Enzymatic Cross-Linking of a Nanofibrous Peptide Hydrogel

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The rheological properties of the environment in which a cell lives play a key role in how the cells will respond to that environment and may modify cell proliferation, morphology and differentiation. Effective means of modifying these properties are needed, particularly for peptide hydrogels which are generally relatively weak and soft. In this report we describe the enzymatic cross-linking of a nanofibrous multidomain peptide hydrogel. When this method was used, the storage modulus, G', could be increased to over 4000 Pa without changes in hydrogel concentration and without dramatic changes in nanostructural architecture. Enzymatic cross-linking represents a mild and simple method for increasing the mechanical strength of peptide hydrogels in applications for which the robustness of the gel is essential. This method should be suitable for a broad array of peptide hydrogels containing lysine such as those currently under study by many different groups.

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Introduction

Self-assembling peptide hydrogels are becoming a popular material choice for a variety of applications including drug delivery, cell delivery, cell culture and as scaffolds for tissue regeneration and engineering.[1−10] Because of their peptidic nature, their synthesis is straightforward and can be modified to tune chemical, biological, and rheological properties. The rheological properties of any matrix, natural or synthetic, play a key role in determining the ease of handling of the material and, in some cases, the response of the cells in contact with the gel.[11−13] We have recently described a series of multidomain peptides (MDPs) that can be cross-linked through either ionic interactions or disulfide bond formation via cysteine residues.[14−16] While these methods and materials allowed the tuning of the storage modulus (G') from 100 to 5000 Pa, the systems with highest G' could only be achieved through the incorporation of cysteine, which is notorious for its difficulty in handling. Thus, an alternate method of oxidation was desired. The possibility of enzymatic cross-linking of residues within a peptide hydrogel solves many difficulties in handling while simultaneously providing an option with a potential for high biocompatibility. In nature, components of extracellular matrix, collagen and elastin, are cross-linked by lysyl oxidase (LO), which oxidizes the primary amine of lysine to an aldehyde. This aldehyde can spontaneously react with another amine to form a Schiff base or undergo an aldol condensation with another aldehyde (Figure 1).[17,18] Here we show that MDP1 (see Table 1), which contains four lysine residues, is able to be cross-linked by either lysyl oxidase (naturally found in serum-supplemented medium) or plasma amine oxidase (PAO, a similar and commercially available enzyme which also functions by oxidation of primary amines). In both cases, cross-linking results in a dramatically increased storage modulus over time.

Experimental Section

Synthesis of the Peptides. Peptides were prepared on a 0.15 mmol scale using modifications of previously published protocols.[15,19] A Rink Amide MBHA low loading resin was used to generate a terminal amide. All the amino acids and other reagents were dissolved in a mixture of 50% MDF (dimethylformamide) and 50% DMSO (dimethylsulfoxide). Amino acid coupling cycles were 60 min in length with the following proportions of reagents: 4 equiv of amino acid, 4 equiv of HATU (*O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*,*N*′-tetramethyluronium hexafluorophosphate), and 6 equiv of diisopropylethylamine compared to 1 equiv of functional group on the solid support. Fmoc (flourenylmethoxycarbonyl) was removed with two 7 min treatments of 20% piperidine, 2% DBU (1,8-diazabicyclo[5.4.0]-undec-7-ene), 39% DMF, and 39% DMSO by volume. After the peptide sequence was completed, the N-terminus was acetylated in the presence of excess acetic anhydride and DIEA in DMF for 2 h. A ninhydrin test was used to monitor the acetylation step. After the reaction was finished, the resin was washed several times with DCM before cleavage from the resin. Cleavage of the peptides was accomplished by shaking the resin with 20 mL of trifluoroacetic acid (TFA)/triisopropylsilane/anisole/ethanedithiol/H_20 (18:0.5:0.5:0.5:0.5 by volume) for 3 h at room temperature. The solution was collected by filtration followed by rinsing the resin twice with 20 mL of neat TFA. All washings were combined and rotoevaporated to a thick solution. The solution was triturated by addition of cold diethyl ether. The precipitate was collected by centrifugation and was washed several times with cold ether followed by drying overnight under vacuum.

Purification of Peptides. MDP1 was subsequently dissolved in deionized water at 5 mg/mL concentration and purified by dialysis using a membrane with a cutoff 100−500 Da (the semipermeable membrane was purchased from Spectra/Por). The water was changed twice a day for 5 days. The solution containing the peptide was frozen and then lyophilized to collect the pure peptide. MDP2 was subsequently dissolved in deionized water at 5 mg/mL concentration with addition of NaOH to achieve a pH of 8, which is required to fully dissolve the peptide. It was purified by dialysis using a membrane with a cutoff of 100−500 Da. The water was changed twice a day for 5 days. The solution containing the peptide was frozen and then lyophilized to collect the pure peptide. MALDI-TOF MS (matrix assisted laser desorption ionization time-of-flight mass spectrometry) analysis was used to characterize the mass of both peptides. MDP3 was purified by HPLC (high performance liquid chromatography) as previously reported.[14]

Preparation of Gels. Gels of MDP1 were prepared by dissolving lyophilized powder in deionized water followed by adjustment of the

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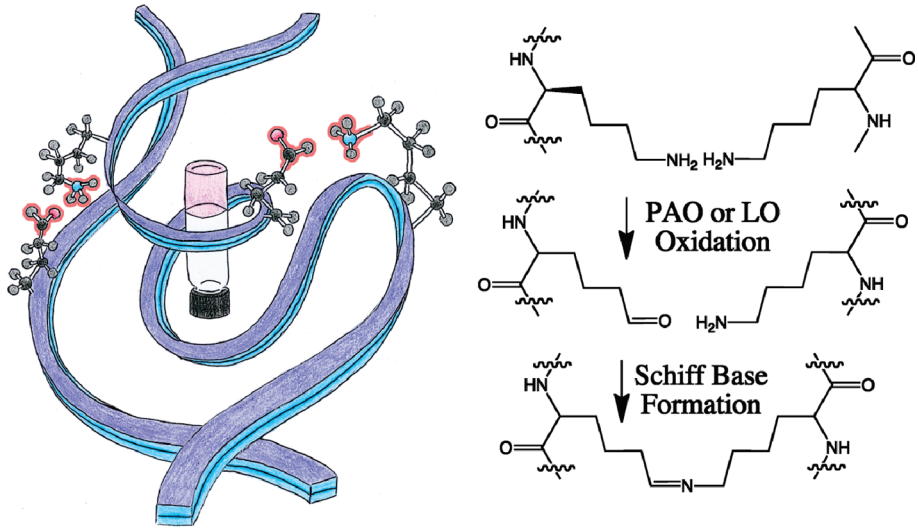

Figure 1. Lysyl oxidase and plasma amine oxidase function by oxidizing primary amines to aldehydes. The formed reactive aldehydes react further to cross-link the extracellular matrix.

Table 1. Peptide Sequences[a]

name	sequence
MDP1	Lys_2-$(Ser\text{-}Leu)_6$-Lys_2
MDP2	Glu_2-$(Ser\text{-}Leu)_6$-Glu_2
MDP3	Lys_2-$(Gln\text{-}Leu)_6$-Lys_2

[a] Peptides are N-terminally acetylated and C-terminally amidated.

pH to approximately 7 by addition of NaOH. Deionized water was added to make a viscous solution with a concentration of 2% by weight. To this solution 2× PBS (phosphate buffered saline) was added in equal volume to make a final solution of peptide at 1% by weight, pH 7.4. The solution was vortexed thoroughly to ensure full mixing. The resulting gel was centrifuged to eliminate bubbles. Gels of MDP2 were prepared by dissolving lyophilized powder in deionized water followed by adjustment of the pH to approximately 7 by addition of NaOH. Deionized water was added to make a viscous solution with final concentration of 2% by weight. To this solution, 4 mol equiv of aqueous $MgCl_2$ was added in equal volume to make a final solution of peptide at 1% by weight. The solution was vortexed thoroughly to ensure full mixing. The resulting gel was centrifuged to eliminate bubbles.

Time Course Oxidation. A total of 100 μL of MDP gel, prepared as described above, was pipetted into individual wells of a 96-well plate. To the top of these gels was added 200 μL of test solution. The test solution compositions included (1) PBS (phosphate buffered saline: 10 mM phosphate, 150 mM NaCl, pH 7.4), (2) medium (α-MEM, 50 μg/mL L-ascorbic acid 2-phosphate, 100 U/mL penicillin, and 100 μg/mL streptomycin), (3) 10% FBS in medium (α-MEM supplemented with 10% fetal bovine serum, 50 μg/mL L-ascorbic acid 2-phosphate, 100 U/mL penicillin, and 100 μg/mL streptomycin), (4) 10% FBS in medium (as above) supplemented with 0.05, 5, or 50 mM βAPN as inhibitor, (5) 10% FBS in medium (as above) with SHED (mesenchymal stem cells from human exfoliated deciduous teeth) cells, and (6) 100% FBS. In addition, there were two conditions that contained no FBS or cell culture medium of any kind: (7) 3 nM plasma amine oxidase in PBS and (8) 3 nM plasma amine oxidase in PBS supplemented with 50 mM βAPN as inhibitor.

The 96-well plate was incubated at 37 °C for the requisite amount of time. One-half of the supernatant was refreshed every second day unless otherwise noted. Gels were carefully transferred to the rheometer at the desired time point with a spatula, taking care not to damage the gel during handling. Gels were not pipetted because the shear-thinning induced by this method would produce a measurable decrease in the elastic modulus of the gel.

Rheology. A TA Instruments AR-G2 rheometer was used to perform the measurements on all the peptides. All experiments were performed at 25 °C. A parallel plate geometry with 8 mm diameter and 250 μm gap was used for all experiments. The gels were placed carefully in the headspace with a spatula, and the geometry was then lowered to the preset gap. Oscillatory strain sweeps were performed at fixed angular velocity of 0.5 rad/s. Oscillatory stress sweeps were performed using a fixed angular velocity of 0.5 rad/s. Oscillatory frequency sweeps were performed using a fixed strain of 0.5%.

Atomic Force Microscopy (AFM). A total of 8 μL of $K_2(SL)_6K_2$ in PBS buffer at pH = 7.4 (with or without added PAO) were dropped onto freshly cleaved mica while spinning at medium speed on a Headway Research, Inc. photoresist spinner. The sample was rinsed with deionized water for 4−5 s and then spun for an additional 10 min. The sample was then placed in a desiccator until imaging. AFM images were collected in air, at ambient temperature, on a Digital Instruments Nanoscope IIIa in tapping mode. Data were collected in height and amplitude channels. All AFM images were flattened using Nanoscope software and height profiles were generated from the flattened images. From these profiles, for each time point measured (0, 1, 4, and 7 days treatment with PAO and without PAO), 20 fiber heights were averaged and a standard deviation was generated.

Results and Discussion

That MDP1 could be oxidatively cross-linked was a serendipitous discovery. As the biocompatibility of various MDP hydrogels was investigated, it became apparent that gels composed of MDP1 that were incubated with dental stem cells,[16] experienced a significant increase in storage modulus (G') over time. This study revealed that the G' increased over 14 days of cell growth to ~6000 Pa (Figure 2a; Figure 4a, blue bars). Histological sections of the cell/hydrogel composite indicated substantial production of natural extracellular matrix (data not shown). Based on these findings, we hypothesized that the newly synthesized extracellular matrix (ECM) was responsible for the increase in mechanical properties, a phenomenon that had also been reported in the literature for similar systems.[20,21] In contrast, parallel experiments run with MDP2, which differs from MDP1 in that it uses the negatively charged amino acid glutamate in the peripheral region to control self-assembly rather than the

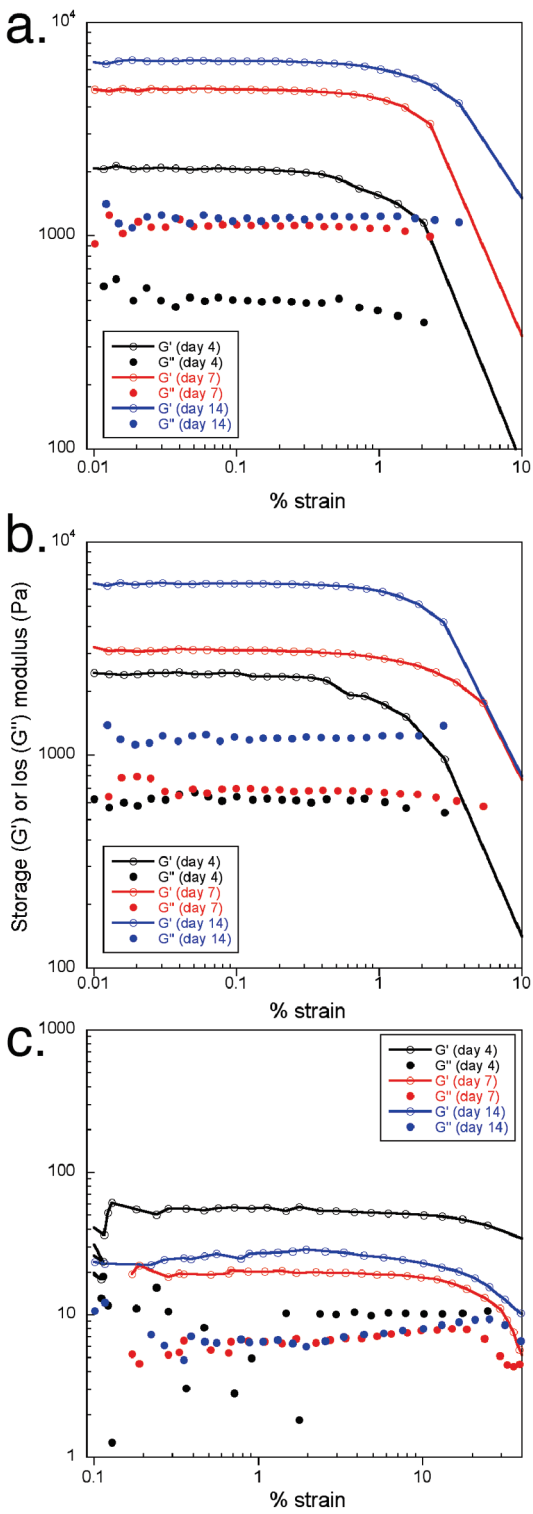

Figure 2. Strain sweep of MDP1 (a) over 14 days of incubations with SHED cells, (b) over 14 days in the presence of medium and 10% FBS, and (c) strain sweep of MDP2 over 14 days of incubations with SHED cells.

positively charged amino acid lysine, were found to *decrease* in G' over time (Figure 2c). In an attempt to understand the difference between these peptides further, a control experiment with MDP1 was run under identical conditions but without cells. Surprisingly, gels prepared from MDP1 showed a similar increase in G' over time despite the absence of cells (Figure 2b; Figure 4a, red bars). Clearly, ECM production was not the cause of the G' increase. To identify the cause of this effect, MDP1 was incubated under several different conditions, including PBS, cell medium, cell medium enriched with FBS, and FBS alone (Figures 3a and 4b). In the absence of FBS, MDP1 did not display an increase in G'. However, in the presence of FBS, G' increased dramatically. These experiments demonstrated that a component of the serum and not production of ECM (or any other cellular component) was responsible for the increase in G'. Furthermore, the increase in G' did not take place with MDP2. Because the increase in G' was seen when a multidomain peptide was combined with FBS together and FBS alone does not form a hydrogel, it can be determined that a component of FBS acts on the MDP leading to the observed increase in G'. Because the difference between the effected MDP1 and uneffected MDP2 is the presence or absence of lysine respectively, it can also be concluded that lysine is a required component of the MDP.

In nature, LO is used to cross-link components of the extracellular matrix. LO oxidizes the amine side chain of lysine to an aldehyde, which can then either react with an amine to form a Schiff base or it can undergo an aldol condensation with another aldehyde, both of which result in the formation of a covalent cross-link between two previously separate residues (Figure 1).[22] These new covalent bonds may also undergo further reaction making more complex cross-links. LO is also found in serum-supplemented cell culture media.[23] One way to determine if this oxidative cross-linking was taking place in our system is to inhibit LO, thereby preventing oxidative cross-linking, while simultaneously monitoring G'. For these experiments, an irreversible inhibitor of LO was chosen: β-aminopropionitrile (βAPN).[24] MDP1 was made into a gel with medium and FBS to which no inhibitor was added (control). Three additional gels were made utilizing medium and 10% FBS to which different concentrations of βAPN were added as inhibitors and G' was monitored for 10 days. As shown in Figure 4b, 50 mM βAPN results in a G' comparable to that of medium with no FBS. Lower concentrations of βAPN result in inhibition to a lesser degree. This strongly suggests that lysyl oxidase is the key component responsible for the change in rheological properties previously observed.

If oxidative cross-linking is indeed taking place, this process should be reproducible by the exogenous addition of enzyme in the absence of serum or cells. Thus, experiments were repeated without any fetal bovine serum or cell culture medium of any kind. As LO is not commercially available, PAO, an enzyme with a similar mechanism of action, was used.[25,26] Both LO and PAO fall into the same class of enzymes, the copper-containing amine oxidases (CuAOs). This class of enzyme is known to be involved in the development and maturation of the extracellular matrix and more specifically, collagen and elastin.[23] Hydrogels of MDP1 were treated with PAO in phosphate buffered saline (but no serum or media) and the G' was monitored over 15 days. As was observed with serum-treated gels, the G' increased dramatically over time. As with serum-treated gels, addition of βAPN, which also inhibits PAO, resulted in the same lack of increase in the storage modulus, indicating that cross-linking did not take place (Figure 5). To test the general applicability of this cross-linking methodology, MDP3 was treated with PAO in the same way and was also observed to have a dramatic increase in G' over time (see Supporting Information, Figure S5). Rheology has historically been used to estimate the degree of cross-linking in polymer

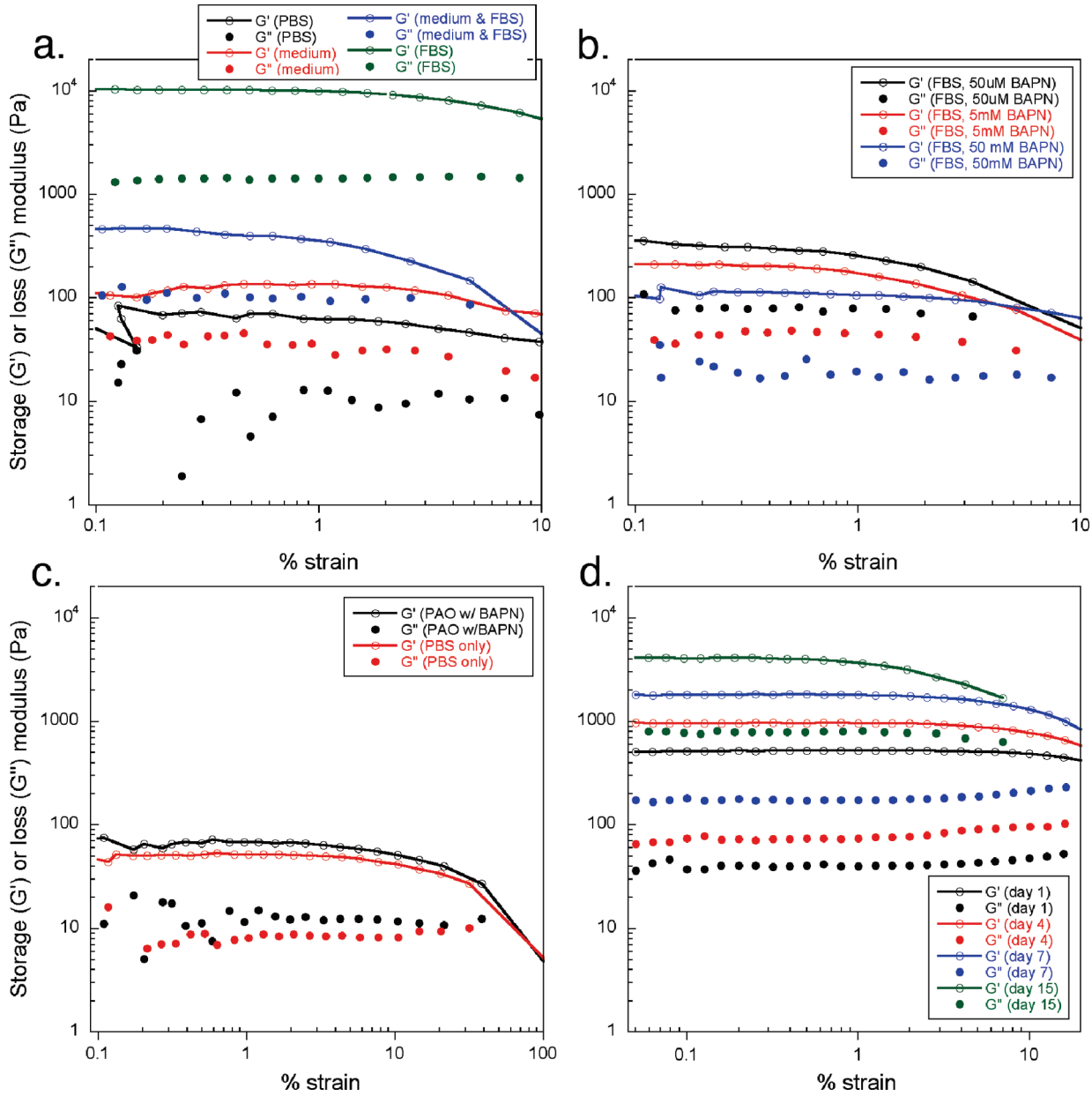

Figure 3. Strain sweeps of MDP1 under various medium compositions: (a) no inhibitor after 10 days of treatment, except FBS, which is after 14 days treatment; (b) after 10 days of treatment with FBS and indicated concentrations of βAPN; (c) after 10 days of treatment with PBS only and after 10 days of treatment with PAO and βAPN; and (d) after 1, 4, 7, and 15 days treatment with PAO.

systems where G is directly proportional to the degree of cross-linking. The clear increase in G' in the presence of active enzyme can be attributed to progressively higher degrees of cross-linking while the lack of increase in the presence of an enzyme inhibitor or in systems lacking the amino acid lysine indicates that no cross-linking is taking place.[27,28]

AFM of MDP1 shows small, discrete self-assembled fibers prior to addition of phosphate buffer (Figure 6a). In the presence of phosphate these small fibers undergo further self-assembly in which they are physically cross-linked by the presence of phosphate (Figure 6b). Treatment with PAO results in the covalent capture of the gel network (Figure 6c). The introduction of covalent bonds between individual peptides results in additional aggregation of peptide fibers that appears as fiber thickening over time which is absent in MDP fibers aged without enzyme (Figure 6d, Table 2). An increase in fiber height is observed over time, from approximately 1.6 nm at time zero to

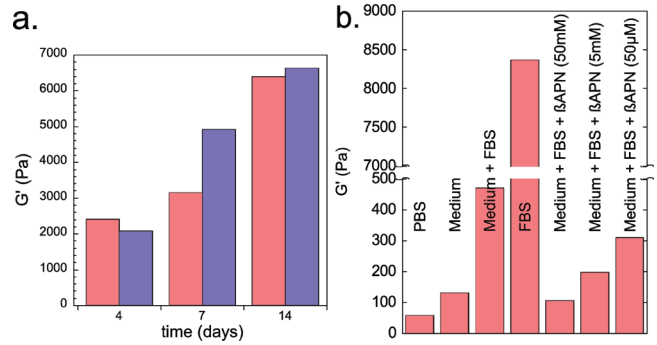

Figure 4. (a) Storage modulus of MDP1 over 14 days in the presence of cells with medium enriched with 10% FBS (blue) or medium enriched with 10% FBS but with no cells (red). In both cases, one-half the supernatant was refreshed every 2 days. (b) MDP1 incubated under indicated conditions for 10 days without cells and without refreshing the supernatant.

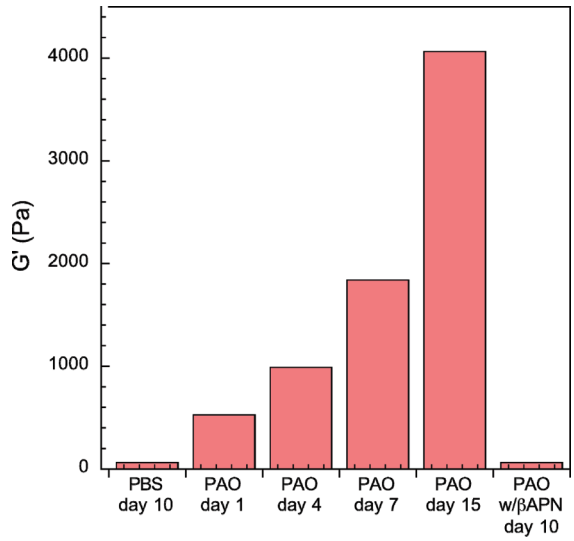

Figure 5. Storage modulus (G') without PAO, with PAO, and with PAO plus βAPN as an inhibitor. The values of G' were chosen at an angular velocity of 0.5 rad/s in the linear viscoelastic region.

Table 2. Comparison of AFM Height Profiles[a]

day	height w/PAO	height w/o PAO
0	1.6 (0.3)	
1	1.9 (0.7)	1.6 (0.2)
4	2.0 (0.6)	1.6 (0.2)
7	2.7 (0.7)	1.5 (0.2)

[a] Height with PAO was significantly higher at time points 1, ($p < 0.05$), 4 ($p < 0.01$), and 7 ($p < 0.01$) compared to control. Heights given in nm with standard deviation in parentheses.

2.7 nm after seven days of treatment with PAO. This suggests fiber bundling occurs during oxidative cross-linking. The general fibrous nature of the gel network, however, is preserved after treatment with the enzyme suggesting that the nanostructure is relatively unchanged.

Conclusion

The results of this study have two important consequences. First, nanofibers formed from multidomain peptides that contain lysine will be oxidatively cross-linked in standard mammalian cell culture conditions (supplemented with FBS); this can have a desirable impact on the handling of the hydrogel, as it becomes more robust with time rather than less so. Additionally, studies that attribute improvements in mechanical properties of hydrogels to the production of ECM should first be careful to rule out covalent cross-linking of the matrix used. This covalent cross-linking in standard cell culture conditions is possible without added enzymes, as lysyl oxidase is found in serum-supplemented media. Second, this study is a proof-of-principle that lysine oxidation using exogenous addition of plasma amine oxidase can be harnessed to cross-link self-assembled nanofibers. Enzymatic cross-linking via aldehyde formation and subsequent condensation represents a mild and simple method for increasing the mechanical strength of peptide hydrogels in applications for which the robustness of the gel is essential. This method should be suitable for a broad array of peptide hydrogels containing a high density of lysine such as those currently under study by many different groups.[29−32]

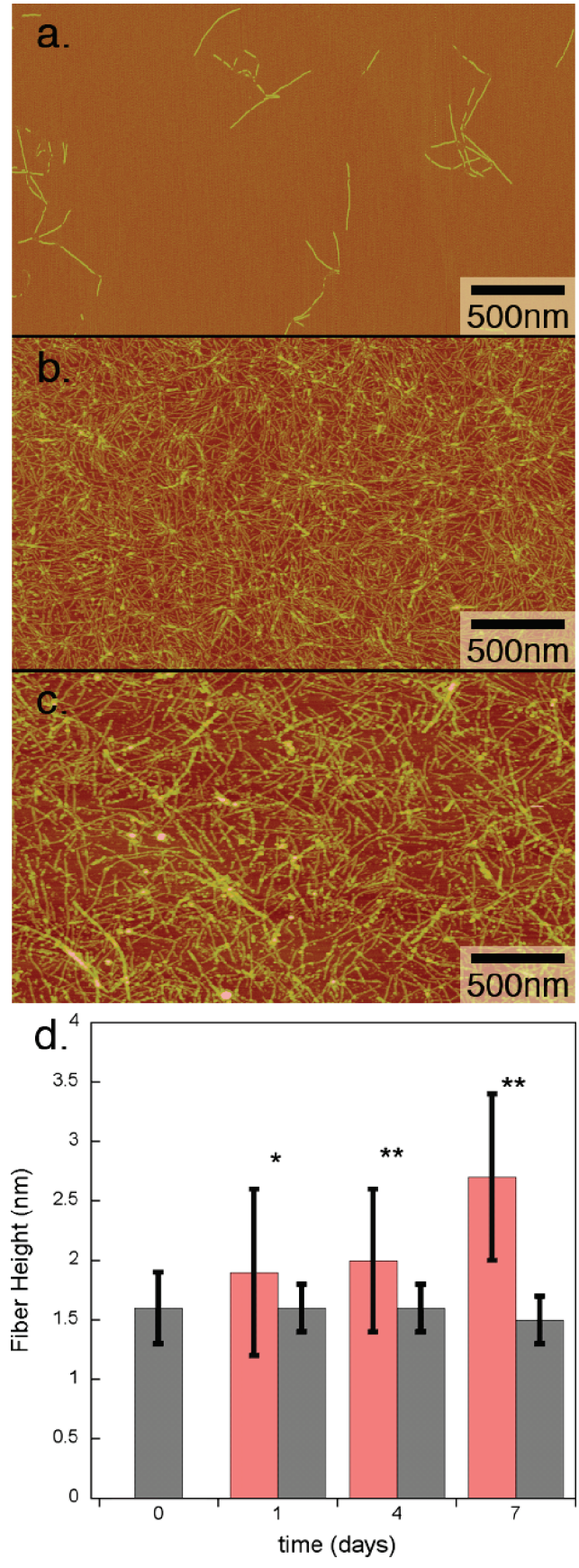

Figure 6. AFM of MDP1 nanofibers (a) before gelation, (b) after gelation, but before treatment with PAO, and (c) after four days of treatment with PAO. (d) Average height of fibers treated with PAO (red) compared to untreated control (gray) as measured by AFM: *$p < 0.05$, **$p < 0.01$. Day 0 are samples before treatment with enzyme.

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Supporting Information Available. Peptide mass spectrometry, HPLC, rheology, and photos of the hydrogel. This material is available free of charge via the Internet at http://pubs.acs.org.

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