaged using each modality.

Figure 4.

Cell attachment, survival, and proliferation on gelatin cryogels in vitro. (A) Representative 2-photon microscopy image of CMFDA-labeled cells at 150 μm depth below the surface of a rhodamine-gelatin cryogel 2 h after seeding ($n=5$, scale bar = 200 μm). (B) SEM micrograph of 3T3 cells spread on crygel surface at 1 d post-seeding ($n=5$, scale bar = 10 μm). Cells are false-colored for emphasis. (C) Staining of F-actin on histological sections showing cell spreading within scaffolds after one day of culture ($n=3$, scale bar = 25 μm). (D) Analysis of the number of live cells, overall metabolic activity as measured by alamarBlue reduction, and viability of cells recovered from gelatin cryogels over time in culture. Values represent the mean and standard deviation ($n=3$). (E) Staining for new DNA synthesis within the scaffold using EdU incorporation ($n=3$, scale bar = 100 μm).

Figure 5.

Enzymatic degradation of gelatin cryogels in vitro. (A) In vitro degradation of rhodamine-gelatin cryogels (n=3) in the presence of 25 U/ml collagenase type II. (B) Zymography with mouse and human pro-MMP-2 and pro-MMP-9 using GelMA-polyacrylamide gels. (C) Quantification of degradation of rhodamine-gelatin cryogels (n=4) in the presence of 10 μ g/ml recombinant mouse and human MMP-2 and -9 for 18 hours. One-way ANOVA with Dunnett's test was performed to compare all MMP-treated conditions with the control gels incubated in buffer alone (n=4, ****p<0.0001). Values represent the mean and standard deviation in all plots.

Figure 6.

In vivo injection of gelatin cryogels. (A) In vivo fluorescence image of rhodamine-gelatin cryogel in a C57Bl/6J mouse immediately following subcutaneous injection. (B) In situ image of rhodamine-gelatin cryogel under the skin at 1 w and 2 m following implant. (C) H&E at 1 w and (D) 2 m following implant at the cryogel-tissue interface (left) and the cryogel interior (right) (n=3, scale bar = 50 μ m). Arrows indicate the cryogel-host border.

Figure 7.

In vivo cell recruitment to gelatin cryogels by sustained release of GM-CSF. (A) Schematic of cell recruitment to GM-CSF-releasing gelatin cryogels. Sustained release of GM-CSF from the cryogel implant creates a chemoattractant gradient to attract host immune cells. (B) In vitro cumulative GM-CSF release and (C) average release rate from gelatin cryogels. (D) Recruited cell numbers in blank and GM-CSF-releasing gelatin cryogels at 14 d post-implant (Student's t-test, $n=3$ mice, $**p<0.01$). (E) Representative H&E staining from blank and GM-CSF-releasing cryogels 14 d after implantation in C57/BL6J mice ($n=3$, scale bar = 500 μm). Inset shows a magnified view of the scaffold interior (scale bar = 20 μm). Arrows indicate the cryogel-tissue borders. Values represent the mean and standard deviation in all plots.

Figure 8.

Gelatin cryogel degradation by recruited cells in vivo. (A) Images and (B) quantification of fluorescence signal from longitudinal in vivo imaging of the degradation of blank and GM-CSF-releasing rhodamine-gelatin cryogels in C57Bl/6J-Tyr^{c-2J} mice (n=5). (C) Gelatin zymography of cellular lysates from 1 w implanted blank and GM-CSF-releasing rhodamine-gelatin cryogels. Representative in vivo (D) fluorescence imaging, and (E) quantitation (paired t-test, n=3, **p<0.01), of MMPsense 750 FAST activation in mice 7 d following injection in opposite flanks with blank and GM-CSF-releasing rhodamine-gelatin cryogels. Scaffold borders are outlined in white in (D–E). Values represent the mean and standard deviation in all plots.