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Drying and Storage Effects on Poly(ethylene glycol) Hydrogel Mechanical Properties and Bioactivity

P.T. Luong, M.B. Browning, R.S. Bixler, and E. Cosgriff-Hernandez

Department of Biomedical Engineering, Texas A&M University, College Station, Texas, 77843-3120, U.S.A

Abstract

Hydrogels based on poly(ethylene glycol) (PEG) are increasingly used in biomedical applications due to the ability to control cell-material interactions by tuning hydrogel physical and biological properties. Evaluation of stability after drying and storage are critical in creating an off-the-shelf biomaterial that functions *in vivo* according to original specifications. However, there has not been a study that systematically investigates the effects of different drying conditions and hydrogel compositional variables. In the first part of this study, PEG-diacrylate hydrogels underwent common processing procedures (vacuum-drying, lyophilizing, hydrating then vacuum-drying) and the effect of this processing on the mechanical properties and swelling ratios was measured. Significant changes in compressive modulus, tensile modulus, and swelling ratio only occurred for select processed hydrogels. No consistent trends were observed after processing for any of the formulations tested. The effect of storage conditions on cell adhesion and spreading on collagen- and streptococcal collagen-like protein (Sc12-2)-PEG-diacrylamide hydrogels was then evaluated to characterize bioactivity retention after storage. Dry storage conditions preserved bioactivity after 6 weeks of storage; whereas, storage in PBS significantly reduced bioactivity. This loss of bioactivity was attributed to ester hydrolysis of the protein linker, acrylate-PEG-*N*-hydroxysuccinimide. These studies demonstrate that these processing methods and dry storage conditions may be used to prepare bioactive PEG hydrogel scaffolds with recoverable functionality after storage.

Keywords

Poly(ethylene glycol) hydrogels; processing; storage; drying; mechanical properties; bioactivity retention

INTRODUCTION

Poly(ethylene glycol) (PEG)-based hydrogels are advantageous for a wide range of biomedical applications due to their established biocompatibility, low toxicity, and highly tunable properties^{1–3}. The ability to tune the physical and chemical properties of PEG hydrogels over a broad range contributes to its versatility as a platform for cell

*Corresponding author: Dr. Elizabeth Cosgriff-Hernandez, Department of Biomedical Engineering, Texas A&M University, 5033 Emerging Technologies Building, 3120 TAMU, College Station, TX 77843-3120, Tel: (979) 845-1771, Fax: (979) 845-4450, cosgriff.hernandez@tamu.edu.

encapsulation⁴, drug delivery^{3, 5–6}, and tissue engineering^{7–8}. Mechanical properties and network architecture are easily modulated by varying macromer concentration, molecular weight, and functionalization, as well as polymerization parameters (e.g., UV exposure time, light intensity^{7, 9–14}). For applications that require a specific biological response, bioactive molecules can be immobilized into the inherently bioinert PEG hydrogel to control cell interactions and promote tissue regeneration^{8, 15–18}. Biomimetic replacements for vascular tissues^{17, 19–21}, cartilage, bone^{22–23}, heart valves²⁴, and neural tissue⁴ have been developed by attaching cell-adhesive ligands, proteins, or growth factors to PEG scaffolds. The presence of these bioactive molecules allows for cell attachment and promotes the production of extracellular matrix (ECM) proteins²¹. Another factor that heavily influences cell adhesion, migration, and phenotype is the mechanical environment. Multiple studies have shown that cells respond differently to substrates with different moduli.^{25–30} For example, two separate studies showed that PEG hydrogels with lower moduli result in improved spreading and migration by preosteoblastic cells²⁹ and enhanced neurite extension by PC12 cells³⁰. Therefore, to elicit the desired biological response, one must precisely tune both the modulus of the hydrogel and the distribution of bioactive molecules within the hydrogel.

Implantable devices undergo processing, sterilization, and storage prior to implantation. These treatments may alter the structure of the scaffold and compromise *in vivo* performance. Although the effects of sterilization on PEG hydrogel properties have been reported in the literature³¹, few studies have addressed how processing and storage may affect hydrogel mechanical properties and bioactivity. Maintenance of scaffold properties and the desired cell/material interactions are especially important for bioactive scaffolds, as cells could respond acutely to small changes in the implant^{25, 32}. Ideally, the hydrogel should rehydrate back to its initial structure and measured properties prior to use. In translating research to clinical and commercial use, it is important to understand how to extend the shelf-life of the bioactive scaffold while retaining desired properties after processing and storage.

The present study investigates the effects of common drying and storage conditions on PEGdiacrylate (PEGDA) hydrogel mechanical properties and bioactivity. PEGDA hydrogels were formed via photopolymerization and processed by vacuum drying or lyophilizing. Tensile properties, compressive modulus, and swelling ratio were evaluated after processing and compared to swollen hydrogel controls. To investigate how storage affects bioactivity, collagen or the streptococcal collagen-like protein, Scl2-2, was incorporated into PEGDA hydrogels. The ability for endothelial cells to adhere and spread on these bioactive hydrogels after storage in dry or hydrated conditions for six weeks was then quantified. Results from this investigation will identify key factors in selecting the proper processing and storage conditions in the preparation of hydrogels for biomedical applications.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Aldrich (Milwaukee, WI) and were used as received unless stated otherwise.

PEGDA and PEGDAA synthesis

PEGDA was synthesized from a protocol adapted from Hahn, et al.³³ PEG-diol (3.4 or 6 kDa) was dissolved in anhydrous dichloromethane (DCM) under nitrogen. Triethylamine (TEA) and acryloyl chloride were slowly added at a 1:2:4 molar ratio of PEG:TEA:acryloyl chloride. The solution was allowed to react while stirring under nitrogen for 24 hours at room temperature, after which it was washed with 8 molar equivalents of 2 M potassium carbonate to neutralize acrylic acid byproducts. Water was removed by stirring the polymer solution with anhydrous sodium sulfate. Finally, the acrylated polymer was precipitated in cold diethyl ether, vacuum filtered, and dried under vacuum for 24 hours.

PEG-diacrylamide (PEGDAA) was synthesized according to a similar protocol, using PEG diamine (3.4 kDa) as the starting material. In brief, a reaction mixture of PEG-diamine, acryloyl chloride, and TEA (1:2:4 molar ratio) were stirred in DCM under nitrogen for 24 hours at room temperature. Acidic byproducts were neutralized with 8 molar equivalents of 2 M potassium carbonate. Water was removed by mixing with anhydrous sodium sulfate. The polymer was then precipitated in cold diethyl ether, vacuum filtered, and dried under vacuum for 24 hours.

Functionalization was confirmed with Fourier transform infrared spectroscopy (FTIR) and proton nuclear magnetic resonance (¹H-NMR) spectroscopy. First, dilute polymer solutions in DCM (5 mg/mL) were applied and dried on potassium bromide pellets. Infrared spectra were then recorded on a Bruker TENSOR 27 spectrometer. Successful acrylation of PEG was indicated by the presence of the ester carbonyl peak at 1730 cm⁻¹ and the loss of the broad hydroxyl peak at 3300 cm⁻¹. Acrylamide functionalization was confirmed by the presence of the amide carbonyl peak at 1645 and 1675 cm⁻¹ and the broad amine peak at ~3500 cm⁻¹.

Polymers were dissolved in deuterated chloroform (CDCl₃, 10mg/mL) and ¹H-NMR spectra recorded on a Mercury 300 MHz spectrometer using tetramethylsilane as an internal reference. The conversions of hydroxyl endgroups to acrylates in the PEGDA synthesis and to acrylamides in the PEGDAA synthesis were over 85%. PEGDA ¹H-NMR (CDCl₃): 3.6 ppm (m, -OCH₂CH₂-), 4.3 ppm (t, -CH₂OCO-), 5.8 ppm (dd, -CH=CH₂), 6.1 and 6.4 ppm (dd, -CH=CH₂). PEGDAA ¹H-NMR (CDCl₃): 3.6 ppm (m, -OCH₂CH₂-), 5.6 ppm (dd, CH₂=CHCON-), 6.1 ppm and 6.2 ppm (dd, CH₂=CH-CON-).

PEGDA hydrogel fabrication

Polymer solutions were prepared by dissolving PEGDA (3.4 or 6 kDa) in distilled water to achieve concentrations of 10, 20, or 30 wt%. The photoinitiator, 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (Irgacure 2959, 1 mg per 10 μL of 70% ethanol), was added to each solution at 1% of the total volume. After vortexing, the hydrogel precursor solution was pipetted into cylindrical molds (3 mm inner diameter, 4 mm outer diameter) or sheet molds (1.5 mm thick) and polymerized by exposure to UV light for 6 minutes (Ultraviolet Products High Performance UV Transilluminator, 365 nm, 1mW/cm²). Hydrogels were removed from the molds and prepared for processing and characterization tests as described below.

Hydrogel processing

Hydrogel samples were subjected to one of four possible processing treatments after photocrosslinking: (i) hydration in water (control), (ii) hydration in water followed by vacuumdrying at -30 psi (swell-dried), (iii) vacuum-drying (dried), or (iv) flash freezing in liquid nitrogen followed by vacuum-drying (lyophilized) (LabConco CentriVap Cold Trap, Kansas City, MO). Dried hydrogels were rehydrated prior to mechanical testing and cell culture analysis. Each step was carried out for 24 hours to achieve equilibrium swollen or dried states.

Swelling ratio

Cylindrical discs (8 mm diameter) were punched from 1.5-mm thick hydrogel sheets for swelling ratio measurements. Discs were processed, soaked in water for 24 hours, and weighed to measure the equilibrium swelling mass (W_s). Discs were then vacuum-dried for 24 hours to measure the dry polymer mass (W_d). Equilibrium mass swelling ratio, Q , was calculated as the ratio of the measured masses:

$$Q = W_s / W_d$$

Dynamic mechanical analysis

Discs (8 mm diameter, 1.5 mm thick) were processed and rehydrated prior to compression testing. Unconstrained compression tests were performed at room temperature using a dynamic mechanical analyzer fitted with parallel-plate compression clamps (RSAIII, TA Instruments). Dynamic strain sweeps (0.001–30% strain) were used to determine the linear viscoelastic range for each hydrogel formulation/processing combination. A strain in the upper end of the linear viscoelastic region was used to perform frequency sweeps. Frequency sweeps were performed between 0.79 and 79 Hz, and compressive modulus for each specimen was recorded at 1.25 Hz.

Tensile testing

Hydrogel tubes were cut into 3 to 5 mm long sections and processed for tensile testing. Following processing and rehydration, samples were mounted onto an Instron 3345 fitted with copper hooks and strained at a constant strain rate (6 mm/min) until fracture. The tangential modulus of elasticity was calculated from the resulting stress-strain plot by fitting a line to the strain region between 9 and 17 kPa (within 30% of physiological blood pressure). The ultimate tensile strength (UTS) and ultimate elongation (UE) were also measured from stress-strain plots.

Protein functionalization for storage study

Non-gelling rat tail collagen type I and Scl2-2 were functionalized with photoreactive PEG crosslinkers to allow for incorporation into the hydrogel network. Scl2-1 is an inherently non-adhesive protein that is amplified and purified from the serotype M28 strain MGAS6274A.^{34–38} The Scl2-2 variant of this gene was produced by inserting the sequence GFPGER, as previously described.³⁹ Each protein has 33 lysines with available ϵ -amino groups that can be reacted with NHS esters. Proteins were functionalized with acrylate-PEG-

N-hydroxysuccinimide (Acr-PEG-NHS, MW 3500, Jenkem Technologies USA, Allen, TX) following a procedure adapted from Sebra, et al.⁴⁰ Briefly, collagen or Scl2-2 was combined with Acr-PEG-NHS in 50 mM sodium bicarbonate buffer (pH 8.5) and allowed to react for 24 hours while stirring at room temperature. Both proteins were functionalized at 0.5X functionalization density (0.5:1 molar ratio of Acr-PEG-NHS:amine). The protein solution was dialyzed against 0.1 M hydrochloric acid for 24 hours to remove basic byproducts and then purified by dialysis against de-ionized water for 24 hours (molecular weight cutoff = 20,000). Functionalized proteins were lyophilized to obtain a dry protein powder, and functionalization was confirmed with FTIR spectroscopy.

Bioactive PEGDAA hydrogel preparation

Collagen and Scl2-2-PEGDAA hydrogel precursor solutions were prepared by dissolving functionalized proteins (4 mg/mL), 10 wt% PEG(3.4 k)DAA, and 1% (w/v) Irgacure 2959 in 20 mM acetic acid. Solutions were crosslinked between 0.5-mm spaced plates and photopolymerized under UV light as described above. Discs (6-mm diameter) were cut from the sheets, sterilized for 24 hours in 70% ethanol, and stored for 6 weeks. Three storage methods were evaluated: (i) storage in PBS at 20°C, (ii) lyophilization then dry storage at -20°C, or (iii) lyophilization then dry storage at 20°C.

Cell culture, seeding, and analysis after storage

Bovine aortic endothelial cells (BAECs, Cell Applications) were cultured *in vitro* at 37°C/5% CO₂ in Dulbecco's Modified Eagle Medium (high glucose, GlutaMAX™, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), and 1% Penicillin-Streptomycin (Gibco). Cells were used between passages 9 and 12 after 7–10 days of culture.

After 1 week or 6 weeks of storage, samples were rehydrated in PBS and seeded with BAECs (10,000 cells/cm²). Following a 3-hour incubation (37°C/5% CO₂), cells were stained with rhodamine phalloidin (F-actin/cytoplasm) and SYBR green (DNA/nucleus). Representative images were taken on a Zeiss Axiovert fluorescent microscope. The smart scissor tool in GIMP was used to select rhodamine phalloidin-stained areas and the number of pixels evaluated using the histogram function. SYBR green-stained nuclei were counted as attached cells. These values were used to calculate cell adhesion and spreading area by:

$$Adhesion = C / A \quad Spreading = P / A$$

where *C* is the number of cells counted on the image, *A* is total image area (converted from pixels to microns based on objective scales), and *P* is the rhodamine phalloidin-stained area in pixels. Spreading was then converted to microns using the same scales.

Statistical Analysis

Data were presented as averages ± standard deviation. Student unpaired t-tests were used to perform statistical analysis of all data with statistical significance taken for *p* < 0.05.

RESULTS

Control hydrogel properties

Two molecular weights and three different concentrations of PEGDA were used to create hydrogels with a range of mechanical properties. Lower macromer molecular weight and higher macromer concentration resulted in higher compressive and tensile moduli at the expense of swelling ratio (Figure 1). The moduli measurements and trends were similar to previously reported values¹⁰. Ultimate tensile strength and ultimate elongation exhibited large variances (Figure 2). Generally, higher molecular weight increases both UTS and UE while higher concentrations increases UTS but decreases UE.

Effects of vacuum-drying after photocrosslinking

Vacuum-drying is often used to dry materials that are sensitive to heat damage. In this study, vacuum-drying introduced significant effects in only a select number of hydrogel formulations. Significant increases were observed in the compressive modulus of the 20% 6kDa hydrogels and the tensile moduli of 10 and 20% 3.4kDa hydrogels (Figures 1A and 2A). Decreases were observed in the compressive modulus of the 10% 3.4kDa hydrogel and the swelling ratio of the 30% 6kDa hydrogel (Figure 1B). Interestingly, vacuum-drying increased the tensile modulus of all formulations, but increased the compressive modulus of only two of the formulations. Taken together, these isolated and opposing results do not indicate that vacuum-drying after fabrication introduces significant or reproducible effects.

Effects of vacuum-drying after hydrating

Swell-dried hydrogels were first hydrated in water after photocrosslinking and then vacuum-dried. This two-step process only produced significant increases on the compressive modulus of the 10% 3.4kDa hydrogel and on the swelling ratio of the 20% 3.4kDa hydrogel (Fig 1A-B). If the swell-dry process was affecting the crosslink density of the network, increases in compressive modulus would be expected to correlated with decreases in swelling ratio. Additionally, compressive modulus and swelling of the other hydrogel formulations, along with tensile properties of all formulations, were unaffected by the swell-dry process. There were no evident trends to suggest that vacuum-drying after hydrating significantly affected hydrogel properties.

Effects of lyophilizing after photocrosslinking

Lyophilization was performed by flash-freezing hydrogel specimens in liquid nitrogen for 20–30 seconds, followed by vacuum-drying on a cold trap for 24 hours. The compressive moduli of all formulations after lyophilization were statistically similar to controls (Fig. 1A). Interestingly, all lyophilized hydrogels exhibited higher UTS and UE than controls, but these values have large variances and were not statistically significant (Figures 2B-C). Only the swelling ratio and tensile modulus of the 30% hydrogels were significantly altered after lyophilizing. The 3.4kDa hydrogel experienced an increase in swelling ratio coupled with a decrease in tensile modulus. In contrast, the 6kDa hydrogel had a decrease in swelling ratio and an increase in tensile modulus. If lyophilization did affect hydrogels of higher concentration more acutely than lower concentrations, the effect would have been mirrored

in both molecular weight hydrogels. These opposing and isolated results are likely anomalies and do not imply that lyophilization affects hydrogel mechanical properties.

Hydrogel bioactivity after storage

To assess how storage conditions affect bioactivity, PEGDAA hydrogels with covalently crosslinked collagen or Scl2-2 protein were fabricated and stored for up to 6 weeks in three different conditions: in PBS at 20°C, lyophilized dry and stored at -20°C, or lyophilized dry and stored at 20°C. Bioactivity was measured by seeding endothelial cells onto the stored hydrogels after 1 week or 6 weeks and quantifying the degree of cell adhesion and spreading. Cell adhesion was maintained at 1 week of storage in PBS at 20°C for both bioactive hydrogels but was reduced by 50% after 6 weeks of storage in PBS (Figures 3–5). Cell spreading on Scl2-2- PEGDAA hydrogels was also significantly diminished after 6 weeks of storage in PBS (Figure 4). These results indicate that long-term storage in PBS at 20°C leads to a significant reduction in cell adhesion and spreading on bioactive hydrogels.

Cells were generally well spread after 6 weeks of storage in dry conditions for both bioactive hydrogels (Figures 3–4). A gradual reduction in cell adhesion and spreading on the collagen-PEGDAA hydrogels was observed, but was only significant for spreading after 6 weeks of storage. In contrast, cell adhesion and spreading on Scl2-2-PEGDAA hydrogels were generally unaffected by dry, 20°C storage after 1 and 6 weeks. Bioactivity of the Scl2-2- PEGDAA hydrogels was preserved best by the dry, 20°C storage condition. These warmer conditions may be suitable for Scl2-2 protein storage but are not advisable for collagen due to the adverse effects on cell spreading.

Dry, -20°C storage conditions resulted in only minor reductions in cell adhesion and spreading for both hydrogels after 6 weeks. Bioactivity at 6 weeks was higher under these storage conditions than under PBS. Cell adhesion and spreading on collagen-PEGDAA hydrogels were preserved best in dry, -20°C storage conditions after 6 weeks.

Lyophilization and storage at cooler (i.e., frozen) temperatures seem to maintain long-term bioactivity for collagen- PEGDAA hydrogels, whereas lyophilization and storage at 20°C maintains long-term bioactivity for Scl2-2-PEGDAA hydrogels.

DISCUSSION

Processing effects on hydrogel mechanical properties

Modulation of the physical, chemical, and biological environment of PEG hydrogels is a useful and facile method for controlling cellular response. However, post-fabrication processes can impact the gel network architecture and result in unintended changes in properties and corollary effects on cell behavior. Hydrogels are often vacuum-dried or lyophilized to achieve a dry state for storage. Previous studies reported changes in scaffold composition after drying and rehydrating,^{41–43} but it has been unclear whether these macroscopic changes affected mechanical properties and function. Both air-dried and freeze-dried oligo(ethylene glycol)fumarate (OPF) hydrogels exhibited reduced swelling that resulted in higher cell attachment⁴³. It was speculated that ice crystals formed during lyophilization could potentially alter the OPF hydrogel microarchitecture.⁴² Examination

showed that compressive stiffness of rehydrated lyophilized hydrogels was lower than freshly synthesized hydrogels, but the reported values were statistically similar. Although these studies indicate that processing may impact gel properties, there has not been a study that systematically investigates the effects of different processing conditions and hydrogel compositional variables.

In the current study, we examined the effect of vacuum-drying and lyophilization on hydrogel physical properties for a broad range of hydrogel formulations. Vacuum-drying immediately after crosslinking or after a 24 hour swelling period were also evaluated to investigate the potential effect of residual reactive species on hydrogel properties. A number of impurities have been found in UV-cured PEGDA/2-ethylhexyl acrylate hydrogels, including free PEG, photoinitiator, and polymer chains not attached to the network⁴⁴. Even though the fraction of impurities is small, there is a possibility that unreacted acrylate endgroups may postpolymerize and induce increased crosslink density and stiffness. Hydrogels are typically soaked in water after curing to remove unreacted macromer⁴⁵ or residual solvents and to reach equilibrium swollen state. Without pre-swelling, components containing unreacted endgroups remain in the network and are brought closer together as the hydrogel shrinks during vacuum drying. This increases the likelihood that acrylate endgroups will react with a corollary increase in the crosslink density that generates a stiffer hydrogel upon rehydration.⁴⁶

Vacuum Drying Effects—Small increases in tensile moduli were observed after vacuum-drying without pre-swelling; however, these differences were only statistically significant for the 10 and 20% 3.4kDa hydrogels. Higher tensile moduli in hydrogels are generally indicative of increased crosslink density. Reduced swelling also suggests increased crosslink density and was observed in all hydrogels except 20% and 30% 3.4 kDa formulations. If crosslinking during vacuum-drying was occurring, then it may only be a significant factor in hydrogel compositions at lower concentrations. Reduced gel fractions from precursor solutions of lower macromer concentrations have been reported in the literature.^{47–49} This suggests that low PEGDA concentration formulations have increased levels of unreacted macromer or sol fraction. Propagation in photo-crosslinking reactions occurs randomly to produce both network-bound chains (gel fraction) and free chains (sol fraction).^{50–51} Unreacted chain ends on macromer or microgels in the sol could then react with dangling network chain ends during vacuum-drying to increase the crosslink density, as observed. However, these are only minor trends that are opposed by decreases in compressive moduli of vacuum-dried hydrogels, which may be linked to the geometry of the hydrogel. Hydrogel discs were used for compression testing and swelling studies, but short hollow tubes were used for tensile testing. In addition, a previous study on the stability of a multilayer vascular graft composed of a PEG inner layer and an electrospun polyurethane outer mesh found no macroscopic effects nor significant changes in biomechanical properties following vacuum-drying¹⁹. As an isolated material and a component of a device, PEG hydrogels appear to maintain macroscopic properties after vacuum-drying.

Soaking the hydrogel for 24 hours before vacuum-drying only affected the compressive modulus of the 10% 3.4kDa hydrogel and the swelling ratio of the 20% 3.4kDa hydrogel. In accordance with the previous discussion, pre-swelling removes unreacted monomer that may

have contributed to the previous changes observed in low concentration formulations after vacuum-drying. Control hydrogels that were soaked in water for 24 hours and then tested without drying displayed similar properties as the vacuum-dried hydrogels. The minimal effects of this processing method suggest that soaking to remove residual unreacted macromers followed by vacuum-drying is a suitable procedure for maintaining properties post-fabrication.

Lyophilization Effects—Lyophilization is commonly used to prepare hydrogels for storage with the intention of preserving network architecture⁴¹ and to improve handling, transportation, and storage.⁵² Studies have shown that lyophilization can introduce micropores into the hydrogel structure, which increases flexibility, reduces strength, and improves swelling ratio and rate.^{41, 53–54} For example, freeze-dried chitosan-poly(ethylene oxide) and polyvinylpyrrolidone hydrogels became highly porous compared to air-dried counterparts.^{39–40} In this processing study, small increases in UE were observed across all lyophilized hydrogels (Figure 2C), which supports the finding of increased flexibility. The UTS of lyophilized hydrogels also displayed small increases relative to controls, which contradicts the observation that lyophilization reduces strength (Figure 2B). Although these trends were consistent across all formulations, the changes were not significantly different from controls, which corroborates findings between freshly synthesized and rehydrated lyophilized OPF hydrogels.⁴² In addition, lyophilization did not produce significant effects on compressive modulus nor have recurring effects on swelling ratio or tensile modulus. Therefore, if micropores were introduced into the hydrogels after lyophilization, their presence was not sufficient to significantly or consistently affect the swelling and mechanical properties.

In total, statistically significant changes after processing were observed for a small number of hydrogel formulations and not reproduced across different concentrations or molecular weight. The data suggest that hydrogel properties are not significantly altered from the as-fabricated, swollen state by the three processing conditions tested. However, there remain a large number of processing parameters not tested here that may change swelling and mechanical properties including moisture, temperature, light, excipients, and oxygen.⁵⁵ It is essential that the final *in situ* conditions be considered when processing a hydrogel product and that these parameters be thoroughly investigated to ensure product stability throughout its service lifetime.

Effects of storage conditions on bioactivity

To produce bioactive hydrogels, proteins are typically immobilized in the matrix to provide cell-binding sites or biological cues. Proteins are capable of aggregating, denaturing, or losing their active conformations if not in their natural environments.⁵⁶ Storage conditions may affect the protein, the matrix, or the linker between the two. Collagen and Sc12-2 proteins in PEGDAA hydrogels were used in this storage study as model systems containing cell-binding proteins. PEGDAA was selected as the hydrogel matrix given its increased stability over PEGDA. It has been shown that these esters introduced into the PEGDA macromer are susceptible to hydrolysis such that PEGDA hydrogels may degrade *in vivo* over several months to years.^{57,58} PEGDAA has statistically similar swelling and

mechanical properties as PEGDA formulations but dramatically enhanced hydrolytic stability which isolates the effect of storage conditions to the functionalized protein.⁵⁷ Lyophilization was selected as the drying condition due to the minimal effects observed on swelling and mechanical properties.

The results of a 6 week study indicate that storage of bioactive PEG hydrogels in dry conditions was preferable over storage in buffer at 20°C to maintain bioactivity. Loss of bioactivity after 6 weeks in PBS was attributed to hydrolysis and protein loss. The hydrogel network is primarily composed of crosslinked PEGDAA, which consists of the hydrolytically stable ether backbone of PEG and the amide bonds from the acrylamide endgroups. After 6 weeks of storage, the hydrogel showed no macroscopic effects of dissolution. Therefore, the reduction in bioactivity was attributed to degradation of the protein linker. In a related study, a significant reduction in protein retention was found on 0.5X Acr-PEG-functionalized collagen and Scl2 PEGDAA hydrogels after 6 weeks of swelling in PBS⁵⁹. The reduction in protein retention led to corollary decreases in spreading for collagen and reduced adhesion and spreading for Scl2-2. It was proposed that the acrylate ends of functionalized collagen and Scl2-2 contain ester bonds which are subject to hydrolysis. The proteins are severed from the hydrogel upon chain scission of this hydrolytically labile bond and diffuse out, reducing the number of cellbinding sites over time. In contrast, the lack of water in dry storage conditions prevents ester bond hydrolysis and maintains bioactivity.

A point of concern is protein stability during storage, which influences bioactivity. Generally, advised storage temperatures for proteins are 2–8°C. This study explored the possibility for dry storage or room temperature aqueous storage of bioactive hydrogels as more accessible storage options than refrigeration or freezing. Collagen stored in aqueous solutions at pH 7 is reported to gel and denature around body temperature, but is capable of refolding into its native triple helix below 30°C.^{60,61} Degradation of hydrated collagen fibrils occurs above 300°C.⁶² Both collagen and Scl2-2 hydrogels were stored in PBS at room temperature, which is much lower than the reported degradation temperature. It is therefore unlikely that the proteins are denaturing or degrading during storage. Additionally, PEGDAA hydrogels containing acrylate-PEG-functionalized collagen or Scl2 (1X) were incubated at 37°C in PBS for 6 weeks with no differences in bioactivity compared to 1 day swelling.⁵⁹ These results indicate that the proteins are not denaturing in the timeframe of this study and that losses in bioactivity can solely be attributed to hydrolytic degradation of the protein linker.

CONCLUSION

Ideally, processing and storage of products seeks to preserve the form and function as close to the manufactured state as possible. The performance of tissue-engineered hydrogel constructs is highly sensitive to changes in macroscopic properties and bioactivity; therefore, stability tests are crucial to determine the scaffold's shelf-life and guarantee quality upon use. For situations where demand for the product exceeds supply, as for tissue implants, the ability to manufacture and store a large amount is desirable to reduce patient wait-time. The goal of these studies was to determine the effect of common drying processes

on PEG hydrogel swelling and mechanical properties. PEG-based hydrogels were photopolymerized and subjected to various processing and storage conditions to investigate possible alterations to mechanical and bioactive properties, respectively. Vacuum-drying, hydration followed by vacuum-drying, and lyophilization only introduced significant changes in compressive modulus, swelling ratio, and tensile modulus of isolated hydrogel compositions. Given that there were no consistent trends in these effects, it was concluded that hydrogel physical properties were not significantly altered after processing. PEG-diacrylamide gels containing acrylate-PEG-linked collagen or Scl2-2 protein were found to retain cell adhesion and spreading better in dry conditions than in buffer after 6 weeks of storage. The significant reduction in cell adhesion after storage was attributed to protein loss due to ester hydrolysis of the protein linker. These initial stability studies demonstrate that different processing routes can be taken to prepare PEG hydrogels for storage without affecting mechanical properties and swelling behavior upon rehydration. Hydrolysis during storage and subsequent reduction in bioactivity can be circumvented by storing the scaffold under dry conditions.

Choice of sterilization, in addition to processing and storage conditions, is also a vital post-fabrication procedure before the product can be used for *in vivo* applications. Considerable attention has already been given to the effects of various sterilization procedures on the fragile hydrogel structure. For example, steam and gamma radiation sterilization severely were shown to compromise PEGDA hydrogel structure, whereas dense-carbon-dioxide sterilization effectively eliminated bacterial spores with minimal or no effects on hydrogel properties^{31,63}. In addition, PEGDA and PEGDA-collagen hydrogels exhibited similar endothelial cell adhesion and morphology after vacuum drying and ethylene oxide sterilization as compared with untreated hydrogels¹⁹. This particular result exhibits the potential for a hydrogel-based medical device to undergo processing and sterilization and be reconstituted without detriment on bioactivity and mechanical performance. By selecting appropriate processing and storage conditions in conjunction with a suitable sterilization method, the properties of an off-the-shelf bioactive scaffold can be maintained throughout its shelf life and ensure quality of function during its service life.

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Figure 1.

(A) Compressive modulus and (B) swelling ratio of PEG(3.4k)DA and PEG(6k)DA hydrogel discs. Hydrogel sheets were fabricated 0.5 mm thick for swelling or 1.5 mm thick for compression testing. Discs 8 mm in diameter were punched from hydrogel sheets. ($n=6$, mean $\pm$ standard deviation, $*p < 0.05$ relative to control of similar concentration (10, 20, or 30 wt%) and macromer weight).

Figure 2.

(A) Tensile modulus, (B) ultimate tensile strength, and (C) ultimate elongation at strain failure of PEG(3.4k)DA and PEG(6k)DA hydrogel rings (3–5 mm long). ($n=6$, mean $\pm$ standard deviation, * $p < 0.05$ relative to controls of similar concentration (10, 20, or 30 wt %) and macromer weight).

Collagen-PEGDAA

Figure 3. Endothelial cell attachment to collagen-PEGDAA hydrogels after 6 weeks of storage. (A) Control (no storage). (B) Lyophilized, then stored dry at 20°C. (C) Lyophilized, then stored dry at -20°C. (D) Stored in PBS at 20°C.

Scl2-2-PEGDAA

Figure 4. Endothelial cell attachment to Scl2-2-PEGDAA hydrogels after 6 weeks of storage. (A) Control (no storage). (B) Lyophilized, then stored dry at 20°C. (C) Lyophilized, then stored dry at -20°C. (D) Stored in PBS at 20°C.

Figure 5. Endothelial cell (A) adhesion and (B) spreading on collagen-PEGDAA and Scl2-2PEGDAA hydrogel discs (0.5 mm thick, 6 mm diameter) after 0, 1, and 6 weeks of storage. Hydrogel discs were stored in PBS at 20°C, lyophilized then stored dry at 20°C, or lyophilized then stored dry at -20°C. (Collagen $n=10$, Scl2-2 $n=9$, mean $\pm$ standard deviation, * $p < 0.05$ relative to control of similar storage condition).