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S-Nitroso-N-acetylpenicillamine (SNAP) Derivatization of Peptide Primary Amines to Create Inducible Nitric Oxide Donor Biomaterials

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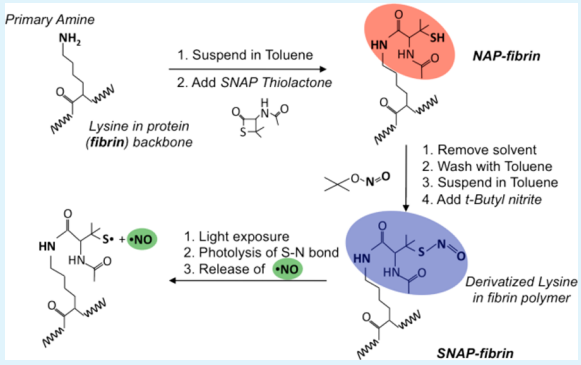
Supporting Information

ABSTRACT: An S-nitroso-N-acetylpenicillamine (SNAP) derivatization approach was used to modify existing free primary amines found in fibrin (a natural protein-based biomaterial) to generate a controlled nitric oxide (NO) releasing scaffold material. The duration of the derivatization reaction affects the NO release kinetics, the induction of controlled NO-release, hydrophobicity, swelling behavior, elastic moduli, rheometric character, and degradation behavior. These properties were quantified to determine changes in fibrin hydrogels following covalent attachment of SNAP. NO-releasing materials exhibited minimal cytotoxicity when cultured with fibroblasts or osteoblasts. Cells maintained viability and proliferative character on derivatized materials as demonstrated by Live/Dead cell staining and counting. In addition, SNAP-derivatized hydrogels exhibited an antimicrobial character indicative of NO-releasing materials. SNAP derivatization of natural polymeric biomaterials containing free primary amines offers a means to generate inducible NO-releasing biomaterials for use as an antimicrobial and regenerative support for tissue engineering.



KEYWORDS: *biodegradable, fibrin, hydrogel, inducible, nitric oxide, tissue engineering*

1. INTRODUCTION

Tissue engineering approaches combining biomaterials with biologics (proteins, genes, and cells) represent an alternative to traditional biological grafts and permanent artificial substitutes. The focus of significant ongoing research is directed in part toward developing competent scaffolding materials capable of fulfilling application specific requirements for biodegradation, biocompatibility, mechanical stability, biofunctionality, and processability.[1−3] An additional consideration for any tissue engineering strategy is clinical diversity (i.e., acute injuries due to trauma or chronic injuries brought on by repetitive stress) and the unique biological, mechanical, and structural challenges posed in each case.[4,5] As a result, it is unlikely that a single approach to tissue regeneration will emerge addressing all clinical problems. However, developing control systems that can be designed into any biomaterial approach could allow for the site-specific tailoring of active treatment elements for the augmentation of wound repair. This work addresses this existing challenge by creating a delivery approach that will allow natural matrix biomaterials (i.e., fibrin, collagen) to be directly modified into inducible nitric oxide release vehicles that can be used as stand alone biomaterials or as part of hybrid materials in combination with other natural and synthetic polymers.[6−8]

Nitric oxide (NO) plays a significant role in tissue turnover and wound healing; and has been implicated as a necessary factor in various wound-injury healing conditions including skin ulcers, diabetic lesions, tendinopathy, and bone fracture healing.[9,10] The beneficial effects of NO in specific tissues and under physiological conditions are tightly bound to its controlled availability, a result of its limited half-life and its spatial and temporal expression.[11] Recent work has revealed that exogenously generated NO (NO-donor therapy) has therapeutic potential for the treatment of infection[12] and modulation of wound healing.[13−16] Beneficial effects include the inhibition of bacterial adhesion, promotion of bacterial cell death, accelerated cell migration and collagen matrix synthesis.[13,15,17] However, if not tightly controlled, elevated or depressed NO availability can lead to potentially detrimental effects including apoptosis, loss of tissue stability, and structural atrophy.[18,19] Thus, even with the efficacy shown by NO-donor therapy (i.e., injected and topical NO-donor group delivery) on soft tissue healing and regeneration,[9,20] practical inducible NO-release systems remain limited.[21] A large effort has gone into developing NO-releasing polymers. Reviews by Frost,[22] Shin,[23] and Varu[24] detail developing hydrophilic and hydrophobic polymers

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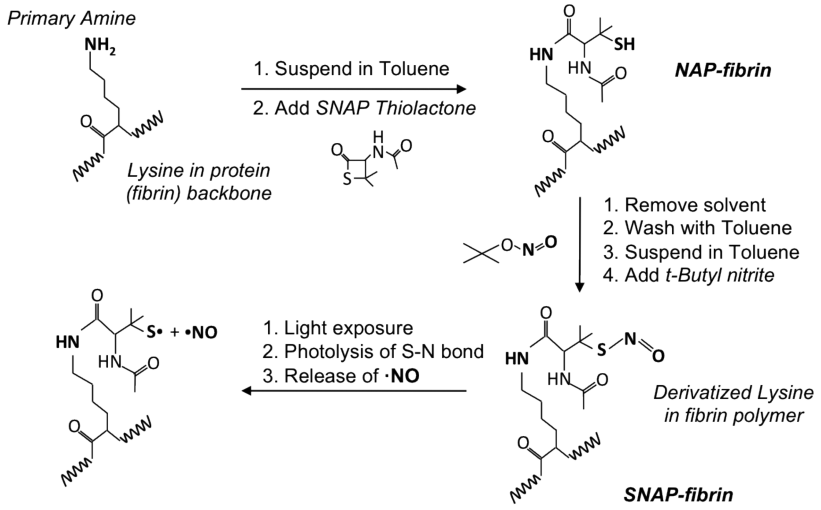

Figure 1. Lysine primary amine derivatization within fibrinogen−fibrin. Pendant primary amines are converted to s-nitrosothiols, the NO-donating groups. This approach can be taken using any polymer containing free primary amines. Subsequent photolytic cleavage of the S−N bond of the nitrosothiols results in controlled NO release.

that can be synthesized to achieve specific NO-release levels and durations. Additionally, hydrogels and biodegradable polymers[25,26] have been synthesized that release NO for application in wound healing.[17,21,27−29]

The characteristics of the polymer used to deliver NO are important, because they will form the underlying framework through which NO will promote wound healing. For this work we chose to use fibrin as the base polymeric biomaterial for derivatization. Fibrin is a highly compatible natural biopolymer (formed during blood coagulation) that can be prepared from autologous sources (i.e., fibrinogen can be isolated from individual patient blood). Fibrin has the unique ability to directly attract and harbor several different cell types, which in turn degrade the fibrin and replace it with extracellular matrix (ECM).[30] Fibrin also maintains favorable mechanical properties that can promote cell expansion and remodeling. Fibrin-based hydrogel materials have been shown to inherently facilitate cell adhesion and promote regeneration through enhanced angiogenesis and cell viability. The ability to alter the architecture of fibrin hydrogels also aids in their use as a tissue engineering biomaterial. Additionally, these materials are resorbable and can be cross-linked to control stability and degradation behavior, further validating their extensive use as injectable tissue-adhesives and scaffolds, and making it a strong candidate for this study.[30−33]

The fundamental approach in this work seeks to derivatize pendant primary amines (lysine units) found in fibrin to form a S-nitroso-thiol adduct and create an inducible NO-releasing hydrogel (S-nitroso-N-acetyl-D,L-penicillamine (SNAP)-fibrin) with tunable NO-release properties, (Figure 1 shows an overall schematic of our approach). S-Nitrosothiols (RSNOs) are NO-donating compounds, which under favorable conditions, liberate nitric oxide (NO•) and a thiyl radical (RS•). RSNOs are particularly sensitive to photolytic cleavage, making them good candidate molecules for the controlled release of NO.[34] Although we chose to use fibrin as the basis for this derivatization study, our approach is readily amenable to other materials containing pendant primary amines. We hypothesized that primary amine groups on any biologic polymer can be readily derivatized with light sensitive RSNOs. That this approach requires active cleavage of the derivatized material for NO release to occur—either via photoactivation or potentially mechanical or thermal stimulus—separates it from other NO-donor approaches. Additionally, spontaneous degradation of the derivatized base material will not elicit a significant active molecule release.

In this work, we develop novel, fibrin-based hydrogels derivatized with SNAP as a controlled NO-release biomaterial system for tissue engineering. We first established and verified SNAP derivatization of fibrin hydrogels. Covalent modification of natural polymers can significantly affect physical properties of the material, so we next examined the affects of derivatization on fibrin hydrogel hydrophobicity and swelling character (swelling ratio); degradation in an aqueous microenvironment; and mechanical properties in compression as well as rheological properties. These properties are important especially if these materials are to be used as injectable scaffolds to promote wound healing and tissue regeneration. We then investigated the active NO release behavior from derivatized hydrogels, evaluating the effect of derivatization time on release kinetics as well as the ability to control release using radiant energy (UV−visible spectra), temperature, and direct mechanical stimulus (compression). Last, with chemical modification, the loss of the inherent biosafety of natural materials can be a concern, and so derivatized hydrogels were evaluated in culture to assess cell viability as well as inherent antimicrobial character of derivatized hydrogels. Additional experiments were completed to demonstrate that the derivatization methodology also created controlled NO-release properties from similarly modified collagen, indicating that this could eventually be a generalized technique to impart NO release character to biopolymers with accessible pendant primary amines for derivatization.

2. MATERIALS AND METHODS

2.1. Preparation of SNAP-Thiolactone. N-acetyl-D-penicillamine, pyridine, acetic anhydride, chloroform, hexanes, hydrochloric acid and magnesium sulfate were purchased from Sigma Aldrich (St. Louis, MO, USA). The N-acetyl-D-penicillamine was converted to a self-protected thiolactone using a procedure previously developed by Moynihan and Roberts.[35] Approximately 5 g of N-acetyl-D-penicillamine was dissolved in 25 mL of pyridine; 10 mL of acetic anhydride was combined with 10 mL pyridine. Both solutions were chilled on ice

for 20 min, combined and stirred for 36 h at room temperature. Pyridine and acetic anhydride were then removed via rotary evaporation. The resultant amber oil was redissolved in 20 mL of chloroform and extracted with 1 M HCl three times. The chloroform solution was collected and dried with magnesium sulfate. The mixture was suction filtered and the chloroform removed through rotary evaporation. The resultant SNAP-thiolactone crystals were collected with hexanes and suction filtration.

2.2. Derivatization of Protein Polymers—Fibrin, Collagen, and Fibrinogen. *2.2.1. SNAP—Fibrin Gels.* Fibrinogen (Sigma Aldrich) (100 mg/mL) was polymerized with equal amounts of thrombin (Sigma Aldrich) (2.5 U/mL) in glass vessels to form 2D fibrin hydrogels (d = 9 cm, t = 2 mm).[36] Gels were soaked in a solution of toluene and SNAP-thiolactone for 24 h. Samples were removed and rinsed with fresh toluene prior to nitrosation with clean t-butyl nitrite (t= 30 min, 1 h, and 2 h) (Figure 1). The t-butyl nitrite is cleaned to remove copper ions that are added to stabilize the organonitrite by extraction with 15 mM cyclam (aqueous). The degree of substitution of N-acetyl-D-penicillamine was determined by Ellman's test for free thiols added after derivatization (see Figure S-1 in the Supporting Information). Covalent attachment and nitrosation were verified separately using FTIR (see Figure S-2 in the Supporting Information). Biopsy punches were used to prepare samples for cell culture and NO-release experiments (d = 10 mm), and surface characterization (d = 20 mm).

2.2.2. SNAP—Collagen Gels. Aqueous collagen (2 mL) (Bio Rad, Hercules, CA, USA) (2%w/v) was reacted with SNAP-thiolactone (25 mg) for 24 h. Derivatized collagen (100 μL) was cast on 12 mm glass disc and allowed to cure for 24 h. Cleaned t-butyl nitrite (100 mL) was applied to the film surface and reacted for 10 min, 30 min, or 2 h.

2.2.3. SNAP—Fibrinogen Fibers. Lyophilized fibrinogen was dissolved in a solution of hexafluoro-2-propanol and phenol red-free Dulbecco's modified Eagle's medium (9:1) at a concentration of 110 mg/mL.[37] Electrospun fibers were generated with a custom-built electrospinning system using the following spinning parameters: voltage, 28 kV DC; needle gauge, 18.5 g; flow-rate, 1.8 mL/h; air-gap, 7 cm; rotational speed, 500 rpm. All samples were spun for 35 min. Electrospun fibers were cross-linked using ethylcarbodiimide hydrochloride (EDC) for 30 min and then soaked in toluene-SNAP-thiolactone for 30 min, and nitrosated using t-butyl nitrite for 5 min.

2.3. Contact Angle and Swelling Ratio. The hydrophobicity of fibrin and SNAP-fibrin hydrogels (d=20 mm) was determined using standard contact angle methods. A Kruss G10 goniometer (Kruss USA, Matthews, NC, USA) system was used to make all measurements. Fibrin and SNAP-fibrin were prepared as described above and stored in deionized water prior to measurements. Samples were removed 1 h prior to measurements and air-dried at room temperature. Using the sessile drop method, ethylene glycol droplets were added in succession to the center of the sample using a micro syringe (5 mm). Following the addition of each droplet, angle measurements of the liquid/substrate surface interface were taken until a contact angle plateau was reached. All measurements were taken at 25 °C to minimize thermosensitive hydrophobicity changes.

Swelling ratio (Q) measures were used to further characterize the effect of derivatization on water affinity and swelling behavior of our hydrogels. Fibrin and SNAP-fibrin hydrogels stored in deionized water (dH_2O) were freeze-dried overnight. After drying, samples were weighed and resuspended in 5 mL of fresh dH_2O for 24 h. After 24 h in dH_2O, the swelling forces were counter-balanced by retraction forces of the cross-linked network to reach equilibrium. Filter paper was used to blot the surface water from each sample and measures were made of the swollen weight. The swelling ratio was estimated by dividing the swollen weight (W_s) by the original dry weight (W_d) of each respective sample (eq 1).

$$Q = W_s/W_d \quad (1)$$

2.4. In Vitro Degradation Behavior. Hydrogels (n = 3) prepared as previously described were dried overnight and weighed (W_i) prior to incubation in 5 mL of phenol red-free DMEM (Invitrogen, Carlsbad, CA, USA) at 37 °C for 14 days. Media was changed each day. For each time-point (1—14 days), fibrin hydrogels were dried and weighed (W_d) as previously described. The percent mass loss (n = 3 for all time-points) was calculated by $(W_i - W_d)/W_i \times 100$.

2.5. Mechanical Testing. Basic fibrin hydrogels were prepared as previously described, using a 2:1 thrombin-fibrinogen mixture. Modified hydrogels (NAP-fibrin) were nitrosated for 30, 60, or 120 min with unmodified fibrin hydrogel materials serving as controls. Uniform 8 mm diameter biopsy punches were used in all control and experimental groups (n = 3). Mechanical testing was performed immediately. The elastic moduli of fibrin gels were determined using unconfined constant strain-rate compression tests performed using a 2 lbs S-beam load cell (Futek, Irvine, CA) attached to a servo-hydraulic uniaxial materials testing machine (model 8872, Instron Corp, Canton, MA). Samples were centered between two force plates and submerged in dH_2O. Force was applied to each hydrogel at a constant rate of 0.333% strain/sec (final strain (ε_f)= 20%) and held constant for 10 min (relaxation period). Elastic moduli were determined over the linear range (approximately up to 5% strain) and stress-relaxation behavior was determined for each group over the full range (n = 3 for all time-points).

Rheological properties of the fibrin gels were determined using a Bohlin C-VOR 200 NF rheometer. An oscillatory frequency (0.1—10 Hz at 0.1 strain) sweep experiment was performed to determine the storage (G') and loss (G'') moduli. All samples were tested using 15-mm parallel plates at a gap size that was 87.5% of individual gel thickness, as measured by a digital caliber. Frequency sweeps were repeated three times per sample and a total of three samples per formulation were tested.

2.6. NO Release Kinetics. For all experiments, NO release was directly measured via chemiluminescence detection using a Sievers 280i nitric oxide analyzer (Boulder, CO, USA) with 200 mL/min N_2 (g) sweep gas at 22 °C. All release measurements were done under hydrated conditions.

2.6.1. Nitrosation Time and Photoinitiated NO Release. Modified fibrin hydrogels (d = 10 mm) were prepared according to the protocol described above. Hydrogels were then soaked in toluene to covalently link the N-acetyl-D-penicillamine via reaction with the SNAP-thiolactone with primary amine groups present in the fibrin hydrogel. The tethered N-acetyl-D-penicillamine was then converted to SNAP by soaking the fibrin hydrogel in t-butyl nitrite for varying time periods (t = 30 min, 1 h, 2 h, 1 day, 2 days, 3 days) to examine the effects of nitrosation time on maximum photoinitiated NO release. Following exposure to t-butyl nitrite, the hydrogels were rinsed to remove unreacted t-butyl nitrite and placed in quartz sample holders and covered with foil to exclude light until NO-release measurements were completed. Photolytic release was initiated using a strong direct incandescent light source (intensity (I) = 116 W/m^2). Derivatized fibrin (SNAP-fibrin) samples were exposed at a distance of 6 cm for 1 h at 22 °C to measure maximum NO release. The derivatized collagen (SNAP-collagen), samples were exposed to 470 nm LED light (6 V, 100 Ω resistor) at a distance of 6 cm for 2 h at 22 °C.

2.6.2. Copper Ion and Ascorbate-Initiated NO Release. Modified fibrin hydrogels (d = 10 mm) were derivatized for 30 min, rinsed to remove residual t-butyl nitrite, and placed in amber glass vessels to prevent photoinitiated NO release. Various solutions (250 μM $CuBr_2$ with 5 mM ascorbate, 250 μM $CuBr_2$, 5 μM $CuBr_2$ with 5 mM ascorbate, ascorbate, MC3T3 cell culture media with 5 mM ascorbate, MC3T3 cell culture media) were injected into the vessel to induce NO release.

2.7. Cell Viability and Antimicrobial Character. Prior to cell culture all controls and SNAP-modified materials were rinsed in 70% ethanol and sterile PBS (3×). To assess cell viability, fibroblasts (L929) and osteoblasts (MC3T3) were cultured directly on fibrin and SNAP-fibrin hydrogels in DMEM containing 10% FBS (changed every 3 days) at 37 °C, 5% CO_2 and a seeding density of 1×10^5 cells/cm^2. In a complementary experiment, fibroblasts (L929 and primary rat tendon-derived) were cultured on cross-linked electrospun SNAP-fibrinogen fibers (diameter ~250 nm) at 2×10^4 cells/cm^2. Cells were allowed to adhere for 24 h, after which derivatized materials were exposed to a strong direct incandescent light (intensity = 116 W/m^2)

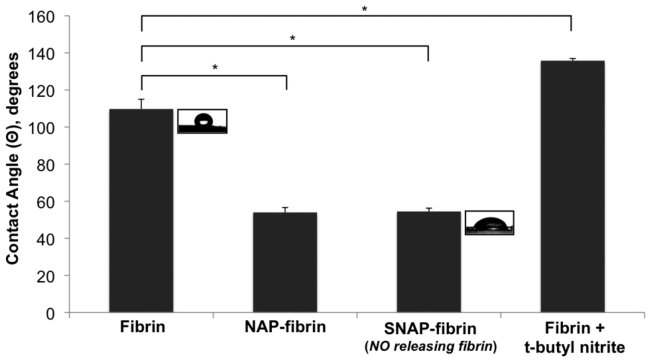

Figure 2. Hydrophobicity changes in derivatized fibrin hydrogels. Contact angle measurements were made using the sessile drop method. Fibrin, NAP-fibrin and SNAP-fibrin hydrogels were compared. Additionally, fibrin that had not been reacted with the derivatization reaction was reacted with t-butyl nitrite to illustrate that formation of the covalently linked acetylpenicillamine is responsible for the change in hydrophobicity. Conversion of the NAP-fibrin to the NO-donating SNAP-fibrin was performed by reaction with t-butyl nitrite for 30 min. (n = 3 for all groups). SNAP-fibrin is the only group capable of NO release. (*) indicate $p < 0.001$.

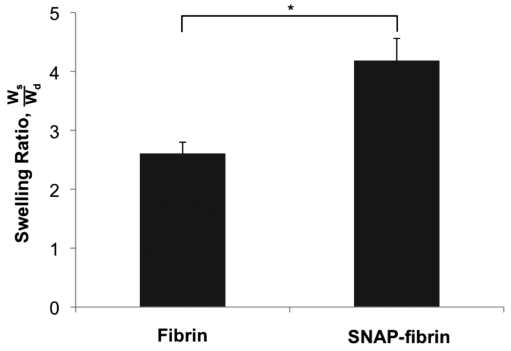

Figure 3. Swelling behavior of SNAP-fibrin hydrogels. Fibrin controls were compared with SNAP-fibrin formed by a 30 min reaction with t-butyl nitrite (n = 3). (*) indicate $p < 0.02$.

for 2 min at 24 and 72 h. Cells were stained with calcein-AM/ethidium bromide and assessed for viability using fluorescent microscopy (Olympus BX51) after a total of 5 days in culture.

To evaluate antimicrobial character, SNAP-fibrin hydrogels (d = 10 mm, t = 2 mm) nitrosated for 30 min, were exposed to a bacterial suspension (5% v/v *S. Epidermidis* (ATCC) in brain-heart infusion media (BD Bioscience)) and incubated for 6 h at 37 °C. Samples stimulated to release NO (d-Fibrin + NO) were exposed to direct incandescent light for 30 min immediately after incubation. All samples were washed with sterile PBS and stained using a bacterial viability assay (BacLight, Invitrogen), imaged using fluorescent microscopy, and assessed for bacterial adhesion and viability (ImageJ) (*$p < 0.05$). Unmodified fibrin hydrogels served as bacterial adhesion controls.

2.8. Statistical Analyses. All experiments were performed in triplicate (n = 3) unless otherwise stated. For quantitative analysis, comparisons were made using ANOVA with a standard t test, with p-values <0.05 considered significant. Error bars represent standard error of the mean (±SEM).

3. RESULTS AND DISCUSSION

3.1. Fibrin-SNAP Derivatization. FTIR data verified covalent attachment of SNAP. This data shows there is an increase in amine signals and the presence of the signal for the cyclic four-member ketone present only in thiolactone indicates ring attachment to the fibrin gel (see Figure S-2 in the Supporting Information)

3.2. Hydrophobicity and Water Affinity of SNAP-fibrin. The hydrophobicity and equilibrium-swelling ratio of NO-releasing SNAP-fibrin hydrogels was determined. Hydrophobicity and swelling behavior of biomaterials can affect among other things cell and protein adhesive character as well as local pH buffering in tissue engineering constructs.[38,39] As previously stated, to generate an NO-releasing hydrogel, samples are soaked in toluene and SNAP-thiolactone prior to nitrosation with t-butyl nitrite. The average contact angle for the NAP-fibrin was significantly lower (54.5 ± 1.8) than that of fibrin controls (109.8 ± 5.3). Similar measures of SNAP-fibrin (53.9 ± 2.6) and fibrin soaked in t-butyl nitrite (135.8 ± 1.1) clearly indicate that the change is most likely due to the covalent linking of acetylpenicillamine to the fibrin hydrogel. It should be noted that the thiolactone formed from the acetylpenicillamine is a self-protected ring that will not allow nitrosation of the thiol group until the ring reacts with a primary amine group and opens to form an amide bond. Therefore, the only means by which the nitrosothiol can be formed to produce the NO-donor group in this material is if the thiolcatone forms the covalent bond and exposes the thiol

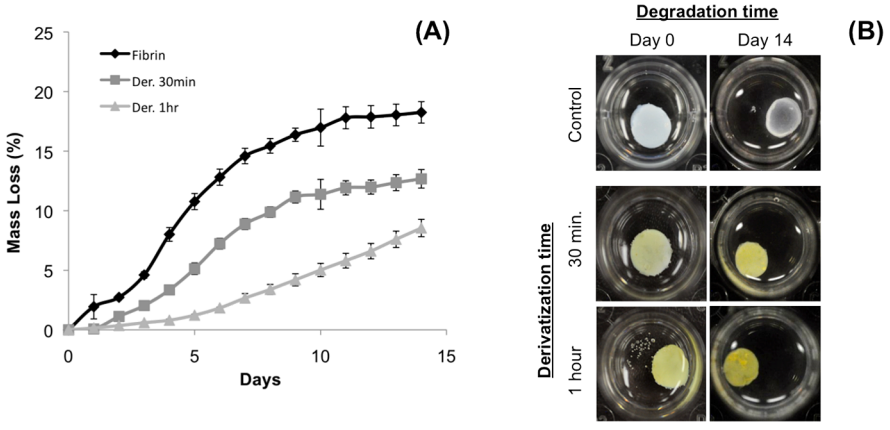

Figure 4. In vitro degradation of SNAP-fibrin hydrogels compared to control fibrin hydrogels. (A) Time-dependent mass loss, where mass loss (%) = $(W_i - W_f)/W_i \times 100$, with W_i = initial weight and W_f = final weight after degradation (n = 3). Significant differences ($p < 0.05$) were seen at all points after 3 days between fibrin, SNAP-fibrin (30 min reaction with t-butyl nitrite) and SNAP-fibrin (1 h reaction with t-butyl nitrite). (B) Images of initial fibrin and SNAP-fibrin hydrogels at day 0 and after 14 days.

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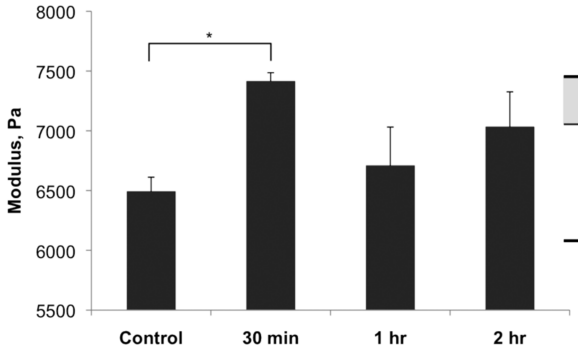

Derivatization Group	$E_{compressive}$ ± SEM, Pa
Control	6490.1 ± 121.5
30 min	7412.3 ± 74.2
1 hr	6706.4 ± 324.9
2 hr	7030.6 ± 295.4

Figure 5. Elastic moduli in compression determined from stress−strain curves for unmodified fibrin (control) and SNAP-fibrin hydrogels reacted for 30 min or 1 or 2 h with t-butyl nitrite, respectively. (*) indicate $p < 0.05$.

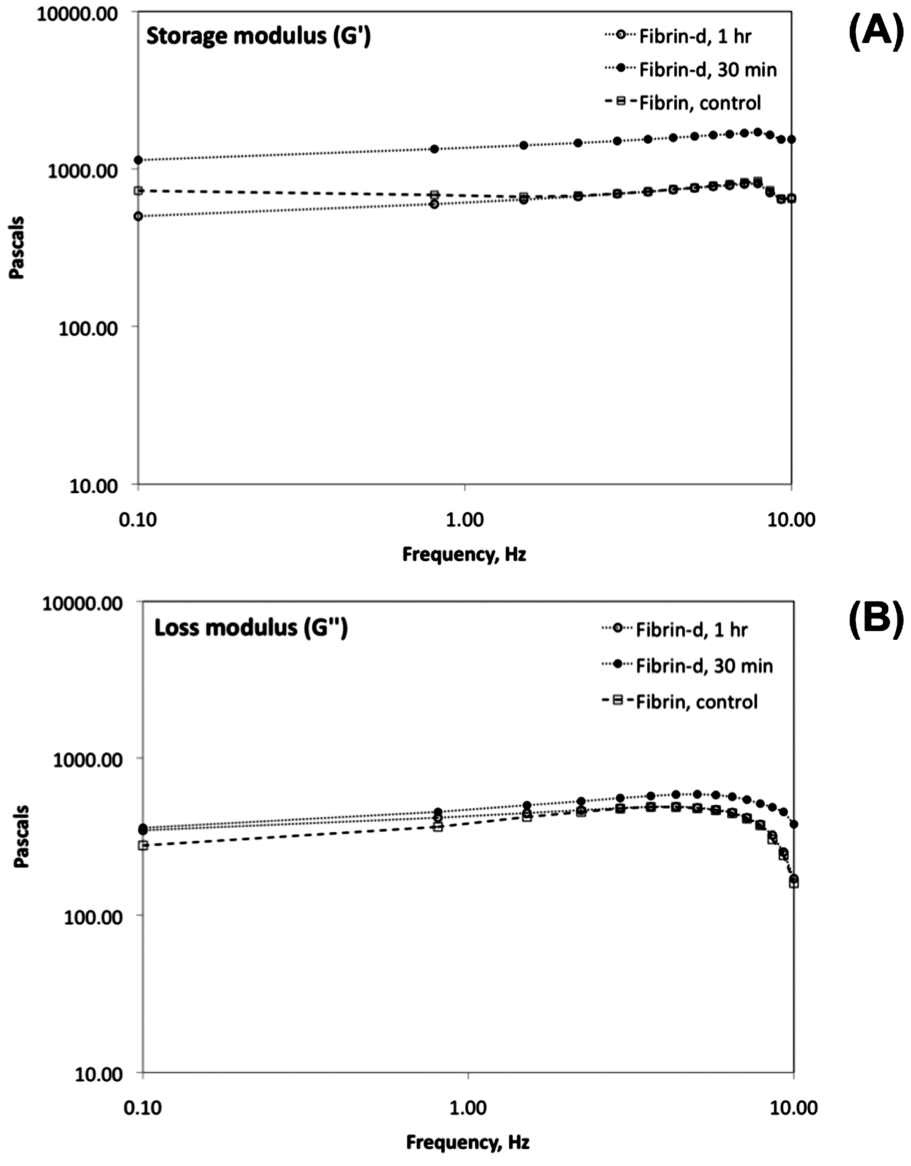

Figure 6. Frequency sweeps of SNAP-fibrin hydrogels. Frequency (0.1−10 Hz at 0.1 strain) sweeps were performed to determine the storage modulus (G') and loss modulus (G'') of control and SNAP-fibrin gels (Fibrin-d) derivatized for 30 min and 1 h.

group (i.e., the acetylpenicillamine is covalently linked to the fibrin and is not simply entrapped). The increased hydrophobicity of fibrin soaked in t-butyl nitrite could be attributed to the formation of a residual t-butanol byproduct after photolytic cleavage of t-butyl nitrite. Even a trace accumulation of t-butanol could cause the increase in the observed contact angle because it is extremely hydrophobic (Figure 2). Introduction of covalently linked SNAP to the fibrin hydrogel likely increases the polarity of the material, thus decreasing the contact angle, because the NO group is polar in nature. With covalent

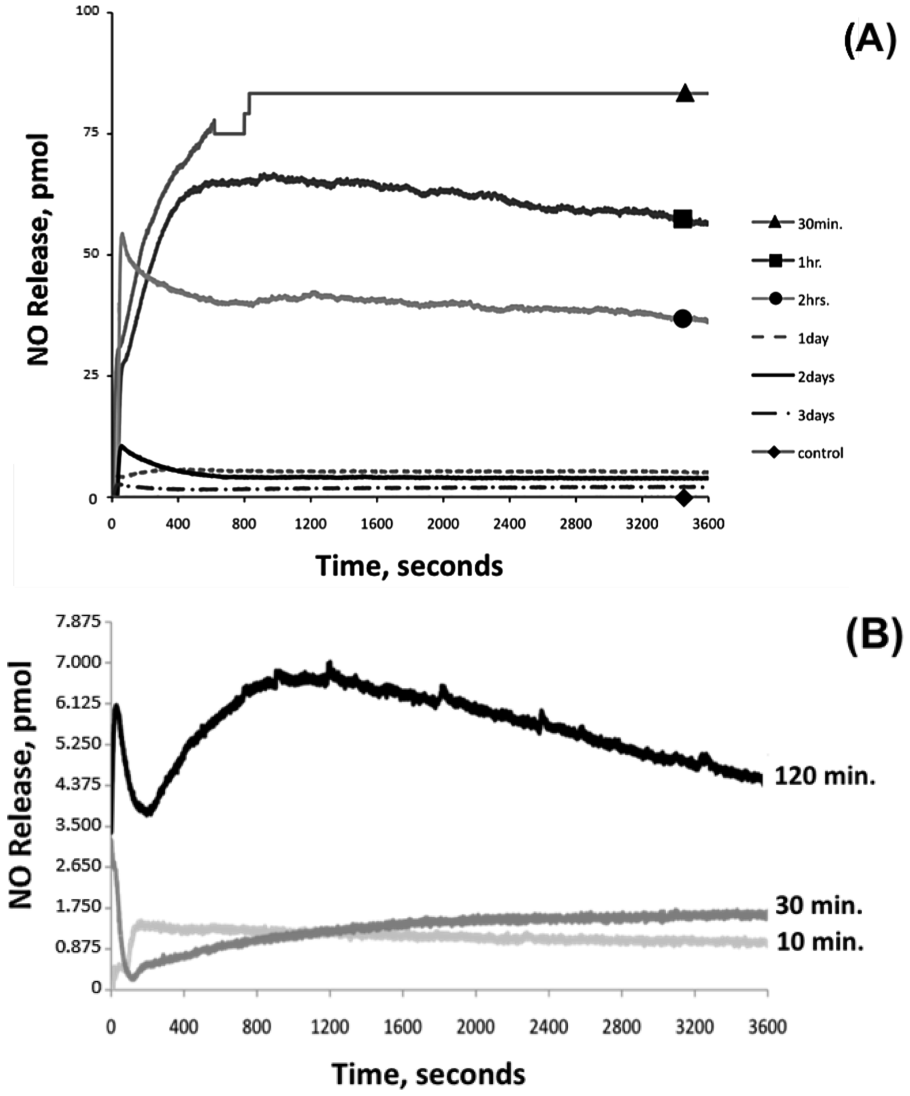

Figure 7. Derivatized hydrogel release behavior and relative release capacity. (A) Measurements of nitric oxide release from SNAP-fibrin gels. Hydrogels were reacted with t-butyl nitrite for different time periods and maximum NO release was measured in response to unfiltered white light (400−700 nm) for 1 h. (B) Measurements of NO release from SNAP-collagen gels reacted with t-butyl nitrite for 10, 30, or 120 min. Samples were exposed to 470 nm LED light (6 V, 100 Ω resistor) or 570 nm LED light (7.5 V, 110 Ω resistor) at a distance of 6 cm for 1 h. NO release was measured with chemiluminescent detection.

modification, a significant increase was also seen in the swelling behavior (swelling ratio, Q) between unmodified fibrin (2.6 ± 0.3) and the SNAP-fibrin (4.2 ± 0.6) respectively (Figure 3). This increased affinity for water may be due in part to the presence of more primary bound water associated with increasingly hydrophilic end-groups in SNAP as well as the disruption of the fibrin cross-linked network resulting from the derivatization process that likely decreases the cross-link density allowing for a greater presence of secondary bound water associated with the material.[40,41]

3.3. Degradation and Mechanical Behavior of Derivatized Hydrogels. The mass loss of fibrin hydrogel formulations was determined as a measure of the degradation (Figure 4). The base fibrin hydrogels exhibited a higher overall degradation at each time-point. The initial degradation rate (slope of degradation curve) and absolute degradation decreased with increasing nitrosation time (week 1), and although the overall degradation remained higher in controls, the rate of mass loss appeared to decrease in controls when compared to derivatized hydrogels by 10 days (Figure 4A).

Mechanical property changes were correlated with changes in physical properties due to the derivatization process by constant strain-rate compression, relaxation testing, and rheometric analysis. The elastic modulus increased significantly at 30 min nitrosation time, but the significance of this difference was lost with increasing nitrosation time (Figure 5). Furthermore, at 30 min hydrogels displayed a characteristic viscoelastic stress relaxation response qualitatively more similar to a standard linear solid model compared to controls and hydrogels of increasing nitrosation time, which displayed behaviors more typical of a Maxwell model system (see Figure S-3 in the Supporting Information). Rheometric data established hydrogel viscoelastic character and the relative change in stiffness with derivatization (Figure 6). Gels are predominantly elastic at low frequencies (G' is independent of frequency and larger than G'', characteristic of elastic solids).[42] Differences in stiffness may be attributed to changes in architecture and water associations in hydrogel structure.[43,44] Typical fibrin hydrogels exhibit nonlinear elastic behavior, where variations in fibrin fiber diameter,

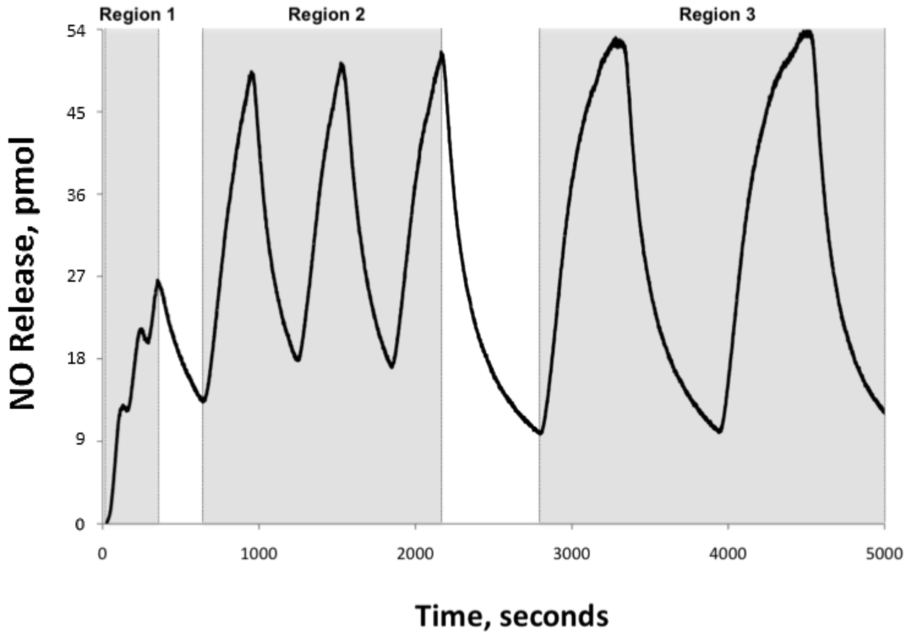

Figure 8. Controllable NO release from SNAP-fibrin hydrogels in response to cycled activating light source. NO release increased when the light was turned on, and decreased in a controlled manner when the light source was turned off. Cycles included 1, 5, and 10 min on/off intervals. NO release was measured with chemiluminescent detection. Region 1 represents on/off cycles of 1 min, region 2 on/off cycles of 5 min, and region 3 on/off cycles of 10 min.

fiber bundling, and density as well as network cross-linking can play a significant role in determining properties.[45] Water-swelling materials such as fibrin are biphasic in nature. These mechanical data coupled with increased swelling and hydrophilic character suggest that with increasing nitrosation time NO-modified hydrogels initially have a stronger swelling character and may contain a greater fraction of internally bound water, contributing a greater degree of drag-force upon compression, making them more difficult to compress and causing them to exhibit a less robust relaxation behavior with time (i.e., polymer molecules gradually accommodating the strain by conformational extension rather than bond distortion). However, increasing nitrosation time also can result in solvent-mediated loss of fiber bundling and network cross-linking. This effect on the fundamental network structure can result in increased porosity and ultimately a change in stress relaxation behavior as seen at a nitrosation time of two hours, where there is a resultant loss in peak applied stress required to achieve an equivalent 20% final strain.[44,46]

3.4. NO-Release Behavior. The relationship between nitrosation time and NO-release behavior was determined for SNAP-fibrin hydrogels. Specifically, the amount and limits to NO availability with nitrosation time, the feasibility of activated controlled release, and the limited contribution of passive NO-release behavior due to copper initiated RSNO decomposition were determined.

Maximum NO-release capacity from SNAP-fibrin is reduced as the nitrosation time is increased (Figure 7A). SNAP-fibrin hydrogels nitrosated for 30 min had the greatest peak release after exposure to direct light for one hour (87.6 pmol). The attenuated release behavior observed with nitrosation time may result from changes in fibrin structural stability (i.e., changes in thickness and cross-linking of matrix thin and thick polymerized fibrin fibrils) that could occur from overexposure to t-butyl nitrite.[33,36,43] The S-nitrosothiol groups may degrade and form disulfide linkages with neighboring groups, consequently diminishing their availability for NO-release.[34] Furthermore, it appears that regardless of nitrosation time, the SNAP-fibrin hydrogels all reach a stable NO-releasing plateau, which suggests that these materials may possess a large enough NO reservoir for long-term controlled release. SNAP-collagen gels also exhibited NO-release, but at dramatically lower levels (maximum 7.01 pmol, that diminished with time) (Figure 7B). This may be due in part to the heterogeneity of the collagen used in this study and the lower level of lysine present in collagen compared to fibrin.

Because the biological effects of NO are highly dependent on its spatial and temporal availability,[9,18,47,48] it was important to establish a robust controlled NO-release behavior from the SNAP-fibrin hydrogels in addition to continuous release. Figure 8 illustrates the photoinitiated controlled NO-release behavior from fibrin hydrogels derivatized for 30 min. NO-release from these hydrogels was responsive to activation from a direct light source. On−off cycles of 1, 5, and 10 min periods were used to determine the resolution in photoinitiated NO-release from SNAP-fibrin hydrogels (Figure 8). Foil was removed from the quartz sample holders during light activation and replaced during periods when the light source was off to minimize background noise from ambient light-induced NO-release. When light was turned on and off at 1 min intervals, NO release from the material was unable to return to baseline levels within the 1 min period of no light exposure, resulting in a continuous increase in the level of NO generated with each subsequent illumination of the light (Region 1, Figure 8). During 5-min cycles of activation, the NO generation from the SNAP-fibrin reached the same maximum level (~48.2 pmol) and was able to return to the same background level of release (~17.5 pmol) when the light source was turned off (Region 2, Figure 8). At 10-min activation cycles, a similar behavior was observed (Region 3, Figure 8), reaching a maximum level of NO-release of ~52.6 pmol and a background level of release near ~13.1 pmol. Preliminary temperature and mechanically induced release data is included in the Supporting Information (see Figure S-4).

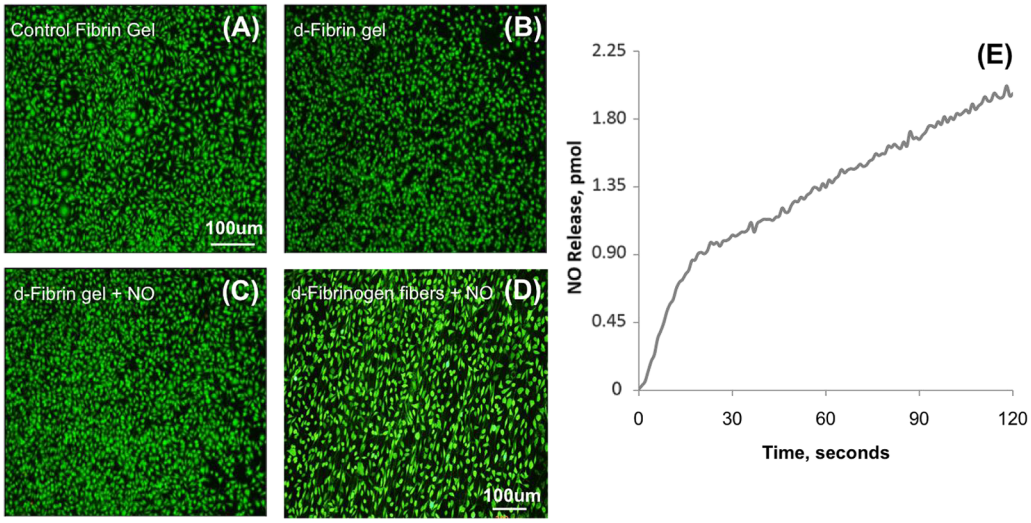

Figure 9. Cell viability on SNAP-activated fibrin and fibrinogen-based materials. Osteoblasts (MC3T3) seeded on (A) unmodified fibrin hydrogel, and (B) SNAP-fibrin hydrogel without exposure to light. (C) SNAP-fibrin hydrogel and (D) SNAP-fibrinogen electrospun fibers seeded with osteoblasts and fibroblasts, respectively; and exposed to light for 2 min after 24 and 72 h in culture. Cells were stained with calcein-AM/ethidium bromide and imaged at 5 days (n = 3). (E) NO release from SNAP-fibrin hydrogels (d = 15 mm, t = 1 mm) over 2 min. NO release measures were used to estimate the level of cell exposure to NO in culture. NO release was measured via chemiluminescent detection (n = 3).

Trace amounts of copper ions that could be present in t-butyl nitrite are capable of initiating the release of NO by degrading available S-nitrosothiols[49] and ascorbic acid supplements to tenogenic and osteogenic cultures can also induce NO release from SNAP-fibrin hydrogels and thus needed to be evaluated.[50−52] In both cases, copper ion- and ascorbate-initiated release profiles are nominal when compared with the controllable, photoinitiated NO release from fibrin-based hydrogels (see Figure S-5 in the Supporting Information). Use of other matrices, however, such as collagen and gelatin, although still potentially useful may require tighter control of the microenvironment in order to maintain controlled release of NO.

3.5. Cell Adhesion, Viability, And Antimicrobial Character. Osteoblasts and fibroblasts cultured directly on unmodified and SNAP-fibrin hydrogel films and cross-linked SNAP-fibrinogen nanofibers were adherent and viable and showed no significant differences compared to controls (>90% survival for all cultures) (Figure 9. A-D). Data indicates that cells remained viable and proliferative on derivatized hydrogels and nanofibers after 5 days in culture. NO release in culture due to ambient light exposure was shown to be insignificant, with measured release not exceeding 2.19 pmol under standard culture conditions (Figure 9E). This indicates that the materials loaded with NO are not causing changes to cell growth and proliferation potential and should serve as a platform upon which studies can be carried out to understand the response of osteoblasts and fibroblasts to controlled release of NO.

Bacterial data indicate a significant reduction in bacterial adhesion and viability on SNAP-fibrin, where the loss of viability is indicated by a significant reduction in the ratio of live to dead (live/dead) cells (Figure 10). It is important to note that the extent to which the loss of adhesion is the result of only ambient NO release is not clear because the modification process also affects changes in material surface properties including hydrophobicity and charge, which could also affect bacterial adhesion as well as survival.[53,54] However, this data indicates that at a minimum the derivatization process promotes

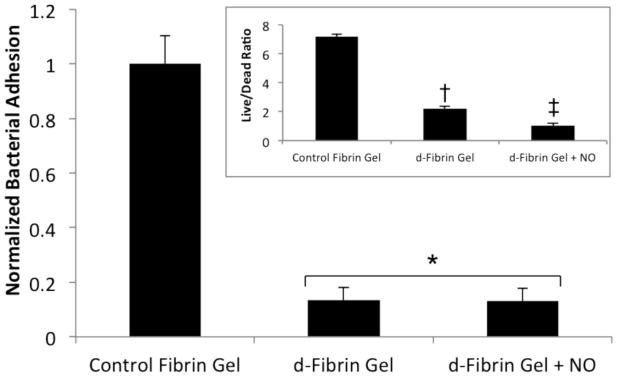

Figure 10. Bacterial (*S. Epidermidis*) adhesion and survival on SNAP-fibrin hydrogels. Fibrin control and NO-releasing SNAP-fibrin materials were exposed to a bacterial suspension (5% v/v *S. Epidermidis* in brain-heart infusion media). Samples were stained using a bacterial viability assay (BacLight-Invitrogen) and imaged. Adhesion and survival (live/dead ratio, inset) of bacteria on fibrin hydrogels assessed using image analysis software (ImageJ), (*) indicates significant differences (p < 0.001) in bacterial adhesion and †,‡ indicate significantly lower (p < 0.05) bacterial viability than control gels and all other groups, respectively.

a less permissive environment for bacterial adhesion and actively reduces survival.

4. CONCLUSIONS

In this study, we developed a novel actively inducible delivery approach that allows fibrin-based biomaterials to be directly modified into inducible NO controlled-release vehicles. This release system requires active cleavage of the derivatized material- either via photoactivation or possibly through mechanical stimulus, pH, or temperature. The spontaneous degradation of the base hydrogel material will not elicit a significant active molecule release. This may allow for tighter spatial and temporal control of NO release that is relatively independent of the degradation kinetics of the underlying natural material, potentially uncoupling a direct relationship

between degradation and NO delivery. The possibility of mechanical activation could also result in a material that has its release kinetics modulated with increasing load transfer to the regenerating native tissue.[55,56] In addition, the materials also have possible use as a general regenerative tissue support that not only aids in stabilizing the metabolic activity of the regenerative site but also provides secondary support through its innate antimicrobial character.[57−61] This approach could result in novel tissue engineering constructs containing both specific and more ubiquitous support elements to promote tissue regeneration.[62] The goal of future investigation is to fully characterize the application and inducible control of these materials in a wound healing or regenerative model system.

■ ASSOCIATED CONTENT

Supporting Information

Data regarding the degree of substitution of N-acetyl-D-penicillamine as determined by Ellman's test, FTIR data showing covalent attachment of SNAP, stress−relaxation behavior of SNAP-fibrin hydrogels, temperature, and compressive mechanical loading induced NO release, and copper ion and ascorbate-initiated NO release data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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