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Alkylation of Human Hair Keratin for Tunable Hydrogel Erosion and Drug Delivery in Tissue Engineering Applications

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Abstract

Polymeric biomaterials that provide a matrix for cell attachment and proliferation while achieving delivery of therapeutic agents are an important component of tissue engineering and regenerative medicine strategies. Keratins are a class of proteins that have received attention for numerous tissue engineering applications because, like other natural polymers, they promote favorable cell interactions and have non-toxic degradation products. Keratins can be extracted from various sources including human hair, and they are characterized by a high percentage of cysteine residues. Thiol groups on reductively extracted keratin (kerateine) form disulfide bonds, providing a more stable cross-linked hydrogel network than oxidatively extracted keratin (keratose) that cannot form disulfide crosslinks. We hypothesized that an iodoacetamide alkylation (or “capping”) of cysteine thiol groups on the kerateine form of keratin could be used as a simple method to modulate the levels of disulfide crosslinking in keratin hydrogels, providing tunable rates of gel erosion and therapeutic agent release. After alkylation, the alkylated kerateines still formed hydrogels and the alkylation led to changes in the mechanical and visco-elastic properties of the materials consistent with loss of disulfide crosslinking. The alkylated kerateines did not lead to toxicity in MC3T3-E1 pre-osteoblasts. These cells adhered to keratin at levels comparable to fibronectin and greater than collagen. Alkylated kerateine gels eroded more rapidly than non-alkylated kerateine and this control over erosion led to tunable rates of delivery of rhBMP-2, rhIGF-1, and ciprofloxacin. These results demonstrate that alkylation of kerateine cysteine residues provides a cell-compatible approach to tune rates of hydrogel erosion and therapeutic agent release within the context of a naturally-derived polymeric system.

Abstract

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Keywords

Recombinant human BMP-2; Recombinant human IGF-1; Antibiotic; Ciprofloxacin; Regenerative Medicine

1. Introduction

Polymeric materials are a key component in tissue engineering/regenerative medicine (TERM) approaches to promote tissue healing and regeneration. Materials used for these applications typically must have minimal toxicity, elicit minimal immune/inflammatory response, have appropriate mechanical properties, and promote favorable cellular responses (attachment and/or infiltration and/or proliferation) [1, 2]. It is generally held that materials should degrade in a manner inversely proportional to the rate of tissue regeneration such that the material provides a cell-support matrix but degrades to avoid impeding tissue regeneration [3]. These materials are also widely used to achieve controlled release of various exogenous therapeutic agents ranging from antibiotics that prevent local infection to growth factors that promote tissue formation or healing [4, 5]. The two general classes of polymeric materials used in TERM applications are natural and synthetic polymers.

Synthetic polymers have the advantage of tunable degradation or controlled release of therapeutic agents via modifications of the polymer backbone constituents, side-chains, and molecular weight [6–8]. The main drawback to the use of synthetics revolves around poor cell attachment, which requires modifications with peptide groups, use of natural-synthetic hybrids, or electrospinning techniques to provide surface topography [9–12]. Conversely, natural protein polymers such as collagen and fibrin have favorable biological interactions because they promote cell attachment and have amino acids as their degradation products [13]. However, the ability to achieve tunable rates of degradation or release of therapeutic agents can be a challenge for natural proteinaceous polymers.

Keratins are a class of intermediate filament proteins that can be derived from various sources including human hair, the source of keratin described in this report. Keratins used without modification or delivery of therapeutic agents have been described for biomedical applications including skin [14], hemostasis [15], bone [16], and nerve [17, 18]. We and others have also reported the use of keratins to promote delivery of therapeutics including model drugs [19], antibiotics [20, 21], and recombinant human bone morphogenetic protein 2 (rhBMP-2) [22–24]

An intriguing characteristic of keratin proteins that might be exploited for controlled erosion and release of therapeutics is the presence of a relatively high percentage of cysteine residues (reported between 7 – 20 mol %) compared to other proteins [25, 26]. Two well-known extraction methods have been developed that lead to physiochemically different forms of keratin. Oxidatively extracted keratin (keratose; KOS) yields a form in which thiol groups on cysteine residues are “capped” with sulfonic acid groups that prevent covalent disulfide cross-linking. Keratose can be formed into hydrogels, but these gels form by chain entanglement and lack covalent disulfide crosslinking. Reductively extracted keratin (kerateine; KTN) yields a form that has chain entanglements and free thiol groups that can spontaneously form covalent disulfide crosslinks. The different levels of disulfide bonding between keratose and kerateine have previously been shown to lead to different rates of hydrogel erosion with KOS erosion occurring in days to weeks and KTN persisting for months [27, 28]. It has also previously been observed that rates of therapeutic agent release correlate with the rates of keratin hydrogel erosion [21, 22]. We hypothesized that thiol groups on KTN could simply be alkylated (“capped”) to modulate disulfide crosslinks, which in turn, would lead to tunable rates of gel erosion and therapeutic delivery.

The approach described in this report uses iodoacetamide as the alkylating agent for keratin cysteine residues [29], as shown in Figure 1A. After assessing levels of alkylation, the effects of the modification on the hydrogel network, mechanical properties, and rate of erosion were also investigated. Cell viability and adhesion were then studied in order to determine if the alkylation had effects on the biological response to the modified kerateines. Lastly, the ability to control the rates of release was assessed for three different therapeutic agents: ciprofloxacin, recombinant human insulin-like growth factor 1 (rhIGF-1), and recombinant human bone morphogenetic protein 2 (rhBMP-2). These molecules have differing physiochemical properties, the most notable being molecular weights of 300 Da (ciprofloxacin), 7 kDa (rhIGF-1), and 26 kDa (rhBMP-2). These agents were selected to assess controlled release due in part to their relevance to tissue engineering. Ciprofloxacin is a quinolone antibiotic that has been used for treatment of Gram-positive and Gram-negative infections in burn wound healing [30–32]. IGF-1 affects multiple types of cells and has been used for regenerative treatments including myocardial infarction [33], nerve defects [34], cartilage [35], and periodontal diseases [36]. BMP-2 belongs to the TGF- β family, is involved in cartilage and bone formation [37], and is used clinically for spinal fusion and dental applications (Infuse/Inductos®, Medtronic) [38–40].

2. Materials and Methods

2.1. Preparation of modified kerateines and characterization of thiol content

Alpha fractions of kerateine (KTN) and keratose (KOS) were obtained as lyophilized, sterile (via 2 MRad gamma irradiation) powders from KeraNetics, LLC (Winston-Salem, NC). KTN was alkylated by adding iodoacetamide (Sigma, St. Louis, MO) at 0, 0.4, 0.9 or 2.5-fold molar ratios of keratin cysteine residues in deionized water at pH 9.6 for 1 hour at room temperature. Table 1 shows the amounts of kerateine, water, and iodoacetamide used for preparation of the materials reported below. In each case, the reaction proceeded for 1 hour at room temperature. The resulting alkylated kerateine was dialyzed in 3500 Da MWCO

dialysis tubing (Spectrum Labs, Houston, TX) against deionized water at pH = 7 with 3 changes of dialysis buffer over the course of 3 days. After dialysis, the kerateine solutions were frozen at -80°C and lyophilized to obtain the final powder form of alkylated kerateine, which we refer to as modified kerateine (MKTN).

The free thiol content was determined by Ellman's assay [41]. In brief, keratin solutions (KOS, KTN, or MKTN) were prepared in water at 1 mg/mL with the concentration confirmed by a modified Lowry method (DC protein assay; Bio-Rad, Hercules, CA). 2 μL of 100 mM Ellman's reagent in DMSO (Sigma, St. Louis, MO) was added to the keratin solution. The resulting mixture was allowed to react for 10 minutes at room temperature and the absorbance was read on a Biotek Synergy HT microwell plate reader (Winooski, VT) at 420 nm and compared to a standard curve of known amounts of cysteine. The free thiol content was used to identify the various formulations (e.g., 7% free thiol is referred to below as 7% S-S MKTN).

2.2. Formation of keratin (KOS, KTN, or MKTN) hydrogels

All keratin gels (KOS, KTN, or MKTN) described for Sections 2.3 – 2.6 and 2.10 – 2.11 were fabricated at 15% (w/v). In a typical preparation, 150 mg of keratin (KOS, KTN, or MKTNs) was placed in a 15mL conical tube. 1 mL of water or water containing therapeutic agents (see 2.11 below) was added. The mixtures were vigorously mixed by vortex and manual agitation. The mixtures were then briefly centrifuged. Typically, the resulting mixtures of keratin were packed into a 1mL syringe, and known volumes (and masses) were placed into 1.5mL tubes (Sections 2.3, and 2.10–2.11), Sylgard molds (Sections 2.4–2.5), or wells of 48-well plates (Section 2.6), depending on the experiments described below. Then, the mixtures were allowed to incubate overnight at 37°C to gel.

2.3. Scanning electron microscopy of keratin materials

We used Scanning Electron Microscopy (SEM) to visualize the porous architecture of the keratin materials. Keratin hydrogels (KOS, KTN, or MKTNs) were fabricated as described in Section 2.2. After loading into a syringe, approximately 400 μL of the keratin was placed into a 1.5 mL tube and allowed to gel overnight at 37°C . The keratin gels were then frozen at -80°C overnight and placed on a Labconco freeze drier (Kansas City, MO) for at least 24 hours. The resulting samples were cut horizontally with a scalpel blade to expose the internal structure, mounted on an SEM stub, immediately sputter coated for 30 seconds at 45 mV (Desk II, Denton Vacuum, Moorsetown, NJ), and imaged at 5.0 kV with a Zeiss Supra 35 (Carl Zeiss Microscopy, Thornwood, NY).

2.4. Swelling and compression testing for characterization of network structure of keratin hydrogels

15% (w/v) keratin gels (KOS, KTN, or MKTNs) were prepared as described in Section 2.2. After placing into the 1mL syringe, the keratin was injected into a 5 mm diameter by 5 mm high mold (made of Sylgard 184) and allowed to incubate overnight at 37°C to gel. The swelling ratio, Q , was determined as described previously by others [42]. In brief, the keratin gel cylinders were weighed after gelation (dry mass, M_d) and placed into phosphate buffered saline until they reached an equilibrium swelling point (swelling mass, M_s). The

swelling time was found to be 2 hours by a pilot experiment, and we noted that keratose gels began to erode after this time point. The swelling ratio was determined from the equation [42]:

$$Q=1+\left(\frac{\rho_k}{\rho_s}\right)\left(\frac{M_s}{M_d}-1\right)$$

where ρ_k is the density of the keratin (assumed to be 0.35 mg/mL for all keratin forms) and ρ_s is the density of phosphate buffered saline (1.006 mg/mL). The swollen gels were measured for diameter and height and then subjected to an unconfined compression test at room temperature (23°C) on an Instron 3344 mechanical tester equipped with Bluehill software and a 100 N load cell. A preload of 0.02 N was used for each kerateine-based gel. A preload of 0.005 N was used for keratose gels, and a 10 N load cell was also used, but reliable data could not be obtained after swelling due to the softness of the gels. A crosshead speed of 1 mm/min was used. The non-linear engineering stress versus stretch ratio data were fit by least squares regression to the one-term Ogden model for an incompressible, isotropic, hyperelastic material to determine the shear modulus (μ) and strain hardening exponent (α) [43]. The compressive modulus (K) was calculated from the optimized shear modulus (μ) as $K = 3\mu$. The effective crosslink density (ν_e) and the molecular weight between crosslinks (M_c) were determined by the two following equations [42]:

$$\nu_e = \frac{K}{RTQ^{1/3}}$$

$$M_c = \frac{\rho_k}{\nu_e}$$

where R is the ideal gas constant (8.314 m³*Pa/K/mol) and T is temperature (296 K). All formulations of keratin were tested in quadruplicate ($n = 4$) for swelling and compression testing on the swollen gels.

2.5. Rheological characterization of keratin hydrogels

15% w/v keratin (KOS, KTN, and 7% S-S MKTN that was made from a 2.5-fold molar excess of iodoacetamide) were formed as described in Section 2.2. For these experiments, the keratin (after loading into a syringe) was injected into a 20 mm diameter by 2 mm height Sylgard mold and allowed to gel overnight at 37°C. The gels were then removed from the mold and placed on the surface of a TA Instruments HR-1 rheometer (New Castle, DE). A 20 mm plate-plate configuration was used to test the hydrogels at a gap distance of 2 mm and a temperature of 25°C. We conducted a frequency sweep from 0.01 to 10 Hz at 1% oscillatory strain. We note that the 1% oscillatory strain was previously found to be in the linear regime for these materials across the frequencies tested. The elastic modulus (G') and viscous modulus (G'') were then measured as a function of frequency. Each formulation was tested in triplicate ($n = 3$).

2.6. Cell proliferation and viability in response to alkylation of kerateine

MC3T3-E1 mouse pre-osteoblasts (American Type Culture Collection, Manassas, VA) grown in α -MEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA) in a humidified atmosphere and 5% CO₂ at 37°C were used for cell viability assays. Cells were sub-cultured every 2 days (prior to reaching 80% confluence) by using standard techniques. Cell proliferation in response to keratin was assessed by coating 96-well tissue culture polystyrene (TCPS) plates with dilutions of keratin proteins prepared in sterile water at a concentration of 150 μ g/mL. TCPS with no keratin was used as a control. After overnight incubation with keratin proteins, wells were rinsed three times with PBS. 2500 cells were seeded into each well and allowed to proliferate. Growth media was replaced every two days. At specified times (24, 48, 72 and 96 hours), culture media was removed and 120 μ L of 5 parts fresh media and 1 part CellTiter 96 Aqueous Nonradioactive MTS (Promega, Madison, WI) was added. After 90 minutes, absorbance was measured at 490 nm in a Biotek Synergy HT microwell plate reader.

The effect of the alkylation chemistry on cell viability was also assessed with a dose-response assay with keratin in solution. In these experiments, MC3T3-E1 cells were seeded on TCPS at 2500 cells/well and incubated for 24 hours at 37 °C in 5% CO₂. Keratin solutions were prepared in sterile water at concentrations ranging from 0 to 1400 μ g/mL. The keratin was then mixed with α -MEM at 7:3 (v/v) ratio, which resulted in keratin concentrations from 0 to 8.4 μ M. Cells were then incubated with the α -MEM:keratin solutions for 72 hours at which time the MTS assay was performed as described above.

A qualitative cell viability assay was conducted with MC3T3-E1 cells in the presence of 15% (w/v) 3-D keratin hydrogels. Keratin hydrogels were formed with water as described above and 50 μ L was loaded into a 48-well plate. The experiment was run in triplicate. 100 μ L/well of serum-free α -MEM was placed onto gels for 4 hours at room temperature. Then, 100 μ L of α -MEM supplemented with 10% FBS and 1% penicillin-streptomycin replaced the serum-free α -MEM and plates were incubated overnight at 37 °C. After overnight incubation, cells were seeded at 40,000 cells/well, incubated for 24 hours, and then subjected to a live-dead assay with calcein-AM and ethidium bromide according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

2.7. Quantitative cell adhesion determined by centrifugation assay

KOS, KTN, MKTN, fibronectin (FN, control) and collagen (CN, control) were dissolved at 50 μ g/mL in sterile water and coated on a 96-well TCPS plate for 2 hours at room temperature. After 2 hours, wells were blocked in 1% BSA for 1 hour at room temperature. Separately, MC3T3-E1 cells were tagged with 2 μ g/mL calcein-AM (Invitrogen) in PBS with 0.1 mg/mL calcium chloride (CaCl₂) for 20 minutes at 37°C. Cells were then seeded at 10,000 cells/well and allowed to attach for 1 hour at 37°C. After 1 hour, the plate was aspirated and refilled with PBS containing CaCl₂ to measure the fluorescence of cells in each well before centrifugation as a means to allow normalization of the data for any well-to-well variability. The plate was covered with sealing tape and centrifuged upside down over a range of detachment forces 12 g – 410 g (where g is the force of gravity) for 5

minutes on a Thermo Scientific ST16R centrifuge with M-20 microplate rotor. The two columns on both sides of the plate were left empty to minimize differences in the centrifugal force across the width of the plate. After centrifugation, wells were aspirated and refilled with PBS containing CaCl_2 to measure a post-spin fluorescence. Pre- and post-spin fluorescence data was measured at 485 nm excitation and 535 nm emission from a Biotek Synergy HT plate reader.

2.8. Immunofluorescent analysis of cell attachment to keratin-coated surfaces

4-well chamber slides (Fisher Scientific, Hampton, NH) were sterilized with ethanol for 15 minutes and coated with protein as described above (section 2.4). MC3T3-E1 cells were seeded onto protein-coated wells at 10,000 cells/well and incubated for 2 days at 37°C. Cells were then fixed with 10% NBF (Fisher Scientific, Hampton, NH) for 20 minutes.

Immunofluorescence for vinculin was performed by standard techniques. Briefly, after washing and blocking, cells were incubated with monoclonal rabbit anti-vinculin antibody (1:100, Abcam, Cambridge, MA) for 1 hour at room temperature. After washing, donkey anti-rabbit secondary with Alexa Fluor 488 (1:200, Abcam) was added to cells for 1 hour at room temperature. Actin filaments were then stained with Alexa Fluor 594 Phalloidin (1:25, Invitrogen) by incubating cells for 20 minutes at room temperature. After nuclear counterstain with 300 nM DAPI (Invitrogen), samples were mounted with Prolong Gold antifade medium (Invitrogen). Images were captured with an Olympus AX-70 microscope equipped with a Nikon D300 camera and MetaVue software in the Miami University Center for Applied Microscopy and Imaging.

2.9. rhBMP-2 and rhIGF-1 binding affinity to keratin

rhBMP-2 (Medtronic, Minneapolis, MN) was dialyzed against deionized water to remove glutamine and other amine-containing groups before biotinylation. Biotinylation of rhBMP-2 and rhIGF-1 (Peprotech, Rock Hill, NJ) was conducted with EZ-Link Sulfo-NHSLC-Biotin (Pierce, Rockford, IL) following the manufacturer's instructions. In brief, 30-fold molar excess of biotin was added to rhBMP-2 or rhIGF-1 in sterile water for 30 minutes at room temperature. The biotinylated rhBMP-2 or rhIGF-1 was then dialyzed at pH 7 in sterile water to remove unreacted biotin. Biotin labeling was quantified by the FluoReporter® Biotin Quantitation Assay kit (Invitrogen). For the solid-phase assay, keratin proteins (KOS, KTN, and 7% S-S MKTN), collagen (CN), or fibronectin (FN) controls were coated at 50 $\mu\text{g}/\text{mL}$ on clear, high-binding polystyrene microplates (R&D systems, Minneapolis, MN) for 2 hours at room temperature, followed by washing, blocking with 1% BSA, and additional washing. rhBMP-2 was added to the plates starting at 2 μM and then diluted serially at 1:2 to a final concentration of 30 nM. rhIGF-1 was added to the plates starting at 26 μM and diluted serially at 1:2 to a final concentration of 405 nM. rhBMP-2 or rhIGF-1 was allowed to bind for 1 hour at room temperature and wells with no rhBMP-2 or rhIGF-1 were used as controls. After washing to remove unbound rhBMP-2 or rhIGF-1, High Sensitivity Streptavidin HRP Conjugate (Pierce, Rockford, IL) diluted to 1:8000 was added to the wells and incubated for 30 minutes at room temperature. Tetramethylbenzidine (TMB) substrate (Sigma, St. Louis, MO) was then added and incubated for 20 minutes, followed by addition of 0.5N HCl stop solution. Absorbance was measured at 450 nm on a BioTek Synergy HT platereader.

2.10. Erosion of modified and unmodified kerateine hydrogels (KTN and MKTN)

~ 100 μ L of 15% (w/v) hydrogels (weighed to account for small differences in volumes) were placed into 1.5 mL tubes and allowed to gel overnight at 37°C. 150 μ L of phosphate-buffered saline (PBS) was layered on top of each gel and PBS was removed then replaced with fresh PBS at specified time points (1.5, 3, 6, 12, 24 hours, then daily for 1 week). The keratin concentrations in the PBS (i.e., keratin that had eroded from the gels) was determined by a modified Lowry method (DC protein assay; Bio-Rad, Hercules, CA).

2.11. Growth factor and antibiotic release from keratin hydrogels

15% kerateine hydrogels were loaded with rhBMP-2, rhIGF-1, or the antibiotic ciprofloxacin in order to assess the effect of alkylation on drug release for drugs of varying molecular weights (~ 300 Da, 7 kDa, and 26 kDa for ciprofloxacin, rhIGF-1, and rhBMP-2, respectively). Gels were formulated with water (control) or loaded with rhBMP-2 (10 μ g/mL) or rhIGF-1 (100 μ g/mL) as described above. For ciprofloxacin-loaded gels, ciprofloxacin was dissolved at 2 mg/mL and pH = 5.2 to prevent its precipitation [21]. All formulations were placed in 1.5 mL tubes in quadruplicate as described above. 150 μ L of PBS was layered on top of each gel and replaced with fresh PBS at specified time points (1.5, 3, 6, 11, 24 hours, then daily for 1 week). The collected PBS was stored at -80°C until analysis. The amount of rhBMP-2 and rhIGF-1 released was determined by ELISA (Peprotech for rhBMP-2, R&D Systems for IGF-1). The amount of ciprofloxacin in each sample was determined by fluorescence at 360 nm excitation and 460 nm emission on a BioTek Synergy HT platereader.

2.12. Statistical Analysis

Where appropriate, statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc testing or Student t-test at 95% confidence interval in SigmaPlot (San Jose, CA). $p < 0.05$ was taken to be statistically significant.

3. Results

3.1. Alkylation of kerateine

The capping of kerateine thiol groups was accomplished by an alkylation reaction in which different mole ratios of kerateine and iodoacetamide (0 – 2.5 of iodoacetamide to kerateine) were simply mixed to react in an aqueous solution followed by extensive dialysis to remove unreacted iodoacetamide. The final MKTN product was obtained as a powder by freeze-drying. The free-thiol content from modified kerateine was examined by Ellman's assay, showing an inversely proportional relationship to the mole ratio of iodoacetamide to kerateine (Figure 1B). Three formulations of MKTN were prepared with initial iodoacetamide:keratin cysteine ratios of 0.4, 0.9, and 2.5 (mole:mole). Data are reported on these formulations in terms of the percent free thiol: 80%, 74%, or 7% free thiol. This means that for the 80% formulation, 80% of thiol groups would be available for disulfide crosslinking (80% of KTN crosslinking) whereas 7% free thiol means that only 7% of the thiol groups would be available for crosslinking (7% of KTN crosslinking).

3.2. Evaluation of pore architecture by SEM

At the macroscopic level, gels formed with KOS, KTN, and each of the MKTNs had similar appearances. KTN was observed to gel more rapidly than KOS and MKTN gels, consistent with the hypothesis that the MKTN gels have fewer available thiol groups to form disulfide crosslinks and rely on chain entanglement or other interactions similar to how keratose forms gels. The freeze-dried samples used for SEM imaging (Figure 2) showed that each formulation had similar porous architecture. These results suggest that the alkylation of the KTN at various crosslink densities (7% S-S, 74% S-S, and 80% S-S) did not lead to major changes in the appearance and porous architecture of the materials.

3.3. Characterization of network structure

Common parameters for network structure (effective crosslink density and molecular weight between crosslinks) were determined for each of the keratin formulations (KOS, 7% S-S MKTN, 74% S-S MKTN, 80% S-S MKTN, and KTN). An initial test indicated that KTN gels were at equilibrium swelling (as determined by two consecutive readings at the same swelling ratio) by 2 hours. Subsequent experiments were performed at 2 hours because the gels were at equilibrium and because KOS formulations were observed to erode at later time points. Unconfined compression testing on the swelled materials showed a non-linear behavior, so we elected to fit the data by a least squares regression to the one-term Ogden model of hyperelasticity in order to determine the compressive modulus. We then computed the effective crosslink density and molecular weight between crosslinks. The results of these measurements and calculations are shown in Table 2. KOS and each of the MKTN formulations had significantly different swelling ratios than KTN (column 2 of Table 2). We note that we were unable to obtain the compressive modulus on the KOS gels because they were too soft to register loads on either a 10 N or 100 N load cell (reported as N/A in Table 2). The 7% S-S MKTN and 80% S-S MKTN showed significant differences compared to KTN for compressive modulus (K), effective crosslink density (ν_e), and molecular weight between crosslinks (M_c). Further, these differences followed the expected trend of fewer disulfide crosslinks leading to lower compressive modulus, lower crosslink density, and larger molecular weight between crosslinks. Interestingly, the 74% S-S MKTN formulation had a higher compressive modulus, higher effective crosslink density, and lower molecular weight between crosslinks than KTN.

3.4. Characterization of rheological properties

To further characterize the hydrogels, we performed rheological testing on the KOS, 7% S-S MKTN, and KTN samples. The results of the frequency sweep for elastic modulus and viscous modulus are summarized in Figure 3. On the log-log plot, all three gel types tested were nearly linear for G' , although frequency dependence was observed for KOS and 7% S-S MKTN for G'' . This frequency-dependent behavior of KOS and 7% S-S MKTN for G'' may be due to greater chain mobility due to larger molecular weight between crosslinks (see Table 2) [44, 45]. KOS had significantly lower elastic modulus (storage; G') and viscous modulus (loss; G'') than KTN ($P < 0.05$ at all frequencies as determined by t-test). Over the frequencies tested, the 7% S-S MKTN hydrogels had elastic moduli in between KOS and KTN, and these differences were statistically significant at all frequencies ($P < 0.05$ as

determined by t-test). The decrease in elastic modulus for the 7% S-S MKTN compared to KTN is interpreted as owing to the loss of the disulfide bonds in these formulations, consistent with results of the network structure analysis of Section 3.3. The viscous moduli of 7% S-S were similar to KTN (not significantly different at any frequency except the 2 lowest frequencies tested) but were significantly greater than KOS at all frequencies tested ($P < 0.05$, t-test).

3.5. MC3T3 pre-osteoblasts cytotoxicity

To determine if alkylation of KTN led to increased levels of toxicity due to changes in the protein or due to the presence of residual iodoacetamide, we conducted three types of cytotoxicity assays. Quantitative cell viability assays (MTS) were performed with keratin coatings and with keratins in solution to assess the effects of the alkylation on toxicity associated with keratin. A qualitative assay for viability was also conducted as a proof-of-concept study by seeding cells in the presence of three-dimensional hydrogels. This latter assay was conducted to assess effects of the actual hydrogels in the form that they would be used for TERM applications. Ideally, cells would have been incorporated directly into the keratin hydrogels for these studies. However, kerateine does not gel in the presence of the salts present in cell culture media, so we were unable to incorporate cells directly into the keratin (though we note that we have recently shown cell viability in keratose gels [46]).

MC3T3-E1 cells cultured on keratin-coated wells showed no statistically different rates of cell viability measured as metabolic activity by MTS compared to coatings of KTN, KOS, or uncoated TCPS (Figure 4A). Cell viability in the presence of soluble (relatively low concentration) keratin proteins was also assessed by MTS assay in a dose-response fashion as shown in Figure 4B. We note that higher concentrations were not assessed due to the possibility that these concentrations of keratin would become insoluble. The results show that cell viability decreases in a dose-dependent manner. However, none of the keratin formulations were significantly different from each other, indicating that the alkylation process did not lead to increases in toxicity from the soluble form of keratin.

For the qualitative assay in the presence of the three-dimensional hydrogels, a live/dead assay (Figure 5) showed no obvious difference in cell density on TCPS in the presence of KTN compared to MKTN. Importantly, the alkylated MKTN did not show (qualitatively) an increase in the number of dead cells compared to KTN or TCPS control, which is consistent with the coating and soluble dose-response assays described above.

3.6. MC3T3 pre-osteoblasts adhesion and immunofluorescence

To assess any effects that alkylation had on cell binding to kerateine, the adhesion strength of pre-osteoblasts to keratins was measured by a centrifugation adhesion assay [31]. Fibronectin and collagen (which is a current clinical rhBMP-2 carrier) were used as comparative controls, with fibronectin a positive control for cell adhesion. The detachment forces tested by centrifugation ranged from 12 – 410 times the force of gravity. The adhesion fraction was calculated based on the ratio of fluorescence before centrifugation to fluorescence after centrifugation following a 1 hour incubation of MC3T3 cells on protein-coated TCPS at 37 °C. Fibronectin and keratins had no different cell attachment until 172 g,

which is shown in Figure 6. There was also no difference between fibronectin and keratin at 320 g (data not shown). However, keratins showed better adhesion profiles than collagen across all detachment forces used in this experiment (only 172 g force is shown in Figure 6). MC3T3 cells showed no differences in fractional adhesion in any of the keratin groups across the forces tested.

To further assess cell adhesion to the modified keratins, we conducted immunohistochemical staining for focal adhesions. MC3T3 cells were incubated on TCPS surfaces coated with KTN, MKTN, fibronectin or collagen for 2 days. Vinculin was stained to assess focal adhesions and actin filaments were stained to assess stress fiber formation. The images shown in Figure 7 indicate that the levels of focal adhesions were similar between all proteins tested. However, actin filaments were noticeably reduced in collagen compared to fibronectin or keratin, indicating a weaker interaction consistent with the centrifugation assay results presented in Figure 6.

3.7. rhBMP-2 and rhIGF-1 binding to keratins

It has previously been observed that release of rhBMP-2 from keratin hydrogels correlates with the rate of gel erosion [22, 23], suggesting a possible interaction between rhBMP-2 and keratin. Here, we determined an equilibrium binding value by a solid phase assay for rhBMP-2 and rhIGF-1 in order to (1) evaluate the strength of the interaction and (2) to determine if alkylation led to a change in the interaction. Biotinylation of rhBMP-2 led to 8.8 ± 1.7 moles biotin per mole rhBMP-2. As shown in Figure 8A, rhBMP-2 followed saturation profile common for ligand-receptor or Michaelis-Menten binding. Based upon this initial data for rhBMP-2 binding to keratin, one rhBMP-2 (biotinylated) concentration was selected for further analysis. At 106 nM rhBMP-2 concentration (Figure 8B), which is near the $\frac{1}{2}$ maximal value, there was no significant difference in binding fraction between any of the keratins (KOS, KTN, or 7% S-S MKTN) or the fibronectin. However, rhBMP-2 showed significantly increased binding affinities for all keratin formulations compared to collagen.

Biotinylation of rhIGF-1 led to 0.1 ± 0.01 moles biotin per mole rhIGF-1. In contrast to rhBMP-2 in which all keratin formulations showed detectable levels of binding, for rhIGF-1 detectable levels of binding were found between rhIGF-1 and KTN only up to a concentration 20 μ M shown (Figure 8C). Other keratins (KOS and MKTN) and proteins (FN, CN) showed a small, linear increases over the same range of rhIGF-1 concentration, indicating little binding and likely no binding beyond non-specific effects. 7.1 μ M rhIGF-1 (near the $\frac{1}{2}$ max value of rhIGF-1 binding to KTN) was used to compare the binding affinity between proteins as shown in Figure 8D. 7% S-S MKTN had lower binding affinity than KTN to rhIGF-1 while both of keratins contained the equivalent binding affinity to rhBMP-2.

3.8. Modified Kerateine hydrogel erosion and growth factor and ciprofloxacin release

Erosion rates of modified kerateine hydrogels were examined by DC protein assay (Figure 9A). These results indicate that the rate of erosion of MKTNs was more rapid than kerateine mainly due to the lower level of disulfide crosslink density. Further, the alkylation led to the

erosion rate of modified kerateine hydrogel that correlated to the amount of iodoacetamide used, but the relationship was not directly proportional to the iodoacetamide:keratin cysteine ratio. In addition, KTN and each of the MKTN formulations had significantly lower levels of erosion than KOS gels (shown in Figure 9A insert due to much higher erosion percentages). KOS does not have disulfide crosslinks and therefore forms gels by chain entanglements and through other forces. The lack of direction proportionality to alkylation levels (in the MKTN formulations) and the lower rates of erosion compared to KOS indicates that factors beyond the free thiol content of the keratins play a role in the rate of gel erosion, as we discuss below.

Release of rhBMP-2, rhIGF-1, or ciprofloxacin from 15% (w/v) keratin gels (KOS, KTN, or three different MKTNs (80%, 74% and 7% S-S MKTNs) was determined at specified time points. rhBMP-2 and rhIGF-1 were quantified by ELISA while ciprofloxacin was quantified by its inherent fluorescence. As shown in Figure 9B and 9C, rhIGF-1 and ciprofloxacin release rates increased as the free thiol content of modified kerateine decreased due to the lower level of disulfide crosslinks achieved by alkylation of the keratin. For ciprofloxacin and rhIGF-1 release, the amounts of release from MKTNs are proportional to the level of disulfide bonds within MKTNs. 7% S-S keratin gels achieved higher levels of ciprofloxacin release than KOS gels while for rhIGF-1 release, 7% S-S keratin gels approached lower release level than KOS gels. While rhBMP-2 release also followed the trend of increasing release with decreased levels of disulfide crosslinking (Figure 9D), statistical differences in cumulative release were not found between any of the formulations after 3 hours of release.

4. Discussion

Polymer systems that promote cell attachment but can also be easily tuned for controlled release of therapeutic agents and erosion of the hydrogel matrix would be beneficial to numerous TERM strategies for tissue repair [47–49]. Keratin is characterized by a relatively high cysteine residue content compared to other ECM-like proteins and has previously been used in various TERM applications as coatings, scaffolds, and hydrogels either with or without the release of exogenous therapeutics. In this report, we used a simple alkylation approach that could be used to exploit the presence of cysteine residues as a means to maintain favorable cell interactions while providing tunable rates of erosion and therapeutic agent release.

Alkylation of thiol groups by iodoacetamide was effective in decreasing the free thiol content of kerateine (Figure 1B). It should be noted that in these studies, the reaction conditions were not optimized (e.g., for reaction time or temperature) and the resulting MKTNs did not show a change in free thiol that was directly proportional to the amount of iodoacetamide. Further optimization of the reaction conditions in future studies would likely allow even finer tuning of the levels of alkylation and resulting hydrogel properties. However, a 2.5-fold molar excess of iodoacetamide did lead to a reduction in thiol content (and thus disulfide crosslinking) to only 7% of pre-modification levels, which is a free thiol level similar to KOS (oxidatively extracted keratin). Based on these results, we were able to pursue proof-of-concept studies related to the effects of the alkylation process on the material properties of and cellular response to the alkylated keratin materials.

While the appearance of the hydrogels at the macroscopic level and microscopic level (Figure 2) were similar, the alkylation was shown to have a functional effect on the hydrogels. The swelling ratios increased with increasing levels of alkylation compared to KTN. Further, the MKTN hydrogels with the lowest levels of S-S crosslinking (7% S-S MKTN) as well as the 80% S-S MKTN had significant differences in compressive modulus (of the swollen hydrogels), effective crosslink density, and molecular weight between crosslinks compared to KTN. The effects of the alkylation were also seen to have clear effects in the visco-elastic properties of the hydrogels as indicated by rheological characterization. Our interpretation of the network analysis (Q , K , v_e , and M_c) and rheological data is that decreasing levels of disulfide in the 7% S-S MKTN gels is responsible for the significant decrease in elastic modulus compared to KTN. The effects of lower thiol content are also consistent with the effects on the observed rates of keratin erosion (Figure 9A). However, the fact that the 7% S-S MKTN has similar levels of free thiol to KOS but much lower rates of gel erosion (Figure 9A insert) and that the 74% S-S MKTN did not show significant changes in Q , K , v_e , and M_c compared to KTN suggests that factors besides disulfide crosslinks play a role. While the specific nature for these effect are not clear, the gel formation is complex and depends on more than just disulfide crosslinks. For example, keratose does not have disulfide crosslinks, yet forms gels, likely through chain entanglements and other intermolecular interactions. The alkylation reaction used could have effects on the resulting keratin hydrogels, thereby affecting the mechanical characteristics of the materials. Also, given the known role of hydrophobic interactions in the assembly of keratin fibers [50], it would not be surprising if hydrophobic interactions are one physiochemical reason for these observations. The results, however, show proof-of-concept to validity and simplicity of this approach.

Given the known toxicity of iodoacetamide in its unreacted form, toxicity of the alkylated keratin proteins was assessed in three different formats: as 2-D coatings, in solution, and as 3-D hydrogels. Only the highest level of alkylation (7% S-S MKTN) was investigated in these studies because this formulation would be the most likely to cause toxicity or other changes that would lead to unfavorable cellular interactions (see below) due to the highest levels of iodoacetamide. For keratin coatings and soluble keratin in culture media (Figure 4) the alkylation of MKTN did not lead to increased levels of toxicity compared to unmodified KTN or KOS, and toxicity levels of KTN and KOS were consistent with other reports [17, 28]. Further, 3-D hydrogels composed of KTN or MKTN (7% S-S MKTN) did not show qualitatively increased levels of toxicity to MC3T3-E1 cells cultured in the same well (Figure 5). These results indicate that procedures to remove excess iodoacetamide following the coupling chemistry (i.e., dialysis) were successful and that leaching of any unreacted iodoacetamide from keratin hydrogels was not a problem on the timescales considered.

Although keratin is known to promote cell attachment [51], the specific domains responsible are not known in all cases. A previous report indicates that the extraction methods that were used to obtain the keratin proteins used in our studies is composed, in part, of keratin 31 and keratin 33 [27], which have been shown to contain the leucine-aspartic acid-valine (LDV) sequence [51]. This sequence is recognized by the $\beta 1$ integrin [52], which is known to be found in various cell types including osteoblasts [53]. Certain keratin fractions are also reported to contain the RGD sequence [54], so it is possible that multiple integrin binding

domains play a role in cell attachment to keratin. The strength of cell adhesion was investigated through both qualitative and quantitative measures of cell attachment (Figure 6 and 7).

The centrifugation adhesion assay (Figure 6) used fibronectin as a positive control due to its known integrin binding domains [55] and collagen was used mainly because its 3-D scaffold form (absorbable collagen sponge) is clinically used as a BMP-2 carrier. This experiment was conducted by increasing forces via centrifugation. At the forces tested, cells attached in higher percentages to keratin than to collagen. However, none of the keratin formulations differed significantly, indicating that the alkylation of KTN did not interfere with whatever binding domains are responsible for cell attachment to these materials.

These quantitative adhesion results confirm immunofluorescence images showing higher levels of stress fibers in MC3T3-E1 cells on fibronectin and keratin surfaces compared to collagen (Figure 7). Collagen is well-known to possess the RGD sequence [56], so the reduction in adhesion of MC3T3-E1 cells could be because the RGD sequences have not been “unlocked” [57]. While the exact integrin binding sequence within KOS, KTN, or MKTN responsible for MC3T3-E1 attachment was not investigated, it seems that the levels of adhesion would be best explained by integrin binding or another cell adhesion molecule rather than some non-specific interaction between cells and keratin.

Given the previously observed correlation between the rate of keratin hydrogel erosion and rate of therapeutic agent release, we suspected that there may be some specific interaction between keratin and the therapeutic agent depending on the physiochemical properties of the therapeutic. Therefore, equilibrium binding between keratin and two growth factors (rhBMP-2 and rhIGF-1) were tested with a solid phase assay (Figure 8) [58, 59]. The assay was not conducted with ciprofloxacin because the biotin (~ 244 Da molecular weight) used for detection would have contributed much more to binding measurements than it would on rhIGF-1 or rhBMP-2.

The solid-phase assay for rhBMP-2 to collagen, fibronectin, and all keratin forms (KOS, KTN, 7% S-S MKTN) followed a traditional saturation curve, indicating some level of specificity in the interaction of rhBMP-2 with keratin through some unknown binding domain. The different materials (KTN, MKTN, KOS, FN, and CN) were compared at approximately the half-max value for KTN (106 nM). At this concentration, rhBMP-2 showed the highest levels of binding to fibronectin, followed by the keratins, and had the lowest binding to collagen. While it is not clear that similar levels of binding are present in 3-D versions of these materials (e.g., the absorbable collagen sponge used clinically for rhBMP-2 delivery in some applications), it is interesting that in this study, rhBMP-2 has the lowest binding affinity to collagen. This could explain some of the known problems related to burst-release and subsequent ectopic bone growth with the absorbable collagen sponge [60, 61] and suggests that keratin might be a suitable alternative carrier for rhBMP-2.

It was also interesting that no binding was detected between rhIGF-1 and KOS or MKTN, though we were able to detect binding of rhIGF-1 to KTN. The levels of interaction between KTN and rhIGF-1 were nearly 2 orders of magnitude greater than the interaction between

KTN and rhBMP-2. This result indicates that rhBMP-2 will not dissociate from keratin as readily as rhIGF-1, which helps to explain the observed rates of delivery of rhBMP-2 and rhIGF-1 as well as ciprofloxacin from the 3-D keratin hydrogels (Figure 9).

Alkylation led to highly tunable levels of ciprofloxacin release, with 74% S-S MKTN having similar release to KOS and 7% S-S MKTN having even more rapid release than KOS. For rhIGF-1, 7% S-S MKTN clearly had a different release profile than KTN with significantly greater release starting at the first (1.5 hour) time point. In contrast, while rhBMP-2 followed this trend of increasing release with decreasing levels of disulfide crosslinking, there was no significant difference between KTN and any of the MKTN formulations.

These results seem to be explained by the aspects of this system: (1) the rates of gel erosion (Figure 9A) due to varying levels of alkylation, (2) the equilibrium binding data between the growth factors and keratin as determined by the solid phase assays in Figure 8, and (3) the physiochemical properties of the drug (particularly molecular weight and isoelectric point). The levels of rhBMP-2 release are very low compared to the overall amount of gel erosion, indicating that rhBMP-2 release may be underestimated, possibly due to loss of bioactivity and thus detection by the ELISA system used. Nevertheless, for each therapeutic agent there is increasing release (only by trend for rhBMP-2) with decreasing levels of disulfide crosslinks. This indicates the role of the rate of keratin erosion on the release of the drug. The molecular weight of the drugs partially explain the differences in the release profiles for ciprofloxacin, rhIGF-1, and rhBMP-2. The smallest molecule (ciprofloxacin) is released the most rapidly whereas the largest (rhBMP-2) has the slowest release. This would be consistent with a diffusion mechanism with increasing rates of diffusion with decreasing molecular weights. We also suggest that the binding affinity, which is affected by properties such as molecular weight and electrostatic interactions (related to isoelectric point of the protein and keratin), plays a role in the variations in release profiles for each therapeutic. Because rhIGF-1 has no detectable binding to KOS or MKTN, it is released much more rapidly. The small (but detectable) affinity of rhIGF-1 for KTN would explain the retardation in its release from KTN gels. Likewise, given that we observed an even higher binding affinity between rhBMP-2 and each form of keratin (KOS, KTN, MKTN), this may partially explain why release of rhBMP-2 from each keratin hydrogel formulation is lower (as a percentage) than rhIGF-1. This can be most readily observed by comparison of Figure 9D (4–8% rhBMP-2 release for the various keratin formulations) to Figure 9C (60% release of rhIGF-1 from keratose, which has a low binding affinity with rhIGF-1).

While keratin biomaterials have previously been used as stand-alone materials for several applications [14, 17], the incorporation of tunable delivery of therapeutic agents could enhance their utility for TERM applications. For example, the delivery of rhIGF-1 for nerve regeneration could further enhance the previously observed regenerative effects of keratin [62]. Similarly, given the effects of keratin in burn wound healing [14], co-delivery of antibiotics may aid in reducing bacterial colonization to provide improved treatment and healing profiles [63]. As noted above, the higher binding affinity of rhBMP-2 for keratin may be advantageous compared to collagen in reducing ectopic bone growth, as we have recently reported [23]. Further, the ability to modulate keratin biomaterials that promote

strong cell attachment while providing temporal control over delivery of therapeutic agents may lend keratin to other TERM applications that require more than a material alone.

5. Conclusions

These studies presented a proof-of-concept that a simple alkylation of cysteine residues on keratine can be used as an approach to modulate erosion of the hydrogels as well as the subsequent release of therapeutic agents. This modification process did not lead to increased cytotoxicity and maintained the ability of cells to attach to the material. Factors affecting release of therapeutic agents appear to include the rate of keratin hydrogel erosion and binding affinity of the drug to keratin, which is impacted by the physiochemical properties of the drug. These studies have implications for controlled delivery of therapeutic reagents as these modified keratin hydrogels wed advantages of natural protein polymer systems such as cell attachment with advantages of synthetics such as tunable erosion and therapeutic agent release.

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Figure 1.

(A) Schematic showing the mechanism of the reaction used to alkylate or “cap cysteine residues of kerateine (KTN) with iodoacetamide, which is referred to in this manuscript as modified kerateine (MKTN). (B) Thiol content of modified kerateines measured by Ellman’s assay after modification with iodoacetamide. Results are the mean with error bars representing standard deviation ($n = 4$). * indicates significant difference compared to KTN, # denotes significant difference from KOS, and ** indicates significant difference from

MKTN ($p < 0.05$ for each) as determined by a one-way ANOVA with Tukey's post-hoc analysis.

Figure 2. Scanning electron micrographs of lyophilized keratin hydrogels showing porous structure of (A) KOS, (B) 7% S-S MKTN, (C) 74% S-S MKTN, (D) 80% S-S MKTN, and (E) KTN. All images were taken at 500× magnification and working distances of ~4.5–7.0 mm. Scale bars indicate 100 μm .

Figure 3.

Rheological characterization of hydrogels. (A) Elastic modulus (G') for KTN, 7% S-S MKTN, and KOS as a function of frequency. (B) Viscous modulus (G'') for KTN, 7% S-S MKTN, and KOS as a function of frequency. 7% S-S MKTN had significantly greater G' values than KOS and significantly lower G' values than KTN at all frequencies ($P < 0.05$, t-test). 7% S-S MKTN had significantly greater G'' values than KOS at all frequencies ($P < 0.05$, t-test) but did not have statistically different G'' values than KTN at all but the two lowest frequencies tested. $N = 3$ for each sample where G' and G'' were determined from the same sample. Error bars indicate standard error of the mean.

Figure 4.

(A) Proliferation of MC3T3-E1 pre-osteoblasts cultured on tissue culture polystyrene coated with keratin (KOS, KTN, or 7% S-S MKTN) at 150 $\mu\text{g/mL}$ of keratin and (B) MC3T3-E1 pre-osteoblast viability relative to untreated control exposed to keratin in solution at indicated concentrations for 72 hours as measured by MTS assay (mean $\pm$ STD, $N = 4$). No statistical differences were determined after one-way ANOVA with Tukey's post-hoc analysis.

Figure 5.

MC3T3-E1 pre-osteoblasts grown in the presence of three-dimension keratin hydrogels composed of (A) 15% w/v KTN, (B) 15% w/v 7% S-S MKTN or (C) on tissue culture polystyrene (control) for 24 hours and stained with a live (green)/dead (red) assay. Scale bar indicates 200 μm.

Figure 6.

MC3T3-E1 pre-osteoblast adhesion to tissue culture polystyrene (TCPS) with adsorbed proteins as determined by centrifugation assay. 50 $\mu\text{g/mL}$ of each protein was adsorbed to TCPS for 2 hours, cells were incubated on the protein-coated surfaces for 1 hour, and the cells were subjected to 172 g detachment forces by centrifugation. Bars represent mean $\pm$ standard error of the mean ($n = 8$). * and # denote values that differ significantly from FN and CN, respectively ($p < 0.05$) as determined by one way ANOVA with Tukey's post-hoc analysis.

Figure 7.

Epifluorescence images of MC3T3-E1 pre-osteoblasts cultured on tissue culture polystyrene pre-adsorbed with (A) FN, (B) CN, (C) KTN, or (D) 7% S-S MKTN illustrating focal adhesion by vinculin (green) and F-actin as determined by phalloidin staining (red). Levels of focal adhesions (vinculin) are similar for all protein coatings. However, actin stress fibers for cells on KTN and MKTN are similar to those on FN whereas CN shows far less intense actin staining. Nuclei are counterstained with DAPI (blue) and scale bar indicates 50 μm .

Figure 8.

(A) rhBMP-2 (biotinylated) binding to proteins coated on TCPS as determined by solid-phase assay. (B) Binding affinity of (biotinylated) rhBMP-2 to these proteins at 106 nM rhBMP-2 concentration (near the $\frac{1}{2}$ max value as determined for KTN in part (A)). There were 8.8 ± 1.7 biotin molecules per rhBMP-2 and error bars represent mean $\pm$ standard deviation ($n = 4$ for FN and CN; $n = 3$ for keratins). # denotes values that are significantly different from CN ($p < 0.05$) and * indicates those significantly different from KTN as determined by student t-test. (C) rhIGF-1 (biotinylated) binding to proteins coated on TCPS as determined by solid phase assay. (D) Binding affinity of (biotinylated) rhIGF-1 to these proteins at 7.1 μ M rhIGF-1 (near the $\frac{1}{2}$ max value as determined for KTN in part (C)). There were 0.1 ± 0.01 biotin molecules per rhIGF-1 and bars represent mean $\pm$ standard deviation

(n = 4 for all groups). # denotes values that are significantly different from CN and * indicates those significantly different from KTN ($p < 0.05$) as determined by student t-test.

Figure 9.

(A) Hydrogel erosion of 15% w/v MKTN formulations and unmodified KTN. Increasing levels of alkylation show increasing rates of erosion. Inset of (A) shows erosion of 15% KOS hydrogels, which were much greater than KTN or MKTN formulations. (B) – (D) show release from 15% w/v keratin hydrogels loaded with (B) 2mg/mL ciprofloxacin (C), 100 μ g/mL rhIGF-1, or (D) 10 μ g/mL rhBMP-2. Ciprofloxacin release was determined by inherent fluorescence of the molecule while rhIGF-1 and rhBMP-2 release were determined by ELISAs specific to each growth factor. #, *, +, **, † denote values that differ significantly from KTN, KOS, 80%, 74% and 7% S-S MKTN respectively ($p < 0.05$) as determined by one way ANOVA with Tukey's post-hoc analysis, after 3 hours of release.

Table 1

Amounts of reagents for alkylation of kerateine proteins.

Experimental Group Name*	Mass of Kerateine	Volume of Water for Kerateine Solution	Mass of Iodoacetamide (added as 0.54 M solution in water)	Molar Ratio of Iodoacetamide to Kerateine
7% S-S MKTN	800 mg	160 mL	133.3 mg	2.5
74% S-S MKTN	800 mg	160 mL	47.9 mg	0.9
80% S-S MKTN	800 mg	160 mL	28.4 mg	0.4

* Experimental group name was based on results of Ellman's assay for free thiol.

Table 2

Experimentally-determined hydrogel characteristics.

Experimental Group	Swelling Ratio (Q)	Compressive Modulus (K) in kPa [*]	Effective Crosslink Density (v_e) in $\mu\text{mol}/\text{cm}^3$	Molecular Weight between Crosslinks (M_c) in kg/mol
Keratose	$1.18 \pm 0.26^{\#}$	N/A	N/A	N/A
7% S-S MKTN	$1.07 \pm 0.01^{\#}$	$10.4 \pm 1.98^{\#}$	$4.12 \pm 0.79^{\#}$	$87.7 \pm 19.9^{\#}$
74% S-S MKTN	$1.06 \pm 0.02^{\#}$	53.0 ± 19.8	21.1 ± 7.90	19.2 ± 9.4
80% S-S MKTN	$1.05 \pm 0.01^{\#}$	$26.4 \pm 2.4^{\#}$	$10.6 \pm 0.96^{\#}$	$33.4 \pm 3.2^{\#}$
Kerateine	1.01 ± 0.01	42.8 ± 8.9	17.3 ± 3.58	21.1 ± 5.2

N/A indicates not available because keratose gels were too soft to obtain reliable values for compressive modulus with 10 N or 100 N load cells. Values indicate average $\pm$ standard deviation. N = 4.

[#] indicates statistically different from KTN ($P < 0.05$) as determined by student t-test for the indicated measurement (Q or K) or calculation (v_e or M_c).

^{*} Compressive modulus was determined by fitting the non-linear stress-stretch results to a one-term Ogden model of hyperelasticity.