Chronic Wound Dressings Based on Collagen-Mimetic Proteins

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Objective: Chronic wounds are projected to reach epidemic proportions due to the aging population and the increasing incidence of diabetes. There is a strong clinical need for an improved wound dressing that can balance wound moisture, promote cell migration and proliferation, and degrade at an appropriate rate to minimize the need for dressing changes.

Approach: To this end, we have developed a bioactive, hydrogel microsphere wound dressing that incorporates a collagen-mimetic protein, Scl2_{GFPGER}, to promote active wound healing. A redesigned Scl2_{GFPGER}, engineered collagen (eCol_{GFPGER}), was created to reduce steric hindrance of integrin-binding motifs and increase overall stability of the triple helical backbone, thereby resulting in increased cell adhesion to substrates.

Results: This study demonstrates the successful modification of the Scl2_{GFPGER} protein to eCol_{GFPGER}, which displayed enhanced stability and integrin interactions. Fabrication of hydrogel microspheres provided a matrix with adaptive moisture technology, and degradation rates have potential for use in human wounds.

Innovation: This collagen-mimetic wound dressing was designed to permit controlled modulation of cellular interactions and degradation rate without impact on other physical properties. Its fabrication into uniform hydrogel microspheres provides a bioactive dressing that can readily conform to irregular wounds.

Conclusion: Overall, this new eCol_{GFPGER} shows strong promise in the generation of bioactive hydrogels for wound healing as well as a variety of tissue scaffolds.

INTRODUCTION

COLLAGEN IS A MAJOR COMPONENT of most skin substitutes due to its structure and bioactive cues.¹ Collagens are abundant extracellular matrix (ECM) proteins, which give structural integrity to many tissues and regulate cell functions through interactions with cellular receptors and additional ECM constituents. There are at least 28 different mammalian collagens, all with a defining left-handed triple helix that com-

prises three polypeptide chains. Each polypeptide chain contains continuous GXY motifs, where X is often proline and Y is usually hydroxyproline.² Proline hydroxylation and protein glycosylation are important in overall triple helix stability and collagen network formation. Unlike many commercialized recombinant proteins, the required post-translational modifications of collagen have made it difficult to generate viable recombinant collagen products. Synthetic collagen

peptides have limited quantities and are cost prohibitive,³ which have limited collagen products to animal sources. These animal-derived materials have inherent disease risk, batch variability, and are often milled with heterogeneous results.⁴

In contrast, bacterial-based collagen mimics have been characterized as triple helical proteins despite a lack of hydroxyproline, which circumvents the need for post-translational modifications and enables facile recombinant expression. Several researchers are currently investigating these proteins as potentially useful biomaterials.^{3,5–16} Their production and purification from bacterial expression systems allows for batch conformity and scale-up.¹¹ One bacterial collagen isolated from *Streptococcus pyogenes*, Scl2, has shown particular promise as a biomaterial for wound care. Scl2 contains an N-terminal globular domain, followed by a collagen-like region comprising GXX repeats,⁸ where X is often proline or a charged residue to provide helix stability.¹⁷ Since bacterial protein engineering is possible in reasonable time frames, Scl2 possesses an additional advantage, in that it can be optimized to possess specific properties.¹⁸ Ligands for Scl2 have not been identified and therefore selective introduction of mammalian collagen sequences can be used to control and tune cell–material interactions. This is a feature unavailable with current animal collagens, which offer a broad array of signals to cells. We previously used site-directed mutagenesis to introduce the GFPGER motif into the Scl2 sequence (termed Scl2_{GFPGER}) and provided evidence that human integrin-binding sites function within Scl2_{GFPGER} to bind and activate $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Integrins are cell surface heterodimers that enable outside-in and inside-out signaling, which regulate numerous processes, such as migration, proliferation, and phenotype, which are key processes in wound healing.^{19–29} The Scl2_{GFPGER} platform provides a unique opportunity to investigate the contribution of collagen-binding integrins to wound healing. Although integrin signaling is not the only event leading to wound closure, our scouting studies in a rodent excisional model demonstrated that soluble Scl2_{GFPGER} application increased wound closure rates. Since soluble protein is not a therapeutically viable option, we have incorporated the Scl2_{GFPGER} into microspheres that should retain the same bioactivity.

The collagen-mimetic protein, Scl2_{GFPGER}, was incorporated into hydrogels utilizing conjugation chemistry developed in our laboratory to generate a robust wound dressing.¹⁴ Specifically, the Lys residues of Scl2_{GFPGER} were conjugated to a

photocross-linkable poly(ethylene glycol) (PEG) linker for incorporation into the hydrogels. We have previously demonstrated that the degree of functionalization has an effect on cell–material interactions, where a higher degree of functionalization imparted steric hindrance of the integrin-binding site.³⁰ Sufficient functionalization is required to anchor proteins within the hydrogel, and too little functionalization results in protein loss and reduction of cell adhesion. In this study, we present a redesigned Scl2 backbone to address these issues—engineered collagen (eCol_{GFPGER}). The eCol_{GFPGER} was designed to have functionalization sites further away from the integrin-binding motif to decrease steric hindrance.

Initial studies on these bioactive hydrogels utilized a PEG-diacrylate (PEGDA) matrix; however, the degradation rate of these hydrogels is slow relative to wound healing (months to years). A biodegradable hydrogel that degrades at a rate complementary to regeneration would eliminate the need for frequent dressing changes and reduce patient discomfort. We have developed modified PEG-based hydrogels that use thio- β ester linkages to provide controlled hydrolytic degradation that can be tuned to match wound healing. The addition of thio- β ester linkages increases the degradation rate of the PEGDA hydrogels by increasing the susceptibility of the neighboring acrylate ester bonds to hydrolysis.³¹ Careful control of the method for thio- β ester introduction allows for the addition of hydrolytically cleavable sites without changing other hydrogel properties that could influence wound care (*e.g.*, water uptake).

Although hydrogel dressings are common in wound care management, slabs or sheets of hydrogels may be difficult to fit in deep or irregularly shaped wounds. Cellular migration is also hampered in these geometries and limited to the rate of hydrogel degradation. In contrast, hydrogel microspheres provide a gel-like dressing that is able to conform to wound shape, offering immediate hydration balance and infection protection to the wound. Cellular infiltration into the microsphere network is facilitated by the gaps between each particle. Moisture control is also expected to be enhanced due to the increased surface area, allowing for more rapid water uptake and diffusion. Typical fabrication of hydrogel microspheres involves water-in-oil emulsions or microfluidics; however, these methods suffer from size heterogeneity and limited batch sizes. Droplet formation using passive coflowing streams can provide greater control over droplet size and uniformity.³² Fluidic methods have shown improved fabrication

processes with the potential to photopolymerize hydrogel microspheres.³³

In this study, integrin affinity and cell adhesion to Scl2_{GFPGER}, eCol_{GFPGER}, or an animal-derived collagen control were determined as an initial assessment of potential for wound care applications. Degradation of the hydrogels as a function of composition was investigated to identify promising compositions that permit reduced dressing changes. Finally, hydrogel microspheres were fabricated using a cofluidics setup, and the water uptake properties of both hydrogel slabs and microspheres were quantified. The potential of this dressing to conform to an irregular wound was then demonstrated in a rat model. Overall, this strategy harnesses biological dressing advantages while providing wound coverage and hydration through the presence of a tunable hydrogel network.

CLINICAL PROBLEM ADDRESSED

Human skin wounds affect 6.5 million patients per year and cost the United States \$25 billion per year.³⁴ These numbers are expected to rise since skin wounds correlate with an elderly population, obesity, and diabetes.³⁵ The impact of wounds on human health is minimized due to their nature as secondary diagnoses following a more severe diagnosis such as a pressure ulcer resulting from brain injury immobilization.³⁴ Skin wounds are classified by causality and include chronic wounds (diabetes and obesity), pressure ulcer, venous ulcers, and acute wounds (surgical and trauma).³⁴ Skin wounds can have grave consequences, such as amputation and infection risk, as well as severe quality of life adjustments.^{34,36–40}

Treatment for skin wounds varies depending on the severity of the wound and type of healthcare facility. If a wound has persisted long enough, more expensive treatments may be considered, such as negative pressure wound therapy, hyperbaric oxygen treatment, and skin substitute dressings.^{36–38} Since wound care bundling is currently being applied to outpatient treatments; the cost of wound care materials is of great concern to clinicians.⁴¹ Skin substitutes are often expensive since they contain animal-derived extracellular matrix (ECM) materials and often cells that require special handling and preparation.⁴² Current skin substitute limitations include low closure rates, difficulties with hydration balance, lack of wound exudate absorbance, and limited active wound healing options. To address these limitations, we propose to create a first-line wound healing therapy using bioactive

hydrogel microspheres to promote active wound healing that balances exudates and wound hydration, promote cell migration and proliferation, and modulate a hydrogel composition to degrade at an appropriate rate to match tissue regeneration and eliminate the need for frequent dressing changes.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma Aldrich and used as received unless otherwise noted.

Protein engineering

Recombinant eCol_{GFPGER} protein was derived from the Scl2-containing integrin-binding motif, GFPGER, at positions 118–123. The C-terminus of Scl2 contains regions where the triple helix is predicted to be less stable and contributes to a lower melting temperature (algorithm used to predict collagen stability is described in Persikov *et al.*¹⁷). Using this algorithm, GXY triplets were identified that may contribute to protein stability, and selected triplets were mutated to more stable triplets. To generate a more thermally stable collagen, two triplets of GKDGKD were mutated to GDRGER (Lys314→Asp314, Asp315→Arg315, Lys317→Glu317, Asp318→Arg318), where D and E at X position and R in Y position of the GXY triplet are predicted to be more stable and are more frequent in eukaryotic collagens. To stabilize functionalization of Scl2_{GFPGER}, single Lys residues were replaced by Arg residues in GKD triplets at Lys positions, 182, 236, 248, 263, 269, 275, 278, 284, 290, 293, 299, 305, 308, and 323 (the Arg residues at the X position of a GXY triplet exist at same frequency in eukaryotic collagens as replaced Lys residues and are similarly thermally stable). The mutations were introduced by gene synthesis (Genewiz). Histidine-tagged recombinant proteins were expressed from a pCold vector in *Escherichia coli* Top3 cells and purified by Ni²⁺ affinity chromatography on a 5-mL column (GE Healthcare). Protein purity was determined by SDS-PAGE, followed by Coomassie blue staining and western blot analysis. SDS-PAGE analysis was used to determine multimer formation as described. Briefly, proteins were denatured by incubation at 95°C for 5 min in the presence of 0.1% SDS and 2% β -mercaptoethanol. Nondenatured samples were incubated in 5% glycerol and kept on ice before electrophoresis on 12% SDS gels. Gels were stained with Coomassie blue, and protein migration as it corresponds to size was determined using protein standard.

Circular dichroism. Circular dichroism spectra of protein samples in 50 mM acetic acid were recorded on a Jasco J720 spectropolarimeter in a thermostatically controlled cuvette with a 0.5-mm path length. Data were collected at ambient temperature in a wavelength range from 250 nm to 190 nm and integrated for 1 s at 0.2-nm intervals with a bandwidth of 1 nm. For each spectrum, ten scans were averaged and the contribution from the buffer was subtracted. For thermal transition experiments, the ellipticity at 220 nm in a cuvette with a 0.5-mm path length was monitored as the sample temperature was increased from 25°C to 50°C, with an average temperature slope of 10°C/h.

Protein functionalization. Scl2_{GFPGER}, eCol_{GFPGER}, and rat tail collagen type I were functionalized with Aam-PEG-I. Briefly, Aam-PEG-I was dissolved in anhydrous dimethylformamide and added dropwise to a collagen solution in phosphate-buffered saline (PBS) and Scl2_{GFPGER} and eCol_{GFPGER} in 50 mM acetic acid at room temperature (acetic acid for Scl2_{GFPGER} and eCol_{GFPGER}). The molar ratio of Aam-PEG-I:NH₂ was 0.1:1 for collagen and Scl2_{GFPGER} and 0.5:1 for eCol_{GFPGER}. After 2 h of stirring, dialysis against RO water for 24 h (MWCO = 20,000 Da) was used to purify functionalized proteins. FTIR spectroscopy confirmed functionalization of proteins with Aam-PEG-I.

Solid-phase binding assays. Microtiter wells were coated with 2 µg per well of recombinant alpha 1 integrin I-domain. The samples were blocked with TBS containing 2% bovine serum albumin (BSA) for 1 h. Solutions of 1 µM Scl2_{GFPGER}, eCol_{GFPGER}, functionalized Scl2_{GFPGER}, or functionalized eCol_{GFPGER} were added to the wells and incubated for 1 h at room temperature. Rabbit polyclonal antibodies raised against Scl2_{GFPGER}, followed by donkey anti-rabbit-HRP and SigmaFast OPD, were used to detect bound proteins. The absorbance at 450 nm was measured using a Thermomax plate reader.

Cell adhesion study. All cell culture supplies were purchased from Life Technologies and used as received unless otherwise noted. To assess adhesion and spreading of HT1080 fibroblasts, 48-well tissue culture polystyrene (TCPS) plates were coated with 10 µg of Scl2_{GFPGER}, eCol_{GFPGER}, rat tail collagen type I, or Scl2 overnight at 4°C. Protein solutions (0.1 mg mL⁻¹ 20 mM acetic acid) were filtered through 0.22-µm PVDF syringe filters before coating (four wells per protein type). Wells were blocked with 4 vol% BSA in PBS for 1 h at

room temperature and rinsed with sterile PBS thrice. HT1080s were cultured in DMEM with 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were adapted to serum-free media for 12 h before seeding at 5,000 cells cm⁻¹ in protein-coated TCPS well plates. After 3 h of exposure, wells were rinsed thrice with warm PBS to remove unadhered cells, and the remaining cells were fixed with 3.7% glutaraldehyde for 20 min at room temperature. Cells were stained with rhodamine phalloidin (actin/cytoplasm; Sigma-Aldrich) and SYBR Green (DNA/nucleus) and imaged (three images per sample, four samples per protein) on a DeltaVision Elite microscope (GE Healthcare). Fluorescent images of SYBR Green- and rhodamine phalloidin-stained cells were utilized to quantify cell adhesion and spreading. Manual counts of SYBR Green-stained cell nuclei in each image provided adhesion density measurements. Spreading, or cell area, was quantified by applying the Photoshop magic wand tool to image backgrounds until all extracellular regions were selected. The histogram function was utilized to determine the number of extracellular pixels (P_{EX}), and the average number of pixels per cell (A_{CELL}) for a given image was quantified as follows:

$$A_{CELL} = \frac{P_T - P_{EX}}{N} \quad [1]$$

where P_T is total image pixels and N is the total number of cell nuclei. Pixels were then converted to microns using known objective scaling.

Polymer synthesis

Synthesis of PEGDA. PEGDA was synthesized according to a method adapted from Hahn *et al.*⁴³ Briefly, acryloyl chloride was added dropwise to a solution of PEG 2 kDa or 6 kDa diol and triethylamine (TEA) in dichloromethane (DCM) under nitrogen. The molar ratio of PEG, acryloyl chloride, and TEA was 1:2:4, respectively. After the addition of acryloyl chloride, the reaction was stirred for an additional 24 h at room temperature. The resulting solution was then washed with eight molar equivalents of 2 M potassium bicarbonate to remove acidic by-products. The product was then precipitated in cold diethyl ether, filtered, and dried under vacuum.

Synthesis of PEGDA with thio-β esters. Poly(ethylene glycol) d,l-dithiothreitol (PEGDTT) was synthesized by adding d,l-dithiothreitol (DTT) and TEA dropwise to a solution of PEGDA (2 kDa) in DCM, Fig. 1. The molar ratio of DTT, PEG, and TEA was 3:2:0.9, respectively. After the addition of the DTT and TEA, the reaction was stirred for 24 h

Figure 1. Synthesis of poly(ethylene glycol) diacrylate with thio- β esters (PEGDTT). PEGDTT was synthesized by adding d,l-dithiothreitol (DTT) and triethylamine (TEA) dropwise to a solution of PEGDA (2 kDa) in dichloromethane (DCM) under a nitrogen blanket (N_2). The molar ratio of DTT, PEG, and TEA was 3:2:0.9, respectively. The stars denote the sites of thio- β esters in the polymer chain, which undergo rapid hydrolysis. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

at room temperature. The resulting solution was then precipitated in cold diethyl ether, washed, filtered, dried under ambient conditions for 24 h, then placed under vacuum to remove any excess solvent.

Characterization of PEGDA and PEGDTT was confirmed using Fourier transform infrared spectra (FTIR) and proton nuclear magnetic resonance (1H -NMR) spectroscopy. Transmission FTIR spectra of control and functionalized polymers were acquired on a Bruker ALPHA spectrometer by solution casting directly onto KBr pellets with 32 scans and a resolution of 2 cm^{-1} . Proton NMR spectra of control and functionalized polymers were recorded on a Mercury 300 MHz spectrometer using a TMS/solvent signal as an internal reference. Percent conversions of PEG diol to acrylate endgroups was greater than 85%. 1H -NMR ($CDCl_3$): δ 3.6 ppm (m, $-OCH_2CH_2$), 5.8 ppm (dd, $-CH=CH_2$), 6.1 (dd, $-CH=CH_2$), and 6.4 ppm (dd, $-CH=CH_2$). PEGDTT number average molecular weight (M_n) was determined by normalizing to the PEG backbone and calculating the number of DTT macromers bound to PEG. The M_n was found to be 6.4 kDa with a percent acrylation of greater than 90%.

Hydrogel characterization

To measure water uptake, eight 8-mm discs were punched from hydrogel sheets. Samples were swollen in RO water for 24 h and weighed to determine the equilibrium swelling mass (W_s). Then, specimens were dried under vacuum overnight to determine dry mass (W_d). The water uptake (U)

was calculated from the equilibrium mass swelling ratio:

$$U = \frac{W_s - W_d}{W_d} \quad [2]$$

Hydrogels of varying composition were fabricated by making (10 wt%) precursor solutions in RO water to demonstrate a tunable degradation rate. A photoinitiator solution (1 mg Irgacure 2959 per 0.01 mL 70% ethanol) was added at 1 vol% of precursor solution. Solutions were pipetted between 1.5-mm-spaced plates and cross-linked by 6-min exposure to long-wave UV light (Intelli Ray Shuttered UV Flood Light; Integrated Dispensing Solutions, Inc., 365 nm, 4 mW/cm^2). The compositions included variable amounts of PEGDTT in PEGDA 6 (kDa) gels (100%, 75%, 50%, and 0%). The hydrogels were punched into 8-mm discs and incubated in 7.4 pH PBS at 37°C for 21 days with weekly solution changes. The swollen masses were recorded daily for the first week, then at 10, 14, and 21 days. The swelling ratio was then calculated and used to characterize the network degradation.

Hydrogel microsphere fabrication

A 30% hydrogel precursor solution was prepared by dissolving PEGDA (6 kDa) in water. The solution was mixed, and a photoinitiator solution (1 mg Irgacure 2950 per 10 μL 70% ethanol) was added at 1 vol% of precursor solution. Microspheres were then fabricated through a fluidics water-in-oil emulsion technique adapted from Gokmen *et al.*⁴⁴ The hydrogel

precursor solution was injected dropwise (KD Scientific-100 Infusion Pump) into an external mineral oil phase (Harvard PHD 2000 Infusion Pump) and passed through UV excitation (UVP High-Performance Transilluminator 365 nm) for 2 min to initiate radical cross-linking. Needle gauge, aqueous flow rate, and hydrogel injection rate were varied to modulate particle size. Collected microspheres were filtered using vacuum aspiration.

Statistical analyses

All statistical analyses are expressed as the mean $\pm$ standard derivation of the mean. Statistical analysis was performed by an unpaired two-tailed Student's *t*-test. Statistical significance was accepted at $p < 0.05$.

Rat excisional wound model

A 3-month-old male Sprague-Dawley rat that had been euthanized less than an hour previously was obtained through the Comparative Medicine Program at Texas A&M University. This was done under AUP IACUC 2014-010 Maintenance and Utilization of Animals for Animal Care and Use Training, which allows for excess animals and an-

imals euthanized under other AUPs to be utilized in a tissue share program to maximize use of all research animals. An $\sim 1.5 \times 2.0$ -cm full-thickness irregularly shaped defect was created on the dorso-lateral aspect of the rat and extended into the underlying muscle layers.

RESULTS

Protein engineering

First, we modified the Scl2 protein to enhance its use as a bioactive component of our hydrogel wound dressing. Efforts were taken to improve Scl2_{GFPGER} thermal stability and reduce steric hindrance of the PEG linkers around the integrin-binding site. Fourteen single Lys in the collagen-like region of the protein were mutated to Arg residues in GKD triplets, which was predicted to have similar thermal stability, but did not support PEG linker attachment. The resulting protein, eCol_{GFPGER}, was then evaluated for retention of a triple helix, thermal stability, and integrin interactions. After recombinant production and purification of eCol_{GFPGER}, circular dichroism was used to assess the protein's triple helix. Figure 2A and B

Figure 2. CD analysis indicates that functionalization does not significantly affect triple helix formation. CD wavelength scans of (A) Scl2_{GFPGER} (black) and F-Scl2_{GFPGER} (red) and (B) eCol_{GFPGER} (black) and F-eCol_{GFPGER} (red) demonstrate the presence of a peak at 220 nm, indicative of a triple helical structure. CD thermal transition of (C) Scl2_{GFPGER} (red) and eCol_{GFPGER} (green). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

showed typical triple helical spectra with a peak at 220 nm, indicating that our protein engineering did not disrupt the triple helix. In addition, SDS-PAGE analysis revealed the presence of multimers (data not shown). A marked increase in melting temperature from 37°C to 45°C was observed in thermal transition studies, Fig. 2C.

The collagen-mimetic proteins were then functionalized with PEG linkers to determine whether eCol_{GFPGER} had improved integrin-binding site availability. FTIR spectroscopy was first used to confirm successful functionalization of Scl2_{GFPGER} and eCol_{GFPGER} with PEG linkers to formulate F-Scl2_{GFPGER} and F-eCol_{GFPGER}, respectively. The functionalized protein spectra contained absorption peaks corresponding to the carbonyl of the amides ($\sim 1,650\text{ cm}^{-1}$) in the protein backbone and ether ($\sim 1,110\text{ cm}^{-1}$) of the PEG linker (data not shown). An enzyme-linked immunosorbent-type assay was then used to measure the effect of protein functionalization on recombinant $\alpha 1$ I-domain binding. We consistently observed a decrease in integrin affinity regardless of which collagen mimetic was analyzed; however, the observed decrease was less pronounced with eCol_{GFPGER}, Fig. 3. Protein binding was measured using polyclonal antibodies raised against Scl2_{GFPGER} in rabbits; however, these polyclonal antibodies displayed similar titers against all collagen mimetics tested (data not shown).

To assess the functionality of the integrin-binding site in eCol_{GFPGER}, HT1080 fibroblast adhesion and spreading on protein coats were assessed at 3 h, Fig. 4. Although cell spreading was similar between the three collagen-mimetic proteins, a significant increase in adhesion was observed on Scl2_{GFPGER} with the introduction of the integrin-binding site into Scl2, as shown previously.¹⁴ A further increase in adhesion was observed on eCol_{GFPGER}, relative to Scl2_{GFPGER}, to a level that is comparable with that on collagen I.

Hydrogel characterization

The final purified polymer structure of PEGDA and PEGDTT was confirmed with both FTIR and ¹H-NMR spectroscopy. For PEGDA, the presence of ester peaks at $1,730\text{ cm}^{-1}$ and loss of hydroxyl peaks at $3,300\text{ cm}^{-1}$ in the IR spectra confirmed successful acrylation. For PEGDTT, the loss of the thiol stretch at $2,600\text{ cm}^{-1}$, the presence of the hydroxyl peaks at $3,300\text{ cm}^{-1}$, and decrease in the 842 cm^{-1} peak correlating with the carbon-carbon double bond confirmed successful coupling, Fig. 5. The ratio of integrated protons of acryloyl to backbone methyl groups in the ¹H NMR spectra confirmed the structure of PEGDA. Percent con-

Figure 3. eCol_{GFPGER} exhibits reduced steric hindrance of PEG linkers. 1 μM collagen-mimetic, Scl2_{GFPGER}, F-Scl2_{GFPGER}, eCol_{GFPGER}, and F-eCol_{GFPGER}, were incubated with immobilized recombinant human $\alpha 1$ I-domains (1 $\mu\text{g}/\text{well}$) with 1 mM Mg^{2+} . Collagen-mimetic binding was detected as described in Experimental Procedures. Data shown are representative of at least three replicates and normalized to the non-functionalized control protein.

versions of PEG-diol to acrylate endgroups was greater than 90%. The PEGDTT structure and number average molecular weight (M_n) were determined by the ratio of protons of the methyl groups in the PEGDA backbone to the number of protons from the DTT backbone. The M_n was found to be 6.4 kDa with an acrylation of greater than 90%, Fig. 6. After characterization, PEGDTT and PEGDA macromers were cross-linked into hydrogel slabs using 1 wt% Irgacure 2959 at a 10 wt% concentration with compositions, including 1.0, 0.85, 0.75, 0.50, and 0.0 PEGDTT/PEGDA (wt/wt). After 24 h in RO water and 24 h of drying under vacuum, no significant changes were observed in the water uptake of the hydrogel slabs by changing compositions from 1.0 to 0.5 PEGDTT, Fig. 7.

Figure 4. HT1080 fibroblast adhesion and spreading on collagen-mimetic protein-coated wells. **(A)** Adhesion numbers on protein coats after a 3-h incubation period. **(B)** Average cell spreading (area) at 3 h. **(C)** Rhodamine phalloidin and SYBR Green-stained HT1080 fibroblasts. $n=4$ wells per protein, 3 images per well for a total of 12 images per sample; mean $\pm$ standard deviation displayed; $\circ \ddagger *$ indicate statistically significant differences between respective samples $p < 0.05$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

Incorporation of DTT into the PEGDA hydrogels significantly impacted the degradation rate. Increasing amounts of PEGDTT from 50% to 100% resulted in a direct increase of the degradation rate of the hydrogels. Both the PEGDTT and PEGDA hydrogel slabs had similar swelling and water uptake values. Hydrolytic degradation of PEGDA hydrogel networks is attributed to the cleavage of ester groups, which reduce the cross-link density with time. As cross-link density is reduced, the equilibrium swelling ratio is increased.⁴⁵ Therefore, the swelling ratio was analyzed to determine the extent of degradation after incubation in PBS for 21 days. PEGDA hydrogels displayed no sig-

nificant change in swelling ratio, as expected. Hydrogels made with 1.0 PEGDTT showed a loss of mechanical integrity at 12 days and complete dissolution within 14 days. The 0.85 PEGDTT compositions lost mechanical integrity after 14 days, while the 0.75 PEGDTT hydrogels displayed an increase in swelling by 45%, but retained mechanical integrity for the full duration of the study. The 0.5 PEGDTT hydrogels remained statistically similar to the PEGDA hydrogels over the course of 21 days, Fig. 8.

Hydrogel microsphere properties

Microspheres fabricated, using the water-in-oil emulsion technique described above, were filtered

Figure 5. Transmission FTIR spectrum of the PEGDTT (Top), PEG(2k)DA (Middle), and DTT (Bottom). Transmission FTIR spectra of control and functionalized polymer neat films were acquired on a Bruker ALPHA spectrometer by solution casting directly onto KBr pellets with 32 scans and a resolution of 2 cm^{-1} .

Figure 6. ^1H NMR spectra of the PEGDTT in CDCl_3 . Proton NMR spectra of control and functionalized polymer solutions were recorded on a Mercury 300 MHz spectrometer using a TMS/solvent signal as an internal reference. ^1H NMR (CDCl_3): δ 3.6 ppm (m, $-\text{OCH}_2\text{CH}_2-$), 5.8 ppm (dd, $\text{CH}=\text{CH}_2$), 6.1 (dd, $\text{CH}=\text{CH}_2$), and 6.4 ppm (dd, $-\text{CH}=\text{CH}_2$).

Figure 7. Water uptake comparisons of PEGDTT and PEGDA hydrogels. Water uptake reported in mL of water per gram of polymer for all hydrogel compositions after 24 h. All samples were statistically similar ($n=5$).

using vacuum aspiration and then characterized, Fig. 9. Fully swollen fabricated microspheres had an average diameter of $878 \pm 45 \mu\text{m}$. Particles of this size were achieved with an $800 \mu\text{L min}^{-1}$ continuous phase flow rate, $0.5 \mu\text{L h}^{-1}$ dispersed hydrogel phase flow rate, and 1.6-mm-inner-diameter PVC tubing (McMaster-Carr). A blunted 30-gauge needle was used to inject the hydrogel precursor solution into the continuous mineral oil phase. Particle size can be tuned by changing the flow rates of the continuous and dispersed phases. Decreasing the flow rate of the continuous phase or increasing the flow rate of the dispersed phase de-

Figure 8. Effect of PEGDTT concentration on the *in vitro* hydrolytic degradation of PEGDA hydrogels. Changes in hydrogel swelling ratio as a function of incubation time in PBS at 37°C . PEGDA and 0.5 PEGDTT gels were statically similar through 21 days.* Loss of mechanical integrity.

creases the particle size. The flow rate of the continuous phase has the most impact on particle size and shape.

Swelling ratio and water uptake of 10% PEGDA (6 kDa) and PEGDTT (5.8 kDa) hydrogel slabs were measured to compare with 30% PEGDA (6 kDa) microspheres, Table 1. Both the 1.0 PEGDTT and PEGDA hydrogel slabs had similar swelling and water uptake values. The microspheres had

Figure 9. Schematic of microsphere fabrication. Using coflow fluidics, hydrogel microspheres were fabricated by injecting dropwise a hydrogel precursor solution into a continuous mineral oil phase, creating a water-in-oil emulsion. Hydrogel microspheres were then cross-linked using UV light while traveling along the tubing.

Table 1. Swelling and Water Uptake Comparisons of PEGDTT and PEGDA Slabs and Microspheres

	Swelling ratio (<i>Q</i>)	Water uptake (mL H ₂ O/g polymer)
PEGDTT Slab ^a	12.64 ± 0.48	11.64 ± 0.48
PEGDA Slab ^b	13.46 ± 2.23	12.46 ± 2.23
PEGDA Microspheres ^{a,b}	9.16 ± 2.94	8.16 ± 2.94

^{a,b}Indicate statistically significant differences between respective samples $p < 0.05$.

PEGDA, poly(ethylene glycol) diacrylate; PEGDTT, poly(ethylene glycol) d,l-dithiothreitol.

slightly lower, but comparable, swelling ratio and water uptake values compared with the hydrogel slabs, which demonstrate the ability of the hydrogel microspheres to absorb wound exudate. Finally, we demonstrated that this tunable microsphere design allows for improved wound filling when applied to irregularly shaped wounds in a rat model, Fig. 10. The defect was packed with hydrated microspheres such that the defect was completely filled to the level of the surrounding undamaged tissue.

DISCUSSION

Protein engineering

The Scl2 backbone has been characterized as a potentially useful recombinant biomaterial and can support the introduction of human integrin sequences to facilitate cell adhesion, migration, and proliferation.^{3,6–11,13–16,18,46} Our results demonstrate that selective triplet substitutions may be used to further optimize the backbone helix to achieve certain properties. Given that it has been established that a triple helical conformation is required for integrin binding and activation,^{22,23,25,47} our demonstrated ability to stabilize triple helices is expected to affect ligand interactions and potential cellular behavior.⁴⁸ To anchor these proteins into the hydrogel, it is necessary to first functionalize the protein with PEG linkers that can photocross-link with the PEG matrix. The

placement of these PEG linkers along the triple helical backbone can have significant implications regarding the integrin-binding site availability to cells due to steric hindrance.³⁰ Our targeted modification of the Scl2 protein to limit the steric hindrance of these PEG linkers during integrin binding was successful. In addition, stability predictions were used to mitigate any changes in the backbone that might result in disrupted interchain interactions. These efforts resulted in a protein construct with an increase in melting temperature of $\sim 7^\circ\text{C}$. The studies here show an increase in cell adhesion on eCol_{GFPGER} compared with Scl2_{GFPGER}, indicating that these substitutions had some effect on cell–material interactions.

Cell–material interactions

Analysis of HT1080 fibroblast interactions demonstrated that eCol_{GFPGER} induced comparable cell adhesion with that of collagen I. Considering the presence of only one integrin-binding site in eCol_{GFPGER} compared with collagen's multitude of integrin-binding sites, this result demonstrates a notable increase in protein functionality. Furthermore, when compared with Scl2_{GFPGER}, significantly higher cell adhesion was induced on eCol_{GFPGER}. These two proteins have the same integrin-binding motif; thus, the increased integrin interactions with eCol_{GFPGER} are either due to its increased thermal stability or an overall triple helical change, resulting in altered integrin interactions. A stable triple helix is required for integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ binding to the GFPGER site. Cells were incubated on protein-coated plates at 37°C , which is the approximate melting temperature of both collagen I and Scl2_{GFPGER}.⁴⁹ Therefore, it is possible that both collagen and Scl2_{GFPGER} experienced some denaturation during this experiment, rendering their integrin-binding sites inactive. eCol_{GFPGER} was well below its thermal transition temperature of $\sim 45^\circ\text{C}$ in this experiment, indicating that it was intact with active integrin-binding sites. However, we cannot rule out the

Figure 10. Hydrogel microspheres filling the wound defect in rat model. (A) Irregularly shaped wound on the dorsolateral aspect of the rat (B) Wound packed with hydrated microspheres, 5 mm scale bar (C) Swollen hydrogel microspheres, 1 mm scale bar. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

possibility that these changes did not have an effect on the interaction with the integrin itself and studies are underway to characterize the binding mechanism.

Hydrogel characterization

Tunable degradation rates of PEG-based hydrogels utilizing the incorporation of β -thio esters have been previously studied.^{31,50,51} However, these approaches often change other properties in addition to the degradation rate, including the cross-link density, swelling, and modulus of the hydrogel.^{31,50} In this study, the PEGDTT and PEGDA polymers had similar molecular weights to allow for a direct comparison of the degradation profile. By utilizing PEGDTT polymers, specifically the incorporation of β -thio esters into the backbone without changing the overall molecular weight between cross-links of the network, the degradation rate of hydrogels can be tuned independently of hydrogel properties. Finally, its fabrication into uniform hydrogel microspheres provides a bioactive dressing that can readily conform to irregular wounds. Future studies will determine if the observed enhancement in cell interaction is conferred to the bioactive gels due to reduced steric hindrance around the integrin-binding site, increased stability of the triple helix, or a combinatory effect. Overall, this new eCol_{GFPGER} shows strong promise in the generation of bioactive hydrogels for wound healing as well as a variety of tissue scaffolds.

INNOVATION

Although hydrogel sheets are popular wound dressings, these passive dressings do not promote regeneration, require frequent changing, and cannot conform to irregularly shaped wounds. Previously, we fabricated bioactive hydrogels through the addition of collagen-mimetic proteins that can regulate key processes in wound healing. In this study, we present a novel collagen-mimetic protein with reduced steric hindrance, reduced melting properties, and increased cell–material interactions. The hydrogel matrix was designed to permit controlled modulation of the degradation rate without impact on other physical properties. Hydrogel microspheres were then fabricated to provide a dressing that can readily conform to irregular wounds and facilitate cell infiltration.

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KEY FINDINGS

- A collagen mimetic, eCol_{GFPGER}, was developed that has improved stability and functionalization profile while maintaining integrin and cell interactions.
- Hydrogel dressings were synthesized that have tunable degradation rates independent of other hydrogel properties (*i.e.*, water uptake).
- Hydrogel microspheres were fabricated using a dual fluidics setup, which were uniform in size and allow for immediate coverage of irregular wounds.

AUTHOR DISCLOSURE AND GHOSTWRITING

Elizabeth Cosgriff-Hernandez, Magnus Höök, and Brooke Russell disclose equity holdings in ECM Technologies, which seeks to commercialize bacterial-derived collagen-mimetic proteins. No competing financial interests exist for the remaining authors. The content of this article was expressly written by the authors listed. No ghostwriters were used to write this article.

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Abbreviations and Acronyms

¹H-NMR = proton nuclear magnetic resonance
 BSA = bovine serum albumin
 DCM = dichloromethane
 ECM = extracellular matrix
 eCol = engineered collagen
 FTIR = Fourier transform infrared spectra
 IR = infrared
 Mn = number average molecular weight
 N₂ = nitrogen gas
 PBS = phosphate-buffered saline
 PEG = poly(ethylene glycol)
 PEGDA = poly(ethylene glycol) diacrylate
 PEGDTT = poly(ethylene glycol) d,l-dithiothreitol
 TCPS = tissue culture polystyrene
 TEA = triethylamine