Solid-Phase Synthesis and Kinetic Characterization of Fluorogenic Enzyme-Degradable Hydrogel Cross-linkers

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Of critical importance in drug delivery and tissue engineering applications is the degradabilty of implanted polymeric materials. The use of peptide-derived cross-linkers in hydrogel design is a valuable approach by which polymeric carriers can be endowed with enzymatic degradability in a predictable, "programmable" fashion. The solid-phase synthesis strategy described herein allows for an expeditious, flexible synthesis of bis-acrylamide-derivatized peptides with complex modifications, as exemplified by the incorporation of fluorophore and quencher moieties into a matrix metalloprotease (MMP)-degradable cross-linker. The crude synthetic product was obtained in high yield and purity and purified by standard methods; it was then used directly for polymerization without the need for tedious and often nonchemoselective solution-phase modifications. Functional appendages incorporated for detection provided a direct, quantitative link between enzymatic activity and hydrogel degradation using routine methods for identification of optimal enzyme-specific degradability.

Introduction

The impact of polymeric technologies on healthcare has been wide-ranging. Far beyond the inert materials that formed the mainstay of early medical applications, the current generation of "bio-mimetic" polymers has heralded sea change in this area.[1−3] For instance, degradable microspheres comprised of poly(lactic acid-*co*-glycolic acid) (PLGA) have attracted much attention for use as drug delivery vehicles with slow-release properties that can be tailored according to their precise chemical composition and particle morphology.[4−6] Along a similar vein, poly(ethylene glycol) (PEG)-modified proteins are substantially increasing the viability of protein-based therapeutics through enhanced pharmacokinetic performance and attenuated immunogenicity.[7−9] With an abundance of examples to be found in the literature, it is clear that novel polymeric technologies can hardly be viewed as an ancillary endeavor in pharmaceutical research, particularly in the burgeoning area of biopharmaceuticals, wherein the marriage of drug delivery with therapeutic agents of high potency and tunable stability is clearly a powerful combination.

We have recently begun a research/engineering program to design and synthesize tissue engineering scaffolds for use in neural tissue regeneration. Specifically, our ultimate goal is to synthesize a cross-linked hydrogel that can serve as a three-dimensional matrix to support neuronal invasion at the site of axonal transection following acute and/or chronic spinal cord injury (SCI). Although this application of biomedical polymer research has been of interest for some time,[10−17] only very recently has the synthesis of enzyme-degradable peptide cross-linkers been mined for its potential to impart desirable degradation properties to polymeric carriers. Such an approach is attractive because it allows for a hydrogel to be designed with a specific rate of degradation "programmed" into its covalent structure. Furthermore, the specific enzyme substrate chosen for development into a cross-linker can be selected in such a way as to capitalize on potential tissue-specific and/or temporally regulated secretion of the target enzyme responsible for hydrogel degradation, thus allowing for tailoring to a specific clinical application.

The past few years have seen considerable developments in the design of "smart" cross-linkers for biodegradable hydrogel design. Exemplary is the family of poly(ethylene glycol) (PEG)-based hydrogels prepared by Hubbell and co-workers using peptide cross-linkers derived from known substrates for matrix metalloproteases (MMPs).[18−22] These enzymes comprise a family of structurally related Zn^{2+}-endoproteases that play key roles in wound repair and tumor metastasis via catabolism of extracellular matrix (ECM) proteins such as collagen and fibronectin.[23,24] In Hubbell's work, bis-cysteine-derivatized peptides have been copolymerized with vinyl sulfone-derivatized PEG macromonomers via anionic polymerization. The hydrogels thus synthesized were degraded in a predictable fashion in response to cell-mediated hydrolysis of the peptide cross-linkers by secretion of appropriate MMPs. This work therefore elegantly demonstrated that synthetic peptides bearing appropriate functional groups can impart a hitherto-inaccessible property of biodegradability to synthetic hydrogels comprised of nondegradable polymers. Toward a similar goal, Healy, Moore, and co-workers prepared degradable hydrogels via installation of the requisite reactive functionalities onto synthetic peptides in aqueous solution.[25,26] This work further demonstrated that nondegradable polyacrylamide-based hydrogels can be rendered biodegradable through rational cross-linker design. To elaborate this approach to more structurally complex peptide-derived cross-linkers, decorated with additional functional groups and appendages, we sought to develop a generalized synthetic route for the synthesis of peptide-derived cross-linkers.

The past decade has seen significant developments in the role of solid-phase peptide synthesis (SPPS), notably through the advent of multidimensional protection tactics, selectively

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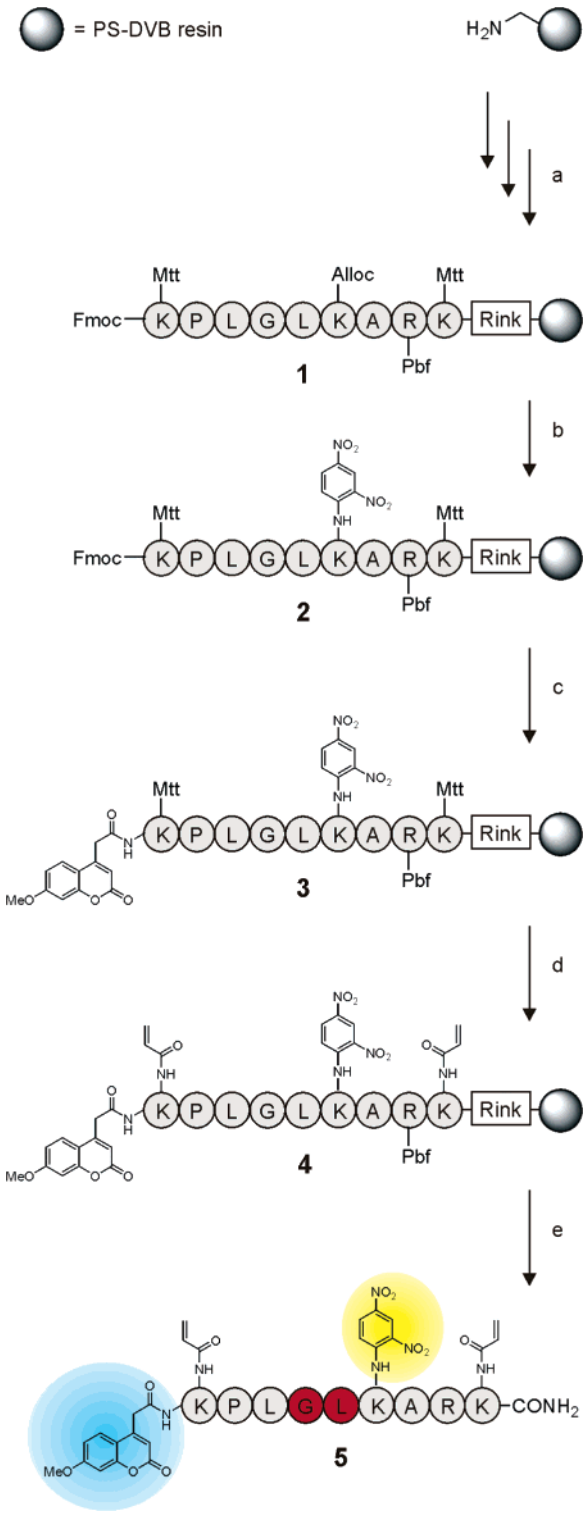
cleavable linkers, and new solid supports.[27,28] SPPS now allows for relatively facile access to elaborate peptide-derived targets by total solid-phase synthesis, obviating the need for solution-phase modifications on often-sparingly soluble intermediates. The cardinal benefit of a total solid-phase synthesis strategy is that so long as manipulations are performed in the proper order of operations and with a judicious selection of reagents, crude products may be obtained in excellent purity and high yield following a single purification step.

In the work described herein, we report the first total solid-phase synthesis of a bis-acrylamide-derivatized peptide cross-linker. Although the installation of two acrylamide moieities within synthetic peptides has been detailed, such reports of solution-phase conjugations onto unprotected peptides are incompatible with many proteinogenic side chains[25] or require specialized reagents.[26] Our all-solid-phase synthesis approach circumvents such solution-phase manipulations and the attending material losses often incurred during subsequent handling and purification steps. Moreover, this strategy allows for an added dimension of structural complexity in cross-linker design, namely incorporation of an internally quenched, fluorogenic substrate, by which peptide cleavage events are linked to a quantifiable increase in fluorescence intensity.[29,30] Thus, we incorporated both fluorophore and quencher moieties into the target peptide cross-linker to allow for both quantitation and visualization of hydrogel degradation in response to MMP-catalyzed cross-linker hydrolysis. This methodology thereby allowed for real-time reporting of this process, and the efficacy of related MMP family members in hydrogel degradation was investigated, whereas kinetic analysis provided useful mechanistic insights.

Experimental Section

Reagents and Materials. Acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were from Sigma-Aldrich (St. Louis, MO). Fmoc amino acids and the Rink Amide Linker were obtained from CS Bio Co. (Menlo Park, CA). Aminomethyl-derivatized poly(styrene-*co*-divinylbenzene) (PS-DVB) resin was obtained from Polymer Laboratories (Amherst, MA) at a loading of 1.11 mmol NH_2/g (75−150 μM). Side chain protections were as follows: Lys (Mtt or Alloc, vide infra), Arg (Pbf); all other amino acids were incorporated without side chain protection. Piperidine was from Sigma-Aldrich, trifluoroacetic acid was from Halocarbon (River Edge, NJ), *N,N*-dimethylformamide (BioAnalyzed) was from J. T. Baker (St. Louis, MO) and EMD (Gibbstown, NJ), dichloromethane (HPLC) was from EMD, and *N,N′*-diisopropylcarbodiimide was from Advanced Chemtech (Louisville, KY). All other reagents, solvents, and chemicals were of the highest purity commercially available and used as received. RP-HPLC was performed using binary gradients of solvents A and B, where A is 0.1% TFA in water and B is 0.09% TFA in acetonitrile. Analytical RP-HPLC was performed using a Vydac 218TP5415 column at a flow rate of 1 mL/min, with detection at 214 nm during a linear gradient of 20−80% B over 30 min. Preparative RP-HPLC was performed using a custom-packed, 2-in. bore C18 column at a flow rate of 15 mL/min, with detection at 220 nm during a linear gradient of 35−65%B over 40 min. In all cases, fractions were analyzed off-line using an ABI/Sciex 150EX single quadrupole mass spectrometer and judged for purity after a consistent summing of 50 scans in multichannel analysis (MCA) mode. For preparative purification purposes, fractions that contained no consistent charged species which accounted for more than 10% of the total ion intensity were designated "pure" and pooled; the homogeneity of this pool was verified by analytical RP-HPLC and was >90%. All purification and synthetic manipulation steps were performed at ambient temperature unless otherwise indicated. MMPs-1,2,7,8,9 and 13 were obtained from Chemicon (Temecula, CA) in zymogen form and were activated with 4-aminophenylmercuric acetate (APMA), obtained from Sigma-Aldrich.

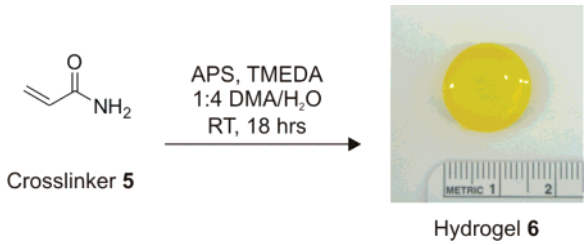
Scheme 1. Synthesis of Fluorogenic Peptide Cross-Linker 5[a,b]



[a] Blue and yellow shading denote fluorophore and quencher moieities, respectively; the red shaded Gly-Leu sequence denotes the MMP cleavage site. [b]Reagents and conditions: a, stepwise Fmoc/*t*Bu SPPS using DIC/HOBt chemistry; b, (i) 0.2 equiv. $Pd(PPh_3)_4$, 15 equiv. *N,N′*-dimethylbarbituric acid, DMF:CH_2Cl_2 (1:1), (ii) 5 equiv. DNP−Fl, 5 eq. NEM, DMF; c, (i) 20% Piperidine/DMF, (ii) 7-methoxycoumarinyl-4-acetic acid, DIC, HOBt (4 equiv. each), DMF; d, (i) TFA:$CHCl_3$ (95:5), (ii) Et_3N in DMF, (iii) 10 equiv. $(Acryl)_2O$, DMF:CH_2Cl_2 (1:1); e, TFA:water:TIPS (95:2.5:2.5).

Peptide Synthesis. The fluorogenic peptide cross-linker **5** was prepared on a 2.0 mmol scale using custom-written DIC/HOBt protocols (available by request from author) for Fmoc/*t*Bu SPPS on a CS Bio 136 automated peptide synthesizer, and is depicted in Scheme 1. At the

Scheme 2. Preparation of MMP-sensitive Hydrogel 6



conclusion of chain assembly, the *N*-terminal Fmoc group was left intact while the Lys-6 Alloc group was removed using 0.2 equiv. $Pd(PPh_3)_4$ and 10 equiv. *N*,*N*′-dimethylbarbituric acid in 1:1 DMF:CH_2Cl_2 for 12 h. The Dnp moiety was then installed using 5 equiv. 2,4-dinitrofluorobenzene and 5 equiv. *N*-ethylmorpholine in DMF for 5 h. After removal of the N-terminal Fmoc group, 7-methoxycoumarinyl-4-acetic acid was coupled according to standard DIC/HOBt protocols. The Lys-1 and Lys-9 ϵ-Mtt groups were simultaneously removed by batchwise treatment with 5% TFA (3 × 1 min); the newly unmasked side-chain amines were then rendered in free-base form with 95:5 DMF:DIEA. Acrylic acid was coupled to the Lys-1 and Lys-9 side chains as the preformed symmetrical anhydride in DMF. This reagent was prepared by preactivating 20 mmol acrylic acid and 10 mmol DIC in 20 mL of DMF for 30 min and addition of the resulting slurry to the peptide-resin. After washing with DMF and CH_2Cl_2 (2 × 20 s flow wash each), the completed peptide-resin was dried in vacuo overnight. Global deprotection and cleavage of the peptide-resin anchorage was accomplished by batchwise treatment with 95:2.5:2.5 TFA:water:triisopropylsilane (TIPS) for 2 h, followed by filtration and removal of TFA by rotary evaporation. The peptide was then triturated from 10 volumes of Et_2O (pre-chilled at −20 °C) and isolated by centrifugation. After likewise washing the pellet two times further with Et_2O, the peptide was extracted into 50% AcOH and purified by RP-HPLC directly. Isolated yield after lyophilization, 845 mg (28%). Similarly, a small batch of Mtt-deprotected resin was set aside and acylated at the Lys-1 and Lys-9 side chains with 1:9 Ac_2O:DMF to afford an acetylated screening construct, **7**, for solution-phase kinetic investigation with the various MMPs. Cleavage and purification of this material were performed as in the case of cross-linker **5**.

Fluorogenic peptide cross-linker, **5**. ESI-MS: Theory, MW = 1499.8; M^{1+} = 1500.8, M^{2+} = 750.9. Observed, M^{1+} = 1500.6, M^{2+} = 751.1.

Acetylated screening construct, **7**. ESI-MS: Theory, MW = 1476.8; M^{1+} = 1477.8, M^{2+} = 739.4. Observed, M^{1+} = 1477.9, M^{2+} = 739.6.

Hydrogel Preparation. Samples of hydrogel **6** were prepared in a Radleys Carousel 12 Place Reaction Station from Brinkmann Instruments (Westbury, NY). Poly(acrylamide-*co*-cross-linker **5**) hydrogels were prepared using a 99.5:0.5 monomer feed ratio as follows (Scheme 2). Acrylamide (213 mg, 3 mmol) was dissolved in 2 mL of Ar-purged water in the reaction vessel and stirred under Ar for 10 min. Cross-linker **5** (23 mg, 0.015 mmol) was then dissolved in 400 μL of *N*,*N*-dimethylacetamide (DMA) and added dropwise to the acrylamide-charged reaction vessel with stirring continued under Ar for 1 h. TEMED and APS were dissolved in water (0.6 and 0.075 M, respectively), and polymerization was initiated by adding first TEMED (200 μL, 0.015 mmol) and then APS (200 μL, 0.12 mmol) to the reaction vessel. The solution was stirred for 15 s, pipetted into 48-well tissue culture plates (8 wells @ 245 μL/well), and allowed to polymerize for 18 h with exclusion of light. Polymerized hydrogels were removed from wells and dialyzed against water three times (4, 4, 18 h) before storing for further use. One of the eight hydrogels thus obtained was weighed before and after lyophilization to determine water content: wet mass = 555 mg; dry mass = 23 mg; wt % water = 95.8%; yield = 71%. The remaining hydrogels were dialyzed against 0.1 M Tris-HCl, 0.1 M NaCl, 10 mM $CaCl_2$, pH 7.5 (hereafter referred to as MMP buffer) for use in enzymatic degradation studies.

Enzymatic Degradation. Six dialyzed hydrogels were placed into separate wells of a 24-well tissue culture plate. For samples employing preactivated MMPs, 100 μL of a 1 mM solution of the organomercurial activator 4-aminophenylmercuric acetate (APMA) in MMP buffer containing 5 vol % DMF (for solubility of APMA) was added to 100 μL aliquots containing 5 μg of MMP-1, 2, 7, 8, 9, and 13 in the same buffer and allowed to incubate at 37 °C for 1 h. These preparations were then added to individual hydrogels. For samples employing MMP zymogens activated in situ, the APMA and MMP solutions were prepared likewise and added directly to individual hydrogels. Fluorescence measurements were taken on a SpectraMax GeminiEM plate reader from Molecular Devices (Sunnyvale, CA). Excitation and Emission were at 325 and 400 nm, respectively, with 2 nm slit widths.

Results and Discussion

Our design of an MMP-degradable bis-acrylamide-derivatized peptide cross-linker was based on the fluorogenic substrate **FS-6**, Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-$CONH_2$.[31] This substrate was chosen because of its known quenching efficiency, catalytic properties, and synthetic simplicity vis-à-vis amino acid composition. Several considerations, however, mandated attention prior to its adaptation as a hydrogel cross-linker. First, the necessarily high synthetic scale for the target cross-linker motivated the substitution of $^{\epsilon}N$-2,4-dinitrophenyllysine rather than $^{\beta}N$-2,4-dinitrophenyl-1,3-diaminopropionic acid in the P_2' position given the exorbitant cost of the latter suitably functionalized, protected amino acid for use in chain assembly. Second, we surmised that installation of the requisite acrylamide functionalities was easiest performed by acryloylation of the P_4 Lys side chain and appending an additional P_5' $^{\epsilon}N$-acryloylysine residue. Clearly, these modifications posed some risk as far as catalytic efficiency was concerned, but were nonetheless necessary as process scalability, cost, and generality are ultimately important goals of the present work.

The synthesis of MMP-degradable cross-linker **5** commenced on a 2.0 mmol scale using PS-DVB resin, with automated Fmoc/*t*Bu chain assembly performed via optimized DIC/HOBt protocols (Scheme 1).[32] Briefly, the resin-bound fully protected sequence **1** was subjected to Pd-catalyzed allyl deprotection, followed by S_NAr coupling with 2,4-dinitrofluorobenzene to afford the Dnp-conjugated intermediate **2**. $^{\alpha}N$-Fmoc deprotection was then followed by coupling with 7-methoxycoumarinyl-4-acetic acid to afford the fluorescence-quenched intermediate **3**. The flanking N- and C-terminal Mtt groups were then simultaneously removed via transient, mild acidolysis (5% TFA) before neutralization and acylated with acrylic acid to afford the fully assembled, protected intermediate **4**. TFA deprotection and RP-HPLC purification yielded the final, purified product **5** in an excellent isolated yield of 28% (Figure 1).

Several noteworthy observations were made during the synthesis of fluorophore-labeled cross-linker **5**. First, our initial route involved Pd-catalyzed allyl deprotection *after* installation of the N-terminal Mca residue. However, this sequence of protecting group manipulations was found to be problematic; alloc deprotection/Dnp conjugation was therefore accomplished with an acidic allyl cation scavenger (*N*,*N*′-dimethylbarbituric acid), whereas the N-terminal Fmoc group was left in place. Second, the installation of the Lys-1 and Lys-9 $^{\epsilon}N$-acrylamide moieties proved to be problematic according to standard DIC/HOBt and HBTU protocols, with HOBt Michael addition byproducts obtained as significant byproducts (data not shown). This side reaction was circumvented through the use of non-nucleophilic coupling chemistry, namely the preformed symmetrical anhydride of acrylic acid. Furthermore, the choice of scavengers used during the final TFA-mediated cleavage/deprotection step early proved to be critical to preserve the

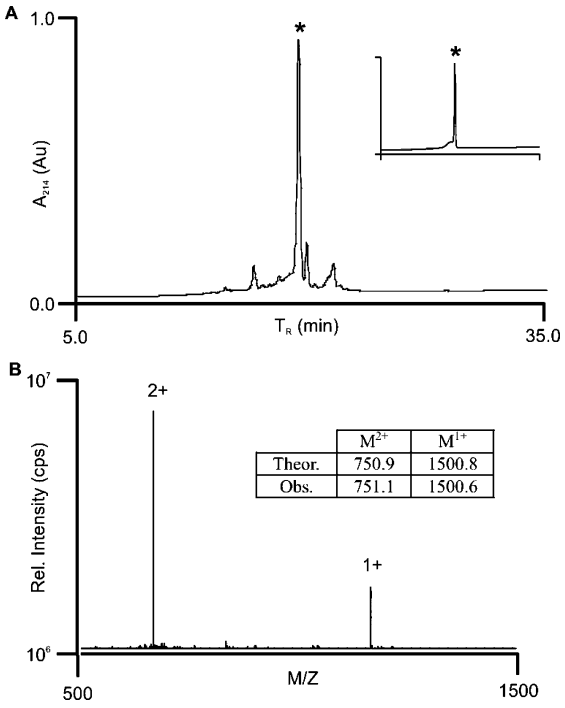


Figure 1. Characterization of internally quenched fluorogenic cross-linker **5**. (A) RP-HPLC trace of crude and purified (inset) **5**, with asterisks denoting retention time (T_R) of the desired product; (B) ESI-MS spectrum of purified **5** with associated charge state information (inset).

integrity of the installed acryloyl moieites. Although it was apparent that obvious strong nucleophiles such as thiols should be omitted from the cleavage cocktail, it became clear that even the weakly nucleophilic thioether moiety of thioanisole formed significant mono- and bis-adducts with the fully deprotected material. Ultimately, triisopropylsilane and water afforded the product in excellent yield and purity, as shown in Figure 1.

The purified internally quenched fluorogenic cross-linker **5** was incorporated into a variety of poly(acrylamide)-based hydrogels to determine optimal conditions with regard to hydrogel rheology for downstream tissue engineering and practical handling purposes. As we and others have found in previous work, the cross-linker molar percentage, concentration of bulk monomer, and polymerization solvent system proved to be of cardinal importance as determinative factors for programming the desired physical characteristics into the hydrogels.[33−35] Optimal polymerization conditions were used to prepare hydrogel **6**, comprised of a 99.5:0.5 acrylamide:cross-linker feed ratio, with a polymerization concentration of 1.5 M acrylamide in a solvent system of 1:4 DMA:water (Scheme 2). It should be emphasized that cross-linker **5** is poorly soluble in neat aqueous media, a problem which is particularly egregious considering the high (ca. 10 mg/mL) concentration demands of polymerization reactions. A variety of mixed solvent compositions were explored, with an aqueous DMA solution providing optimal solubilization of both organic and inorganic components in the polymerization milieu.

Progress curves of observed fluorescence over a 24 h period were obtained for the various MMP enzymes under both preactivation (activation with APMA prior to addition to hydrogels) and activation in situ (activation with APMA in the presence of the hydrogels) conditions. Enzyme activation in situ produced an initial lag period during which zymogen activation preceded the evolution of catalytic activity (data not shown); as this artifact would complicate kinetic analysis, the preactivation panel was used for this purpose (Figure 2). The system was complicated by the slight instability of the fluorophore; under the experimental conditions, the 7-methoxycoumarinyl-4-acetic acid (Mca) fluorophore photobleached with a rate constant of 2.5×10^{-3} min^{-1}. As shown in Figure 2, all MMP progress curves obtained in this study display biphasic kinetics, with both phases exponential with respect to time. Phase 1 results from an enzyme-dependent product formation/substrate loss and phase 2 is the enzyme-independent loss of substrate and product (due to photodegradation and/or any other causes). The simplest kinetic model consistent with these observations is described by the equation (see Supporting Information)

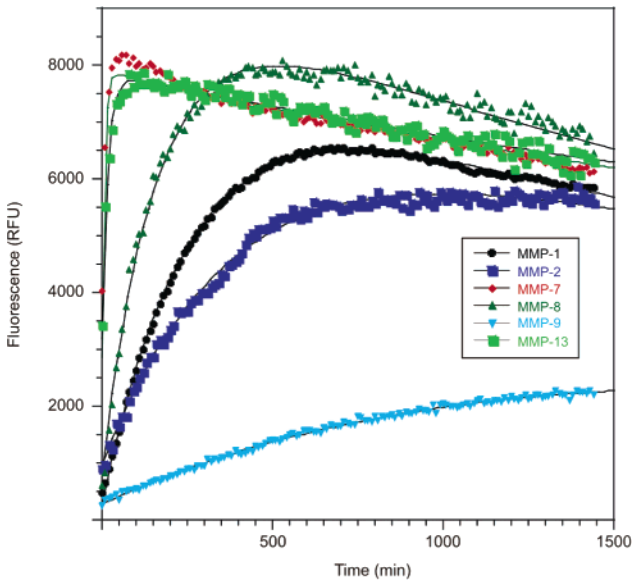


Figure 2. Fluorescence progress curves for MMP-catalyzed degradation of hydrogel **6** samples. Nonlinear least-squaresd fitting of eq I (see Results and Discussion) to each set of data points is superimposed.

Table 1. Rate Constants for MMP-catalyzed Cross-linker Hydrolysis in Hydrogel **6**

enzyme	2nd order rate constant (M^{-1} s^{-1})
MMP-1	600 ± 3
MMP-2	400 ± 5
MMP-7	23 000 ± 1600
MMP-8	1000 ± 12
MMP-9	80 ± 3
MMP-13	9600 ± 340

$$F = F_{max}(e^{-k_d t} - e^{-(k_H E_T + k_d)t}) + F_{initial} \qquad (I)$$

where k_d is the 1st order rate constant for photobleaching of substrate and product, k_H is the 2nd order rate constant for enzyme-catalyzed peptide hydrolysis, and E_T is the concentration of enzyme present. From a nonlinear least-squares fitting of this equation to the progress curves in Figure 1 (k_d fixed at 2.48×10^{-3} min^{-1}), specificity constants (apparent 2nd order rate constants) for cross-linker hydrolysis were obtained for the various MMPs examined in this study (Table 1).

As indicated by fluorescence progress curves, there is significant variation in hydrogel cross-linker hydrolysis by the various MMPs examined, in the order 7 > 13 ≫ 8 > 1 > 2 ≫ 9. Notably, all enzymes reached maximal hydrolysis in several hours, after which photobleaching of the substrate became operative. This rapid rate of enzyme penetration of the hydrogels correlates well with previous observations from our laboratory

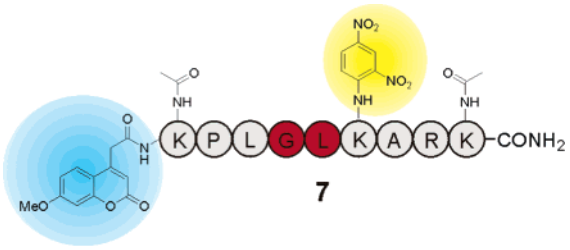


Figure 3. $^{\epsilon}$*N*-Acetylated screening construct **7**.

Table 2. Comparison of MMP Catalyzed Cleavage Rates for Noncrosslinked Substrates

enzyme	screening construct **7** velocity (a.u.)	**FS-6** substrate velocity (a.u.)
MMP-1	4.3	8
MMP-2	65	16
MMP-7	670	850
MMP-8	70	130
MMP-9	210	510
MMP-13	750	1200

on the release of proteins encapsulated in hydrogels of varying cross-linking density.[34,35] In this earlier work, nearly 75% of an encapsulated 66 kDa protein (comparable to MMPs) was found to be released in 12 h from a 0.5 mol % cross-linked acrylamide-based hydrogel; thus, the present finding is an accord with relevant precedent.[34] However, the observed rates and relative ranking of MMP-catalyzed hydrogel cross-linker hydrolysis are incongruous with those observed for MMP-catalyzed hydrolysis of the parent fluorogenic substrate (**FS-6**) from which the cross-linker was designed, which was ranked in the order 13 > 2 > 9 > 8 > 7 > 1.[31]

To determine if this discrepancy in kinetic parameters stemmed from the polymeric carrier or to modifications of **FS-6** necessary for its adaptation into cross-linker format, screening construct **7** was prepared (Figure 3). This construct is a moderately isosteric analogue of cross-linker **5** bearing flanking $^{\epsilon}$*N*-acetamide moieties in place of reactive acrylamides (which would likely have reacted with the MMPs being tested), and was compared side-by-side with the **FS-6** substrate under homogeneous, solution-phase conditions. Construct **7** was prepared from peptide-resin **3** by acetylation of the Mtt-deprotected intermediate using standard protocols, and purified to homogeneity for comparison with **FS-6** hydrolysis by various MMPs. The MMP activity ranking thus obtained is similar for both substrates (Table 2). Notably, MMP-13 is the most active, whereas MMP-8 and MMP-9 exhibit mid-level activity, and MMP-1 is sluggish. On the other hand, we observed poor catalytic activity for MMP-2 and elevated activity for MMP-7, basically the reverse of Neumann and co-workers.[31] When comparing construct **7** with **FS-6** at 5 μM against our panel of MMPs, we see essentially no difference between the two substrates; both exhibit the same rank order of MMP activity and nearly identical initial velocities. The use of Lys in place of Dpa at the P_2' position, the appendage of an additional Lys residue at the C-terminus, and acylation of the flanking Lys side chains appear (in total) to have no selective impact on MMP activity. In addition, when cross-linker **5** was polymerized into a hydrogel, we find roughly the same order of MMP activity as was observed in monomeric form with few exceptions. The order of the two most active matrix metalloproteases is reversed, with MMP-7 being 3-fold more active than MMP-13. The largest deviation in MMP activity between cross-linked (**5**) and non-crosslinked (**7**) substrates occurs with MMP-9, which loses virtually all catalytic activity when its substrate is incorporated into the cross-linked polymer used in this work.

Conclusions

In this work, we have demonstrated that a solid-phase synthesis strategy allows access to structurally complex peptide-derived cross-linkers for use in hydrogels. Several critical observations were made during the execution of our synthetic route regarding the installation of the internally quenched fluorogenic and acrylamide moieties, both of which proved surprisingly labile to conditions normally used in the handling of synthetic peptides. These problems were circumvented through the use of appropriate changes in the sequence of protecting group manipulations, coupling, and deprotection conditions, and in total this strategy now presents a useful, generalized route to appropriately functionalized hydrogel cross-linkers bearing reporter moieties for real-time quantitation and visualization of hydrogel degradation. Kinetic analysis revealed a biphasic model for cross-linker hydrolysis within the hydrogel microenvironment, with the intrinsic fluorescent label allowing for specificity constants to be determined in a high-throughput format without the need for exogenous fluorescence agents. This strategy for hydrogel synthesis is being further developed in our laboratory, notably for the design of a three-dimensional synthetic extracellular matrix surrogate that will allow for in vivo monitoring of neural tissue repair.

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