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Biomaterials 24 (2002) 509–523

Biomaterials

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Synthesis and physicochemical analysis of gelatin-based hydrogels for drug carrier matrices

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Received 4 June 2002; accepted 6 August 2002

Abstract

This study examined the interrelated effect of environmental pH, gelatin backbone modification and crosslinking modality on hydrogel morphology, surface hydrophilicity, in vitro swelling/degradation kinetics, in vitro drug release kinetics and in vivo degradation, inflammatory response and drug release activity. The percent glutaraldehyde fixation had a greater impact on the morphology of the dehydrated hydrogels than gelatin modification. Any decrease in percent glutaraldehyde fixation and/or modification of gelatin with polyethylene glycol dialdehyde (PEG-dial) and/or ethylenediaminetetraacetic dianhydride (EDTAD) increased hydrogel surface hydrophilicity. Swelling/degradation studies showed that modification of gelatin with PEG-dial generally increased the time to reach the maximum swelling weight ratio (T_{max}) and the time to failure by hydrolysis (T_{fail}), but had little effect on the maximum swelling weight ratio (R_{max}) and the weight ratio at failure (R_{fail}). Modification of gelatin with EDTAD generally had no effect on T_{max} and T_{fail}, but increased R_{max} and R_{fail}. Modification of gelatin with PEG-dial and EDTAD increased R_{max}, but had no effect on T_{max}, R_{fail}, or T_{fail}. Decreasing percent glutaraldehyde fixation generally increased R_{max} and R_{fail} but decreased T_{max} and T_{fail}. Decreasing environmental pH from 7.4 to 4.5 had no effect on any swelling/degradation properties. In vitro drug release studies showed that modification of gelatin with PEG-dial and/or EDTAD generally decreased the maximum mass ratio of drug released (D_{max}) and the time to reach D_{max} (T_{dmax}). Percent glutaraldehyde fixation did not significantly affect D_{max} or T_{dmax} (except for EDTAD-modified gelatin hydrogels). In vivo studies showed that gelatin-based hydrogels elicited comparable levels of acute and chronic inflammatory response as that of the empty cage control by 21 d.

Keywords: Biomaterials; Polyethylene glycol; Glutaraldehyde; Swelling/degradation; Drug delivery; In vivo biocompatibility

1. Introduction

A hydrogel is a three-dimensional network composed of a polymer backbone, water, and a crosslinking agent to produce a complex network of high molecular weight. A plethora of hydrophilic polymer backbones have been explored for use as hydrogels, including polyethylene glycol (PEG) [1] and dextran [2]. Crosslinking modalities range from covalent chemical crosslinking via low molecular weight, difunctional compounds (i.e. glutaraldehyde or formaldehyde) [3,4] to radical polymerization via radical-forming polymer end-groups or difunctional compounds (i.e. oligo(PEG fumarate) [1], methacrylated dextran [2], acrylated copolymers of poly(lactic acid) and PEG [5]) to self-crosslinking via inter- and intra-polymer chain condensation due to exposure to high temperatures, reduced pressures and dehydration [4]. Because of the complex, three-dimensional hydrophilic structure, hydrogels are capable of absorbing large amounts of aqueous solution and undergoing degradation via erosion, hydrolysis, solubilization, and other biodegradation mechanisms. Thus, hydrogels have been explored for many uses, including drug delivery devices, wound dressing materials, contact lenses, and cell transplantation matrices [6].

Because of the wide range of unique combinations of polymer backbone and crosslinking agent, properties such as hydrogel swelling/degradation, mechanical strength and drug release kinetics are interrelated in a

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0142-9612/02/$ - see front matter
PII: S0142-9612(02)00369-1

complex and dynamic fashion. However, these characteristics could potentially be tailored to meet a specific biomedical application by modulating hydrogel compositions. Gelatin, obtained by partial degradation of water-insoluble collagen fibers [6], has been chosen as the hydrogel polymer backbone for our study because of the following physicochemical properties of gelatin: (i) great capacity for modification at the level of amino acids, (ii) low level of immunogenicity and cytotoxicity, (iii) FDA approval as a clotting agent and exudate-absorbing construct, (iv) hydrogel formation by facile procedures [7], and (v) ability to biodegrade. Specifically, we have explored modification of the gelatin backbone with PEG-dialdehyde and/or ethylenediaminetetraacetic dianhydride (EDTAD) to alter the physicochemical properties of the gelatin, and to affect the subsequent release, degradation and solubility of model drugs from and within the hydrogel. PEG is widely accepted as having low immunogenicity and cytotoxicity, and PEG-conjuated proteins have exhibited an enhanced biocompatibility and a reduction in degradation rate [8,9]. EDTAD has low toxicity because the only reactive group introduced into the network is the carboxyl group, and lysyl residues of gelatin can be modified with EDTAD in a relatively fast reaction [10]. Furthermore, modification of gelatin with EDTAD introduces polyanionic molecules into the gelatin chain, increasing the hydrophilicity of the gelatin backbone with the addition of charged groups, and thereby potentially improving the swelling capability of the resulting hydrogel. Additionally, we modulated the crosslinking modality (i.e. percent glutaraldehyde or self-crosslinking via exposure to dry heat) of unmodified and modified gelatin to affect the solubility and density of the resulting matrix, which contributed to the swelling/degradation and the release mechanism of therapeutic agents.

By controlling the gelatin backbone structure in tandem with crosslinking modality and environmental pH, our goal is to elucidate the overall and differential effects on the hydrogel morphology, surface hydrophilicity, swelling/degradation kinetics, in vitro drug release/degradation/solubility and in vivo degradation, inflammatory response and activity of drug released. We hypothesized that by modifying the gelatin backbone and modulating the crosslinking modality, the physicochemical properties of the resulting matrix would be varied and controlled to provide a material system with tailored swelling/degradation and drug release kinetics.

2. Materials and methods

2.1. Gelatin backbone modification: acylation

PEG-dialdehyde (PEG-dial) was synthesized by reacting PEG-diol ($M_n = 2$ kDa, Sigma-Aldrich, St. Louis, MO) with acetic anhydride in dimethyl sulfoxide (DMSO) at a molar ratio of 1:80:140 for 4 h at 25°C (Fig. 1). PEG-dial was analyzed with reversed-phase high performance liquid chromatography (10–100% acetonitrile gradient at a flow rate of 1 ml/min in 60 min with Jordi 500 Å column, Gilson, Madison, WI). This reaction produced a mixed product of PEG-monoaldehyde and PEG-dialdehyde. PEG-dial had an elution time of approximately 11.5 min, was approximately 80 wt% of the final product and was used without additional purification. The gelatin (G; Type A: derived from porcine skin, 300 bloom, cell culture tested, Sigma-Aldrich) lysyl amino group was acylated by PEG-dial to form PEG-dial-modified gelatin (PG) and/or EDTAD (Sigma-Aldrich) to form EDTAD-modified gelatin (EG) and/or PEG-dial-and-EDTAD-modified gelatin (P/EG). Specifically, EG was synthesized by adding EDTAD to a 1% (w/v) gelatin solution at pH 10 in a weight ratio of gelatin: EDTAD of 1:0.034 and stirring the solution at 40°C for 3 h (Fig. 2a) [10]. The theoretical maximum percent modification of gelatin lysyl residues using this method is 38%; therefore, modifications larger than this suggest that both functional groups of EDTAD may have participated in modifying lysyl residues. PG or P/EG was synthesized by adding PEG-dial dissolved in 10 ml of dd H_2O (Milli-Q synthesis, 18.2 MΩ cm, Millipore, Bedford, MA) and $NaCNBH_3$ (Sigma-Aldrich) dissolved in 10 ml of dd H_2O separately and simultaneously to a 5% (w/v) gelatin or EG solution at 50–60°C for 24 h in a weight ratio of gelatin/EG: PEG-dial: $NaCNBH_3$ of 1:0.66:0.186 (Fig. 2b) [11]. The theoretical maximum percent modification using this method is 100% modification of gelatin lysyl residues, based on the average 300 bloom gelatin molecular weights and the average lysine content of the gelatin [12].

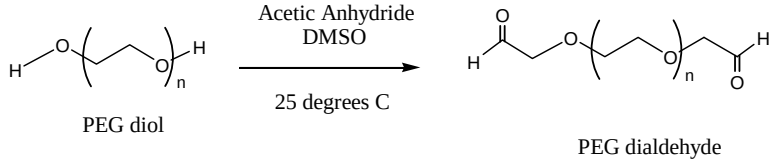


Fig. 1. Reaction scheme for converting PEG-diol to PEG-dialdehyde.

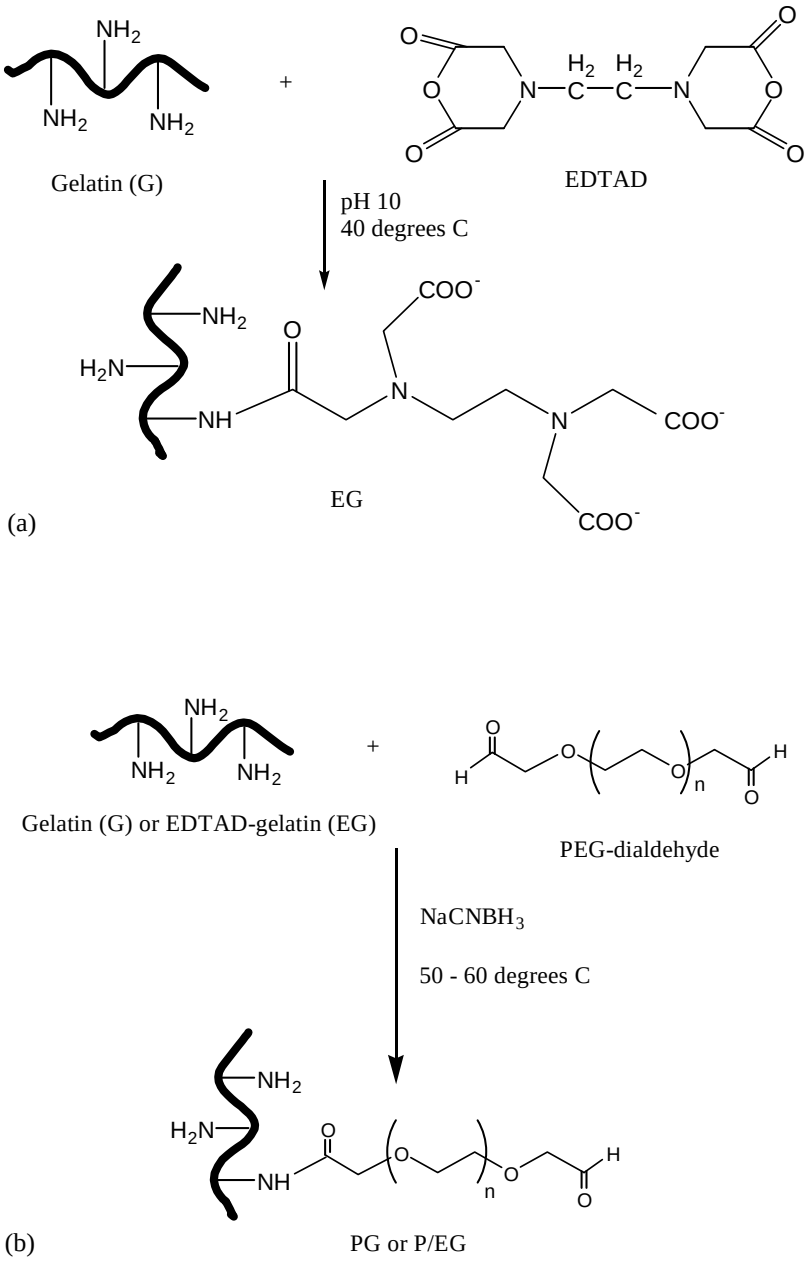


Fig. 2. Reaction schemes for gelatin modifications. (a) EDTAD-G, (b) PEG-G and PEG-EDTAD-G.

The extent of gelatin modification was quantified by using the established 2,4,6-trinitrobenzene sulfonic acid (TNBS) spectrophotometric method to determine the lysyl content of unmodified and acylated gelatins [3,10]. Briefly, 0.5 ml of a 1% protein solution (i.e. G, PG, EG, or P/EG) was added to 1 ml of a 4% $NaHCO_3$ solution (blanks were created following the same procedure excluding the 0.5 ml of 1% protein solution), followed by the addition of 0.2 ml of 12.5 mg/ml TNBS (picrylsulfonic acid, 5% (w/v) aqueous solution, Sigma-Aldrich). The resulting solution was incubated for 2 h in a 40°C water bath. Hydrochloric acid (12 N) was added (3.5 ml), followed by 3 h of incubation at 110°C in a dry oven. The solution was cooled and worked up to 10 ml with dd H_2O. The solution was then extracted twice with 10 ml of cold diethyl ether (Fisher Scientific, Pittsburg, PA) using a separatory funnel, retaining the aqueous layer. The aqueous solution was placed in a 40°C water bath for approximately 30 min to facilitate the removal of traces of ether. The absorbance of the solution was measured at a wavelength of 415 nm against the blank using a spectrophotometer (Genesys 8 UV–Vis spectrophotometer with 2 nm-spectral slitwidth, deuterium UV lamp,

Thermospectronic, Rochester, NY). Absorbance/optical density (OD) was converted to number of lysyl residues using Eq. (1) [3]:

$$\#\text{lysyl residues} = \frac{\text{mol lysyl residues}}{\text{mol gelatin}} = \frac{(\text{OD} \times \#\text{ dilutions})}{(\text{molar extinction coeff} \times \text{path length} \times \text{concentration of gelatin})}, \quad (1)$$

where the number of dilutions following this procedure is 20, the molar extinction coefficient of gelatin is 1.5×10^7 ml/mol cm, path length is 1 cm, and the molar concentration of gelatin is 3.455×10^{-7} mol/ml, as estimated by the bovine serum albumin (BSA) standard curve, assuming a gelatin molecular weight of 10,000 Da for this calculation. Due to the distribution of molecular weights in a given gelatin bloom number, we used a BSA standard curve to correlate absorbance of a 1% gelatin solution to one representative molecular weight and molar concentration of gelatin. The number of lysyl residues for unmodified gelatin was determined and this number was used to obtain percent gelatin modification using Eq. (2):

$$\%\text{ modification of gelatin} = \left[1 - \frac{\#\text{ lysyl residues in modified gelatin}}{\#\text{ lysyl residues in unmodified gelatin}}\right] \times 100. \quad (2)$$

The extent of gelatin modifications and corresponding nomenclatures are summarized in Table 1.

2.2. Hydrogel synthesis

Ten percent (w/v in dd H_2O) solutions of G, 15%-PG, 45%-EG and 65%-P/EG were heated to approximately 70°C and poured into polystyrene petri dishes (60×15 mm, Cole-Parmer, Vernon Hills, IL) to a thickness of 6 mm and allowed to set at rt overnight. Hydrogels were cut into circular discs (diameter = 1 cm) or into squares of 0.5×0.5 cm, and crosslinked with 0.1, 0.01 or 0.001% (v/v in dd H_2O) glutaraldehyde (EM grade, 10% (v/v) aqueous solution, Electron Microscopy Sciences, Ft. Washington, PA) for 6 h with gentle shaking (i.e. approximately 60 rpm on a bi-directional platform shaker). Crosslinked hydrogels were immersed in dd H_2O for 3–5 min, replaced with fresh dd H_2O, and repeated ten times. Washed hydrogels were left overnight in dd H_2O to remove any residual glutaraldehyde, dried at rt in ambient air for 48 h and weighed. Not all hydrogel formulations withstood the crosslinking, gentle washing and drying steps, and were deemed unsuitable for practical biomedical application thus not utilized in the current study. Separately, 10% (w/v in dd H_2O) hydrogels of gelatin were not subjected to glutaraldehyde fixation but dried in ambient air for 48 h, frozen in liquid nitrogen for 30 s to 1 min, then heated at 130–135°C for 8.5 h (self-crosslinked; LN_2-heated G). Preliminary tensile testing showed that gelatin hydrogels crosslinked in 0.1% or 0.01% glutaraldehyde had a Young's Modulus of 1.26 ± 0.14, 1.41 ± 0.16 MPa, a maximum stress of 0.39 ± 0.10, 0.50 ± 0.05 MPa, and a maximum strain of 0.49 ± 0.07, 0.20 ± 0.03 m/m, respectively ($n = 2-3$).

Table 1
Percent modification of gelatin lysyl amino groups

Modified gelatin	% modification	Nomenclature
PG	15.0 ± 5.0	15%-PG
EG	45.6 ± 2.0	45%-EG
P/EG	63.4 ± 0.4	65%-P/EG

2.3. Scanning electron microscopy (SEM) analysis

Hydrogels were dried through a standard graded ethanol series, stored in a desiccator (4-A molecular sieves, EM Science, Gibbstown, NJ), sputter-coated with gold (Autoconductavac IV, See-Vac) for approximately 3 min to a thickness of approximately 20–30 nm, and imaged using scanning electron microscopy (SEM; S570 with LaB_6 emitter, Hitachi, Tokyo, Japan) at 5 or 10 kV.

2.4. Underwater air-captured surface contact angle analysis

Surface hydrophilicity of selected hydrogel formulations was characterized by measuring the underwater air-captured surface contact angle using a modified, computerized video contact angle system (VCA2500, AST Products, Inc., Billerica, MA). Two hydrogels of each formulation with two air bubbles per hydrogel and two angles per bubble were measured. The hydrogel was secured to the underside of a glass slide using strips of stainless steel wire mesh and was placed within a chamber of distilled water at rt. An air bubble was placed on the submerged and exposed surface of the hydrogel and the angle was measured. Based on our measuring system, a larger surface contact angle signifies an increasing hydrophilicity [13,14].

2.5. Swelling/degradation kinetics studies

Dried hydrogels were placed in 5 ml of aqueous solutions of pH 4.5, 7.0 or 7.4 in a water bath of 37°C. Aqueous solutions were created by adjusting the pH of dd H_2O with diluted HCl (0.1 N) and NaOH (0.1 N). Hydrogels were transferred to fresh aqueous solutions at approximately 3 and 6 weeks. Swollen hydrogels were weighed at 2, 4, 6 h, 1, 2, 3, 4, 5 d, and 1, 2, 3, 4, 5, 6, 7, 8 weeks to characterize the swelling/degradation kinetics. Extreme care was taken to preserve the integrity of the hydrogels at every step in the weighing process. The swelling weight ratio at each time point for each hydrogel was calculated as: $(W_s - W_d)/W_d$, where W_s is the weight of the swollen gel (g) and W_d is the original weight of the dry gel (g). The maximum swelling weight ratio that occurred over 8 weeks and the time it occurred was calculated (R_{max}, T_{max}). The last attainable swelling weight ratio (due to hydrogel dissolution) and the time it occurred was also calculated (R_{fail}, T_{fail}). Since both swelling and degradation are occurring from the onset of the study, the resulting change in the gravimetric measurement cannot differentiate the contribution of either phenomenon.

2.6. In vitro drug release studies

Selected dried hydrogel formulations were loaded with a model drug, chlorhexidine digluconate (CHD; 20% (w/v) aqueous solution, Sigma-Aldrich, Fig. 3), using a loading density of 150 μg/kg/day for 21 d (i.e. 630 μg/hydrogel for 0.2-kg animal) as that of our concurrent in vivo study. Based on the maximum swelling weight ratios from the swelling studies, each hydrogel was loaded with 35 μl of CHD (18 mg/ml), a volume well below the maximum volume the hydrogel could absorb. Individual, dried hydrogels (swollen dimensions: $0.5 \times 0.5 \times 0.6$ cm) were placed into individual wells in a 48-well tissue culture plate. Thirty-five μl of CHD or 35 μl of aqueous solution (pH 7.4) for "no drug control" was added to each well (in triplicates), and the hydrogels were allowed to absorb the drug solution or no drug control overnight with gentle shaking (i.e. approximately 30 rpm on a bi-directional platform shaker). Each hydrogel loaded with or without CHD was then placed in a test tube of 5 ml of aqueous solution (pH 7.4, created following the same method as that used for swelling/degradation studies) and placed into a water bath of 37°C. The drug release study was examined at pH 7.4 only since pH had no significant effect on swelling/degradation properties. The mass ratio of drug released, m_t/m_o (mass of drug released at time t divided by the original mass of drug loaded into the hydrogel) was quantified at 2, 4, 6 h, 1, 2, 3, 4, 5 d, and 1, 2, 3, 4 weeks to characterize the drug release kinetics. At each time point, each hydrogel was carefully transferred to a test tube of fresh aqueous solution, and the absorbance of the remaining solution was measured at a wavelength of 258 nm [15–17] using a spectrophotometer (Genesys 8, Thermospectronic). A calibration curve was developed for CHD at 258 nm, using CHD concentrations of 0.5, 1, 5, 10, 50, 100, 125, 150, 200, 300, 400, 500, and 1000 μg/ml. The curve was linear from 0 to 125 μg/ml, and this was fit to a linear

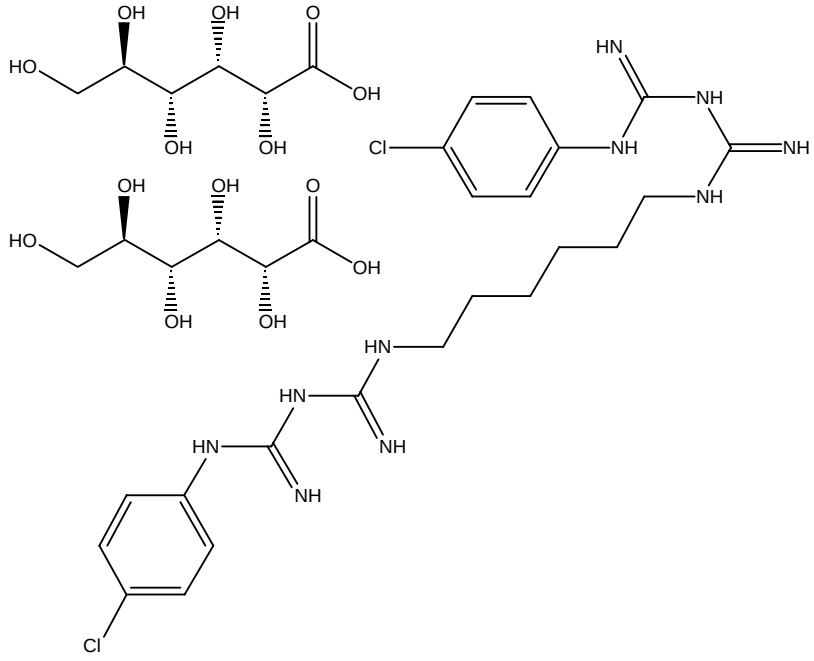


Fig. 3. Chlorhexidine digluconate (CHD); chemical structure: $C_{22}H_{30}Cl_2N_{10} \cdot 2C_6H_{12}O_7$; formula weight = 897.8 Da; $\rho = 1.06$ g/ml.

equation: absorbance = 0.0262 × concentration (μg/ml) + 0.0229 ($R^2 = 0.99$). The highest concentration possible (630 μg in 5 ml) was 126 μg/ml; therefore, all concentrations in the study lie in the linear region of the calibration curve and absorbance can be converted to concentration using this calibration equation. The average absorbance of the no drug controls was subtracted from each absorbance of the CHD test samples, which were then averaged. The resulting absorbance value was converted to concentration using the calibration equation. The concentration at each time point was then added to the concentration at all previous points to obtain a cumulative concentration of drug released at each time point. The resulting cumulative concentration was then multiplied by the volume (5 ml) and divided by the original mass of CHD loaded ($m_o = 630$ μg/hydrogel), to obtain m_t. The mass ratio of CHD released at each time point was calculated by dividing m_t by m_o. The maximum mass ratio of CHD released (D_{max}) and the time this maximum was attained (T_{dmax}) was determined for each hydrogel formulation.

2.7. In vivo degradation, inflammatory response and drug activity characterization

Unmodified gelatin crosslinked in 0.1% or 0.01% glutaraldehyde (G-0.1%, G-0.01%) loaded with or without anti-inflammatory dexamethasone (Fig. 4) at a commonly used dosage of 150 μg/kg/day for 21 d [18–22] were tested in vivo, following the established cage implant system [23]. Samples were placed inside a cylindrical cage (3.5 cm long × 1 cm diameter) constructed from medical grade stainless steel wire mesh. Empty cages were implanted as controls. All cages were implanted subcutaneously at the back of 3-month-old female Sprague-Dawley rats. At 4, 7, 14 and 21 days post-implantation, the inflammatory exudates that collected in the cages were withdrawn and analyzed for the quantitative evaluation of cellular and humoral response to implantation using standard hematology techniques. The distribution of lymphocyte, monocyte, and neutrophil subpopulations in the exudates were determined. Concurrently, the implanted materials were retrieved for analysis of changes in the sample physico-chemical composition (i.e. percent mass loss).

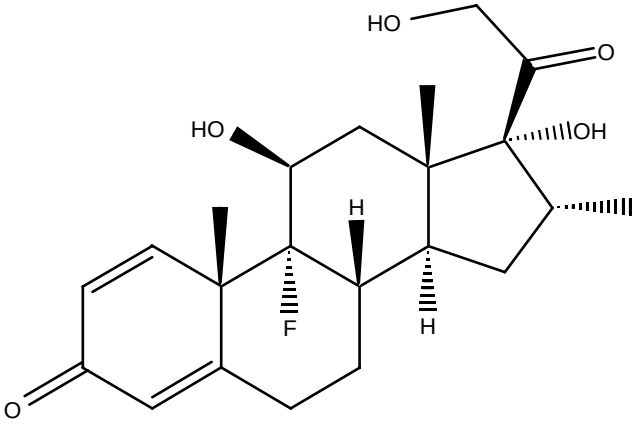


Fig. 4. Dexamethasone; chemical structure: $C_{22}H_{29}FO_5$; formula weight = 392.5 Da.

2.8. Statistical analyses

Statistical analysis for surface contact angle data was performed using paired t-tests ($n = 2$, $p < 0.05$); for swelling/degradation kinetics using two-way ANOVA and Tukey multiple comparisons test for each level of glutaraldedhyde fixation and gelatin backbone modification ($n = 2-7$, $p < 0.05$); for in vitro drug release kinetics using one-way ANOVA and Tukey multiple comparisons test for each level of glutaraldedhyde fixation and gelatin backbone modification ($n = 2$ in triplicates, $p < 0.05$); and for in vivo leukocyte concentration using unpaired equal variance t-tests ($n = 1-7$, $p < 0.05$).

3. Results and Discussion

3.1. SEM analysis

Hydrogels were imaged in the dehydrated state as necessitated by the SEM sample preparation procedure. However, the observed features were substantially different when hydrogels of varying chemistry were qualitatively compared. The surface of the unmodified gelatin hydrogels is featureless or mildly wrinkled for all four crosslinking modalities (0.1%, 0.01%, 0.001% glutaraldehyde and LN_2-heated) (Fig. 5). However, when the cross-section of G-0.001% is imaged, a greater morphology of pits and grooves on the order of 2–5 μm is observed (Fig. 6) when compared to the surface. The morphology of G-0.01% resembles a combination of a typical surface texture and a typical cross-section, with an equal distribution of both pits on the order of 10 μm and a smooth, mildly wrinkled surface. The LN_2-heated G has a smooth surface with a striated and cracked texture near the edge of the gel. PG shows increasing surface morphology with decreasing gluataraldehyde crosslinking concentration at 300× (not shown). At 1000×, a great disparity is observed in the texture of PG of varying crosslinking concentrations (Fig. 7). PG-0.1% is still smooth, while PG-0.01% has a surface texture of small bumps on the order of 10 μm. PG-0.001% has a surface texture of rounded pits on the order of a 2–5 μm, formed by a network of grooves. EG also shows increasing morphology with decreasing crosslinking concentration (Fig. 8). EG-0.1% at

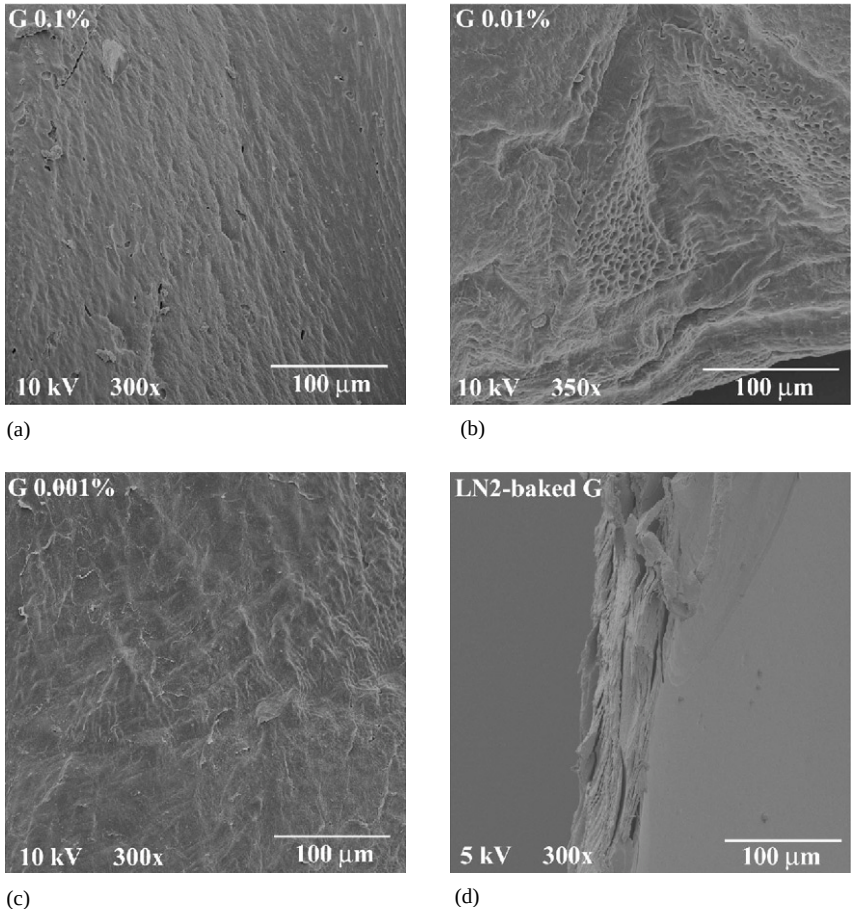


Fig. 5. Scanning electron photomicrographs of unmodified gelatin hydrogels at 5 or 10 kV and 300×. (a) G-0.1%, (b) G-0.01% at 350×, (c) G-0.001%, (d) G-LN_2-heated.

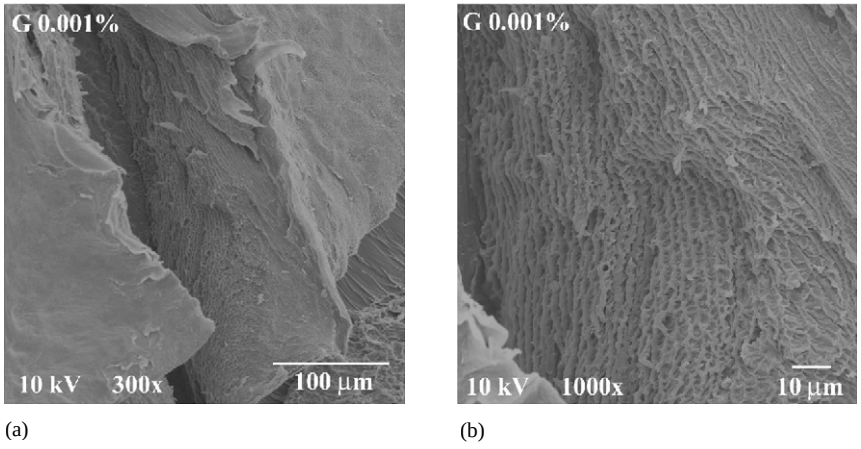


Fig. 6. Scanning electron photomicrographs of G-0.001% hydrogels in cross-section at 10 kV. (a) 300× and (b) 1000×.

2000× shows a relatively smooth surface with a few divots on the order of 10 μm, while EG-0.01% at 1000×, an even lower magnification, shows a highly regular surface of rounded pits on the order of 5 μm. P/EG-0.1% shows surface morphology similar to that of the other forms of gelatin crosslinked with 0.1% glutaraldehyde, a relatively smooth and featureless surface texture (Fig. 9). Again, the cracks in the surface of P/EG-0.1% show increasing texture and morphology, but even at 2000×, the texture of the crack surface is that of shallow ridges and not at all reminiscent of the pits observed of the other modified gelatin hydrogels when crosslinked in lower concentrations of gluataraldehyde. Thus, the percent glutaraldehyde fixation has a greater

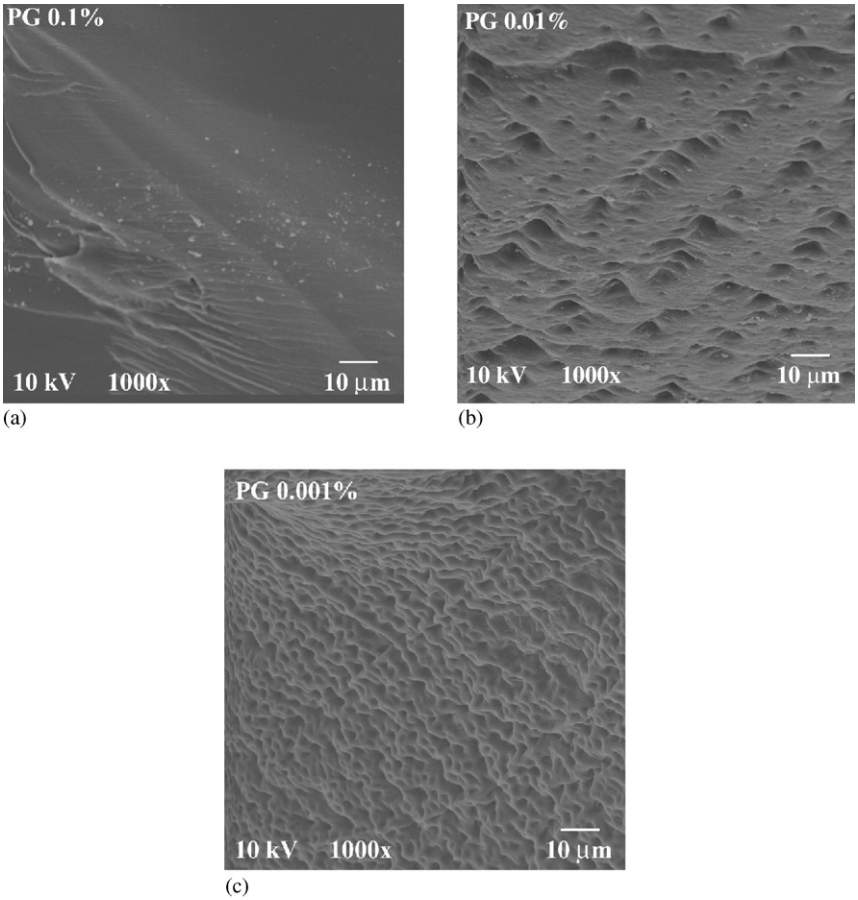


Fig. 7. Scanning electron photomicrographs of PEG-modified gelatin hydrogels at 10 kV and 1000×. (a) PG-0.1%, (b) PG-0.01%, and (c) PG-0.001%.

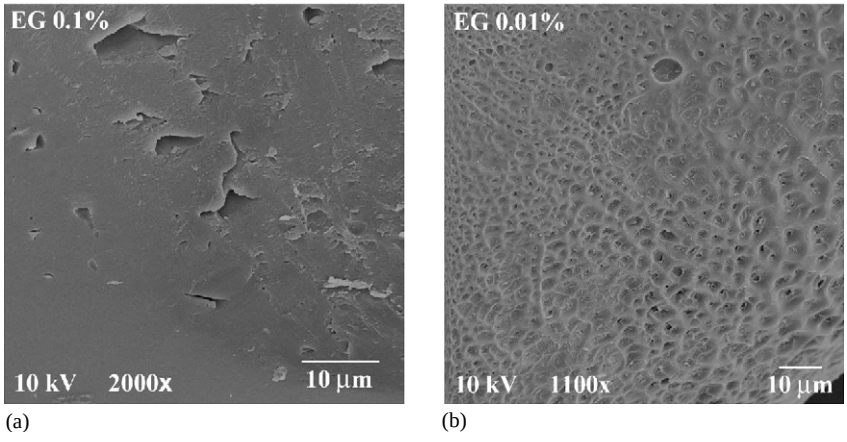
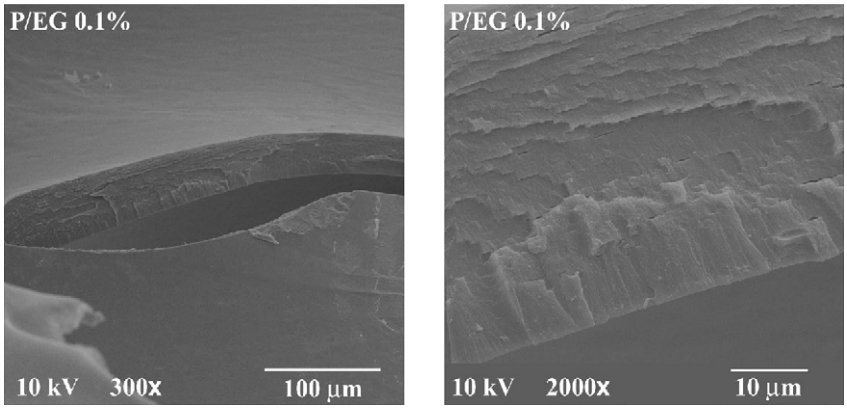


Fig. 8. Scanning electron photomicrographs EDTAD-modified gelatin hydrogels at 10 kV. (a) EG-0.1% at 2000×, (b) EG-0.01% at 1100×.

impact on the morphology of the dehydrated hydrogels than gelatin modification, as observed with SEM.

3.2. Underwater air-captured surface contact angle analysis

Underwater air-captured surface contact angles for gelatin-based hydrogels are shown in Table 2. Surface contact angles range from $140.5 \pm 0.7^{\circ}$ for G-0.1% hydrogels to $171.3 \pm 0.7^{\circ}$ for PG-0.001% hydrogels. These results show that any decrease in percent glutaraldehyde fixation and/or modification of gelatin with PEG-dial and/or EDTAD increased hydrogel surface hydrophilicity. Since modifiying gelatin with EDTAD introduces a polyanionic structure into the gelatin backbone [10] at 45% of the gelatin lysyl



(a) (b)

Fig. 9. Scanning electron photomicrographs of P/EG-0.1% hydrogels at 10 kV. (a) 300× and (b) 2000×.

Table 2
Underwater air-captured surface contact angle for gelatin-based hydrogels

Hydrogel formulation	Surface contact angle (°; mean ± s.e.m.; $n = 2$)
G-0.1%	140.5 ± 0.7
PG-0.1%	162.2 ± 2.3[a]
EG-0.1%	169.3 ± 0.7[a]
P/EG-0.1%	168.1 ± 0.4[a]
G-0.01%	163.2 ± 1.4[a]
PG-0.01%	167.3 ± 3.2
EG-0.01%	168.1[b]
G-0.001%	153.2 ± 4.6
PG-0.001%	171.3 ± 0.7[a]

[a] Significantly different than G-0.1%; all modified gelatin hydrogels not significantly different from one another (paired t-test; $p < 0.05$).
[b] $n = 1$.

residues, we hypothesized that a hydrogel composed of EG would have these anionic characteristics throughout the matrix, thus increasing surface hydrophilicity and water uptake as observed. Modification of gelatin with PEG-dial occurs at 15% of the gelatin lysyl residues; thus, we would not predict that PEG-dial would significantly alter the bulk hydrogel properties, but because PEG-dial is a long hydrophilic molecule that can easily migrate to the surface of the hydrogel and interact with the aqueous environment [9], the modification of gelatin with PEG-dial increases the surface hydrophilicity as observed. The combination of PEG-dial and EDTAD gelatin backbone modification did not result in an additive effect on surface hydrophilicity, potentially due to the highly hydrated state of these hydrogels. As percent glutaraldehyde fixation decreased, the hydrogels became so hydrated that the effect of gelatin backbone modification was not apparent. Thus, significant differences in surface hydrophilicity attributable to gelatin backbone modification were only observed at a glutaraldehyde concentration of 0.1% (v/v) (Table 2).

3.3. Swelling/degradation kinetics

As the samples swell and degrade concurrently when exposed to the aqueous environment, the change in sample mass should be attributed to these two phenomena. The representative swelling/degradation kinetics for gelatin-based hydrogels at pH 7.4 are shown in Fig. 10. The values for defined kinetic parameters, R_{max}, T_{max}, R_{fail} and T_{fail}, for all levels of glutaraldehyde concentration, pH and gelatin backbone modification are shown in Table 3.

First, the effect of gelatin modification within a given percent of glutaraldehyde fixation was evaluated. Swelling/degradation results showed that when hydrogels were crosslinked in 0.1% glutaraldehyde, modification of G with PEG-dial significantly increased T_{max} ($p < 0.05$) but had no significant effect on R_{max}, R_{fail} or T_{fail}, whereas modification of G with EDTAD significantly increased R_{max} and R_{fail} ($p < 0.05$), but had no significant effect on T_{max} or T_{fail}, and modification of G with PEG-dial and EDTAD significantly increased R_{max} ($p < 0.05$), but had no significant effect on R_{fail}, T_{max}, or T_{fail}. Thus, P/EG hydrogels exhibited swelling properties more similar to that of EG hydrogels than PG hydrogels, which was expected since approximately 45% of the 65%-modified P/EG is due to modification with EDTAD. When hydrogels were crosslinked in 0.01% glutaraldehyde, modification of G with PEG-dial decreased R_{max}, increased T_{max} and T_{fail} ($p < 0.05$), but had no significant effect on R_{fail}, whereas modification of G with EDTAD increased R_{max}, R_{fail} and T_{fail} ($p < 0.05$), but had no effect on T_{max}. When hydrogels were crosslinked in 0.001% glutaraldehyde, modification of G with PEG-dial decreased R_{max}, T_{max} and T_{fail} ($p < 0.05$); however, degradation was so rapid with these samples that accurate characterization of these parameters was difficult. The values listed in Table 3 show R_{max}, R_{fail}, T_{max} and T_{fail} values much lower than that of the other glutaraldehyde concentrations, but we

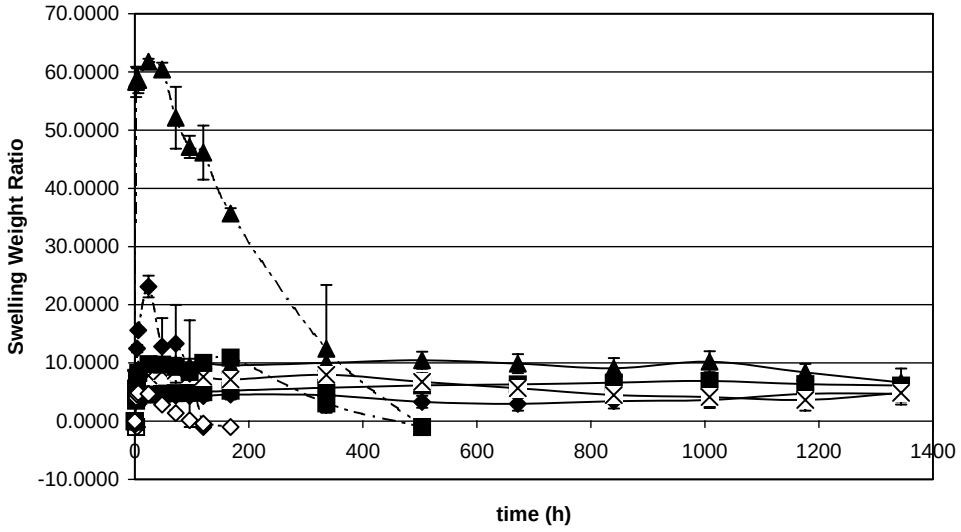


Fig. 10. Swelling weight ratio for G, 15%-PG, 45%-EG, and 65%-P/EG hydrogels crosslinked in 0.1, 0.01, and 0.001% glutaraldehyde and LN_2-heated G hydrogels at pH 7.4. -◆- Gelatin-0.1%, -·◆–·Gelatin-0.01%, -◇- Gelatin-0.001%, –◇—- Gelatin-LN_2-heated, -■- PEG-Gelatin-0.1%, -·■—· PEG-Gelatin-0.01%, -□– PEG-Gelatin-0.001%, -▲- EDTAD-Gelatin-0.1%, -·▲—· EDTAD-Gelatin-0.01%, -X- PEG-EDTAD-Gelatin-0.1%

hypothesize that R_{max} and R_{fail} values for hydrogels crosslinked in 0.001% glutaraldehyde would actually be larger than the R_{max} and R_{fail} values for hydrogels crosslinked in 0.1 or 0.01% glutaraldehyde, but that this phenomenon was not observed due to the rapid hydrolysis of G-0.001% and PG-0.001% hydrogels. These trends were observed at pH 4.5, 7.0 and 7.4.

Second, the effect of percent glutaraldehyde fixation and self-crosslinking within a given type of gelatin modification was evaluated. Hydrogels composed of unmodified G, PG or EG showed an increase in R_{max} and R_{fail} and a decrease in T_{max} and T_{fail} ($p<0.05$) with decreasing percent glutaraldehyde fixation. As noted previously, Table 3 shows that G-0.001% and PG-0.001% hydrogels achieved lower R_{max}, R_{fail}, T_{max} and T_{fail} values than G-0.1% or G-0.01% and PG-0.1% or PG-0.01% hydrogels, respectively, but this is due to rapid hydrolysis of the hydrogels crosslinked in 0.001% glutaraldehyde. For hydrogels composed of unmodified G, the heat treatment of LN_2-heat resulted in an R_{max} and R_{fail} very similar to that of G-0.1% hydrogels, but a decreased T_{max} and T_{fail}; thus, this self-crosslinking procedure produced a hydrogel with swelling properties in between that of G-0.1% and G-0.01%. These trends were observed at pH 4.5, 7.0 and 7.4; pH did not have a significant effect on swelling/degradation properties.

Therefore, we have found that by modifying gelatin with PEG-dial and/or EDTAD and varying the crosslinking modality, we were able to significantly alter the hydrogel swelling capability and stability in water. The effect gelatin backbone modification and glutaraldehyde concentration have on swelling capability is evident in a photograph of G, PG and EG hydrogels crosslinked in 0.1% or 0.01% glutaraldehyde (Fig. 11).

The presence of EDTAD in the gelatin backbone increases the concentration of ionizable groups, thus, increasing the hydrophilicity of the network and the swelling capability of the resulting hydrogel. The proximity and prevalence of the anionic carboxyl groups in the gelatin backbone would likely elicit electrostatic repulsion within the network, thereby increasing the distance between adjacent segments of the gelatin backbone and the resulting volumetric potential for swelling (R_{max} and R_{fail}). Modification with PEG-dial has been shown to stabilize proteins and inhibit hydrolytic and enzymatic degradation. Because PEG-dial chains are lower in concentration (modification of gelatin lysyl residues is 15%), when compared to gelatin modified with EDTAD, PEG-dial may not be homogenously distributed throughout the bulk of the hydrogel, but rather may migrate to the surface of the hydrogel in an aqueous environment, forming the brush regime typical of PEG [9]. Hence this brush regime is a surface phenomenon, and there is no significant effect on bulk hydrogel swelling properties as we had observed. Furthermore, the repulsive characteristic of the PEG brush regime [9] may result in a stabilizing effect as manifested in an increase in T_{max} (i.e. in PG-0.1% and PG-0.01% hydrogels) and T_{fail} (i.e. in PG-0.01% hydrogels). Modification of gelatin with both PEG-dial and EDTAD produced a hydrogel of swelling properties more like that of EG hydrogels. Moreover, the concentration of glutaraldehyde fixation played a greater role in slowing hydrogel degradation than gelatin backbone modification with either PEG-dial and/or EDTAD due to crosslinking density. This was especially evident in P/EG-0.1% hydrogels when compared with G-0.001% and PG-0.001% hydrogels.

Table 3
R_{max}, T_{max}, R_{fail}, and T_{fail} for all levels of glutaraldehyde/heat treatment, pH and gelatin backbone modification

% fixation	PH	Hydrogel	n	Max swelling ratio (R_{max})	Time to maximum (T_{max})	Swelling ratio at failure (R_{fail})	Time to failure (T_{fail})
0.1	4.5	G	7	6.491±0.313	89±28.1	4.443±0.342	1344±0.0
		15%-PG	7	7.652±0.815	1128±120.0	7.002±0.879	1344±0.0
		45%-EG	7	9.343±1.790	354±185.7	6.945±1.829	1272±72.0
		65%-P/EG	7	10.452±1.220	281±88.4	5.346±1.510	1200±93.0
	7.0	G	7	6.241±0.283	507±218.7	4.474±1.300	1296±48.0
		15%-PG	7	7.243±0.470	1080±109.1	5.608±0.825	1344±0.0
		45%-EG	7	13.871±1.580	775±158.0	9.696±1.805	1296±31.0
		65%-P/EG	7	11.010±0.310	418±131.4	6.650± 1.166	1344±0.0
	7.4	G	7	5.154±0.883	442±234.1	3.285±1.276	1152±129.2
		15%-PG	7	7.248±0.446	1080±80.8	6.097±0.778	1344±0.0
		45%-EG	5	11.855±1.235	797±215.9	8.721±1.436	1310±33.6
		65%-P/EG	7	9.376±1.044	254±90.4	3.542±1.224	1152±77.1
0.01	4.5	G	7	34.723±2.169	31±4.4	11.909±2.571	96±15.7
		15%-PG	7	11.954±0.917	110±45.6	7.768±1.564	336±63.5
		45%-EG	4	49.965±8.447	37±11.0	14.790±5.907	378±95.2
		65%-P/EG	0	—	—	—	—
	7.0	G	7	41.717±11.872	34±7.1	13.906±3.120	65±12.5
		15%-PG	7	11.383±0.575	120±43.5	6.451±1.887	408±72.0
		45%-EG	4	51.255±4.481	67±35.2	30.555±8.521	210±42.0
		65%-P/EG	0	—	—	—	—
	7.4	G	7	24.486±2.231	28±7.7	16.001±2.682	55±10.1
		15%-PG	7	11.774±0.797	110±27.6	8.015±1.882	250±42.2
		45%-EG	2	61.750±0.518	24±0.0	30.186±2.306	252±44.9
		65%-P/EG	0	—	—	—	—
0.001	4.5	G	7	0.084±0.057	1±0.4	0.022±0.094	1±0.4
		15%-PG	7	0.000±0.000	0±0.0	0.000±0.000	0±0.0
		45%-EG	0	—	—	—	—
		65%-P/EG	0	—	—	—	—
	7.0	G	7	0.284±0.185	1±0.4	0.192±0.227	1±0.4
		15%-PG	7	0.000±0.000	0±0.0	0.000±0.000	0±0.0
		45%-EG	0	—	—	—	—
		65%-P/EG	0	—	—	—	—
	7.4	G	7	0.306±0.199	1±0.4	0.306±0.199	1±0.4
		15%-PG	7	0.000±0.000	0±0.0	0.000±0.000	0±0.0
		45%-EG	0	—	—	—	—
		65%-P/EG	0	—	—	—	—
LN2-heated	4.5	G	7	6.064±0.798	21±2.6	1.354±0.626	151±34.2
	7.0	G	7	5.240±0.279	19±3.3	–0.083±0.189	96±9.1
	7.4	G	7	4.897±0.378	9±2.6	0.229±0.356	99±11.0

All values expressed in mean±s.e.m.; significant differences omitted for clarity.

3.4. In vitro drug release kinetics

The mass ratio of CHD released from gelatin-based hydrogels at pH 7.4 was quantified (Fig. 12). The values for D_{max} and T_{dmax} for all levels of glutaraldehyde/heat treatment and gelatin backbone modification at pH 7.4 were determined (Table 4).

First, the effect of gelatin backbone modification within a given level of glutaraldehyde fixation was evalulated. In vitro drug release studies showed that when hydrogels were crosslinked in 0.1% glutaraldehyde, modification of G with PEG-dial had no significant effect on D_{max}, but significantly decreased T_{dmax} ($p<0.05$), whereas modification of G with

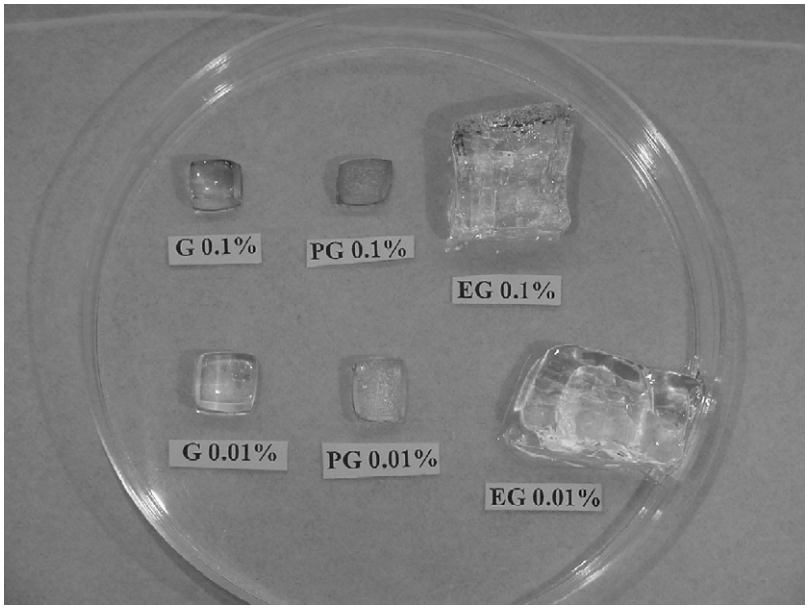


Fig. 11. Photograph of gelatin-based hydrogels: G, 15%-PG and 45%-EG crosslinked in 0.1 and 0.01% glutaraldehyde. Diameter of petri dish base is 85 mm.

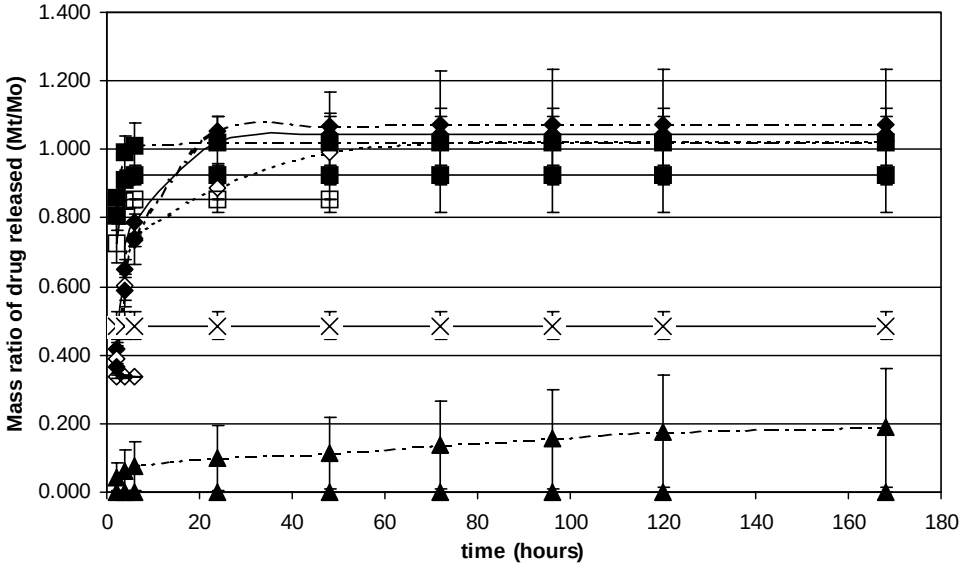


Fig. 12. In vitro chlorhexidine digluconate mass ratio for G, 15%-PG, 45%-EG, and 65-% P/EG hydrogels crosslinked in 0.1, 0.01, and 0.001% glutaraldehyde and LN_2-heated G hydrogels at pH 7.4. -◆- Gelatin-0.1%, - · ◆– · Gelatin-0.01%, -◇- Gelatin- 0.001%, - -◇ – - Gelatin-LN_2-heated, -■- PEG-Gelatin-0.1%, – · ■– · PEG-Gelatin-0.01%, -□– PEG-Gelatin-0.001%, -▲- EDTAD-Gelatin-0.1%, - · ▲ –· EDTAD-Gelatin-0.01%, - · × ·- PEG-EDTAD-Gelatin-0.1%.

EDTAD with or without PEG-dial significantly decreased D_{max} and T_{dmax} ($p<0.05$). D_{max} for P/EG hydrogels lies intermediately between that of PG and EG hydrogels. When hydrogels were crosslinked in 0.01% glutaraldehyde, modification of G with PEG-dial had no effect on D_{max}, but decreased T_{dmax} from 60 to 24 h, whereas modification of G with EDTAD significantly decreased D_{max} ($p<0.05$), but significantly increased T_{dmax} from 60 to greater than 168 h (i.e. the mass ratio of CHD released from EG-0.01% was still increasing at 672 h). When hydrogels were crosslinked in 0.001% glutaraldehyde, modification of G with PEG-dial significantly increased D_{max} ($p<0.05$), but had no significant effect on T_{dmax}.

We observed that swelling weight ratios (i.e. R_{max} and R_{fail}) for G and PG hydrogels were not significantly different at 0.1% glutaraldehyde, but that PG hydrogels had a significantly lower T_{dmax}. Since the drug loading

Table 4
D_{max} and T_{dmax} for all levels of glutaraldehyde/heat treatment and gelatin backbone modification

% fixation	Hydrogel	n	Max of mass ratio of drug released (D_{max})	Time to max mass ratio (T_{dmax})
0.1	G	2	1.042±0.004	48±0.0
	15%-PG	2	0.927±0.029	14±10.0
	45%-EG	2	0.000±0.000	2±0.0
	65%-P/EG	2	0.486±0.041	2±0.0
0.01	G	2	1.072±0.047	60±12.0
	15%-PG	2	1.020±0.077	24±0.0
	45%-EG	2	0.188±0.174	>168±0.0
	65%-P/EG	0	—	—
0.001	G	2	0.339±0.004	2±0.0
	15%-PG	2	0.853±0.070	4±2.0
	45%-EG	0	—	—
	65%-P/EG	0	—	—
LN_2	G	2	1.024±0.209	60±36.0

All values expressed as mean±s.e.m.; significant differences omitted for clarity.

procedure was diffusion-mediated, physically entangled PEG-dial chains may act as a diffusion barrier for CHD resulting in a drug-loaded hydrogel with the drug predominantly surface-associated. Thus, release of CHD from the PG hydrogels would occur more rapidly than that from G hydrogels, leading to a reduced T_{dmax} as observed. Modification of gelatin with EDTAD increased the resulting hydrogel swelling capabilities, which would lead to the drug being immobilized throughout more of the bulk of the hydrogel than that of unmodified gelatin. As expected, we observed very little and slow CHD release. The result suggests that there is a strong electrostatic interaction between the tridentate carboxyl groups of EDTAD in the gelatin backbone and the two guanidinium groups of CHD. At physiological pH, these guanidinium groups are protonated; however, because of resonance stabilization throughout the guanidinium groups and benzene rings of CHD, this positive charge is relatively spread out in the molecule and would not lead to a strong salt-bridge formation. Nonetheless, the carboxyl groups furthest apart on the EDTAD molecule would most likely still form an electrostatic interaction with the guanidinium groups on CHD. Due to this noncovalent interaction between EDTAD and CHD, no CHD release from EG-0.1% hydrogels and a slightly larger release from EG-0.01% hydrogels were observed since the less-crosslinked hydrogels were degrading more. When gelatin was modified with PEG-dial and EDTAD, we observed a phenomenon intermediate to that of PEG-dial or EDTAD; that is, a substantial amount of drug is released from the matrix and early on ($T_{dmax} = 2$ h), which is reminiscent of PG, but only 50% of CHD loaded was released ($D_{max} = 0.486$), suggesting that the PEG-dial modification does not disrupt the electrostatic interaction between EDTAD and CHD.

Second, the effect of glutaraldehyde/heat treatment within a given type of gelatin modification was determined. Hydrogels composed of unmodified gelatin showed that glutaraldehyde/heat treatment had little effect on D_{max} or T_{dmax}. G-0.1%, G-0.01% and G-LN_2-heated all had similar D_{max} values, but all were significantly greater than that of G-0.001% ($p<0.05$). Hydrogels composed of PG showed that percent glutaraldehyde fixation had no significant effect on D_{max} or T_{dmax}. Hydrogels composed of EG showed that percent glutaraldehyde fixation had no significant effect on D_{max}, but that decreasing percent glutaraldehyde fixation from 0.1% to 0.01% had a great significant impact on T_{dmax} ($p<0.05$). Therefore, with the exception of EG, gelatin backbone modification had a much greater impact on drug release kinetics than glutaraldehyde/heat treatment. Thus, although crosslinking indeed affects hydrogel solubility/density/swelling, the effect crosslinking had on polymer relaxation/swelling necessary for drug release was nominal when compared to the effect observed due to G modification. Because of this interaction between EDTAD and CHD and a lower glutaraldehyde concentration, EG-0.01% hydrogels displayed controlled and gradual release kinetics (Fig. 12), thus demonstrating that the release kinetics of the gelatin-based hydrogels can be modulated by varying gelatin backbone modification in tandem with glutaraldehyde crosslinking concentration.

3.5. *In vivo degradation, inflammatory response and drug activity*

Total and differential leukocyte concentrations in the inflammatory exudates of the empty cage and gelatin controls, G-0.1%, and G-0.01% with or without dexamethasone are shown in Table 5. The presence of a high concentration of neutrophils in the exudates indicates an acute inflammatory response, which occurs at the onset of implantation and attenuates with time. The presence of a high concentration of monocytes and lymphocytes in the exudates is indicative of the chronic inflammatory response. Gelatin hydrogels without glutaraldehyde fixation elicited comparable levels of acute inflammation that resolved within 4 d and chronic inflammation that attenuated by 21 d as the empty cage control. G-0.1% hydrogels elicited a slightly enhanced acute inflammatory response at 4 d, and an enhanced chronic inflammation at 7 and 14 d when compared to that of the empty cage control ($p<0.05$). G-0.01% transiently elicited a slightly enhanced chronic inflammatory response at 7 d when compared to that of the empty cage control ($p<0.05$). G-0.01% hydrogels

Table 5
In vivo subcutaneous cage implant results: total and differential leukocyte conentrations in the inflammatory exudates of G-0.1% and G-0.01% with and without dexamethasome

Sample	Day sample retrieved	Exudate cell concentration ($\times 1000$ cells/μl)			
		Total leukocyte	Lymphocyte	Monocyte	Neutrophil
Empty cage (no sample)	4	1.8±0.3	1.7±0.2	0.2±0.1	0.0±0.0
	7	0.6±0.1	0.5±0.1	0.1±0.0	0.0±0.0
	14	0.5±0.1	0.4±0.0	0.1±0.0	0.1±0.1
	21	0.9±0.7	1.0±0.5	0.2±0.2	0.0±0.0
Gelatin control	4	1.3±0.3	0.7±0.4	0.6±0.5	0.0±0.0
	7	0.6±0.2	0.5±0.2	0.0±0.0	0.0±0.0
	14	0.4±0.0	0.3±0.1	0.1±0.0	0.0±0.0
	21	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0
G-0.1%	4	6.0±3.9[a]	2.5±1.2	1.3±1.1	2.2±2.1
	7	1.8±1.3[a]	0.8±0.4	0.3±0.1	0.8±0.7
	14	2.4±0.7[a]	1.2±0.3[a]	0.4±0.2	0.8±0.7
	21	2.0	1.7	0.3	0.0
G-0.01%	4	4.8±2.0	4.1±1.7	0.6±0.3	0.1±0.0
	7	1.8±0.8[a]	1.6±0.8	0.2±0.0[a]	0.0±0.0
	14	0.7±0.4	0.6±0.3	0.1±0.1	0.0±0.0
	21	0.9±0.0	0.7±0.1	0.1±0.0	0.1±0.1
G-0.01% with dexamethasome	4	11.4±10.9	0.9±0.9	0.1±0.1	0.1±0.2
	7	23.0±19.5	20.1±17.2	1.1±1.0	1.8±1.3
	14	2.8±0.7[a]	2.3±0.4[a]	0.2±0.1	0.3±0.2
	21	0.7±0.4	0.6±0.4	0.1±0.0	0.0±0.0

[a] Significant difference between sample and empty cage control at that time point (unpaired equal variance t-test; $p<0.05$).
All values expressed as mean±s.e.m. ($n=1-3$).

Table 6
In vivo subcutaneous cage implant results: percent mass loss, comparing mass of hydrogels prior to and post-implantation

% glutaraldehyde fixation	Day 4	Day 7	Day 14	Day 21
G-0.1	31.9+0.59	34.4+1.24	39.9+3.6	38.1+2.0
G-0.01	43.2+2.0	46.9+0.63	59.6	79.3+10.1

All values expressed as mean±s.e.m. ($n=3-7$).

loaded with anti-inflammatory dexamethasone transiently increased lymphocyte and total leukocyte concentration at 14 d ($p<0.05$), then returned to levels comparable to that of empty cage controls at 21 d. This apparent lack of anti-inflammatory activity of dexamethasone may be due to the quick early release of the drug within 4 d, which was also observed in the in vitro drug release study and/or the use of an ineffective dosage. By day 21, all samples showed a comparable level of chronic inflammation when compared to that of the empty cage and gelatin controls that proceeded toward resolution. Percent mass loss of samples increased with increasing implantation time and was further increased with decreasing percentage of glutaraldehyde fixation (Table 6).

4. Conclusion

By modifying gelatin lysyl groups with PEG-dial and/or EDTAD and varying the crosslinking modalities of gelatin-based hydrogels, we were able to modulate and correlate the structure-function relationship associated with the surface hydrophilicity, swelling and drug release characteristics and in vivo response.

Acknowledgements

This work was supported in part by NIH Grant HL-63686/EB-00290 and Whitaker BRG RG99-0285.

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