Biomolecular Hydrogels Formed and Degraded via Site-Specific Enzymatic Reactions

Martin Ehrbar,[†,‡] Simone C. Rizzi,[†,‡,||] Ronald G. Schoenmakers,[§] Blanca San Miguel,[†] Jeffrey A. Hubbell,[§] Franz E. Weber,[†] and Matthias P. Lutolf*[,§]

Oral Biology, Bioengineering Section, and Department of Cranio-Maxillofacial Surgery, University Hospital Zurich, Zurich, Switzerland, Institute of Bioengineering, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

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We present polymeric hydrogel biomaterials that are biomimetic both in their synthesis and degradation. The design of oligopeptide building blocks with dual enzymatic responsiveness allows us to create polymer networks that are formed and functionalized via enzymatic reactions and are degradable via other enzymatic reactions, both occurring under physiological conditions. The activated transglutaminase enzyme factor XIIIa was utilized for site-specific coupling of prototypical cell adhesion ligands and for simultaneous cross-linking of hydrogel networks from factor XIIIa substrate-modified multiarm poly(ethylene glycol) macromers. Ligand incorporation is nearly quantitative and thus controllable, and does not alter the network's macroscopic properties over a concentration range that elicits specific cell adhesion. Living mammalian cells can be encapsulated in the gels without any noticeable decrease in viability. The degradation of gels can be engineered to occur, for example, via cell-secreted matrix metalloproteinases, thus rendering these gels interesting for biomedical applications such as drug delivery systems or smart implants for in situ tissue engineering.

Introduction

The protein- and sugar-rich hydrated milieu that harbors tissue cells in our body not only keeps cells in the right place, but also regulates many of their functions.[1] That is, cells express receptors that allow them to bind soluble and tethered signaling cues from this extracellular matrix (ECM) environment. In turn, these receptor−ligand interactions trigger complex cascades of intracellular enzymatic reactions that regulate gene and protein expression, defining the fate of a cell in a tissue. Simultaneously, cells send out signals to actively construct and degrade their microenvironment by secreting ECM-forming and -degrading proteins.[2] The rapid increase in the molecular understanding of this cell−ECM crosstalk in recent years has opened the door for the design of novel families of smart synthetic polymer hydrogels, termed artificial ECMs (aECMs), recapitulating several facets of natural ECMs.[3,4]

Among the main challenges in creating potent aECMs is a suitable chemical strategy to enable (i) the cross-linking of the matrix in the presence of cells and biomolecules and (ii) the well-controlled and stable tethering of desired biomolecule signals to the matrix. These two characteristics are advantageous in many biomedical applications, including delivery systems for protein therapeutics or three-dimensional (3D) tissue engineering matrixes for encapsulated cells, in which materials need to be formed or brought in contact with sensitive biomolecules or cells in tissues.[3,5]

The first challenge, the development of mild cross-linking schemes to form aECMs in the presence of cells, has been successfully pursued in the past few years. For example, gelation mechanisms based on concepts from supramolecular chemistry have been exploited to form nanofibrillar gels[6] via the self-assembly of low molecular weight building blocks such as peptides[6] or ureidopyrimidinone[7,8] and moderate molecular weight amphiphilic block copolymers.[9−11] On the other hand, several mild chemical cross-linking schemes are now available that permit the encapsulation of viable cells.[12] The second challenge, the well-controlled tethering of desired protein signals to an aECM has proven more difficult. In particular, there is hardly any formulation that would allow the site-specific and stable immobilization of cell regulatory proteins into a gel network without compromising the protein's bioactivity. One of the caveats of standard chemical bioconjugation schemes to attach a synthetic polymer such as poly(ethylene glycol) (PEG) to a protein is their limited selectivity.[13] Except for some aldehydes that show selectivity for N-terminal α-amino groups because of their lower pK_a, electrophilic functional groups on polymer precursors, such as the most frequently used *N*-hydroxysuccinimide ester of PEG carboxylic acids, react with ϵ-amino groups of lysine side chains. The polymer thus attaches randomly at multiple sites, resulting in poor control over protein immobilization and potentially compromising protein activity. An alternative scheme is the PEGylation of thiol groups via 1,4-addition reaction on a conjugate unsaturated system, as demonstrated by some of us earlier.[14] Specificity can be obtained, since several unsaturated groups, such as maleimide, vinylsulfone, or acrylate, exist that react significantly faster with thiols than amines. However, free cysteines are less abundant in proteins and are often critical for their activity, in which case polymer attachment would impair function. We have previously used protein engineering (site-directed mutagenesis) to produce vascular endothelial growth factor (VEGF) bearing an exog-

* Corresponding author. Address: Institute of Bioengineering and Laboratory of Stem Cell Bioengineering, Bldg. AI 3138, Station 15, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland; e-mail: matthias.lutolf@epfl.ch; tel: +41 (0)21 693 18 76; fax: +41 (0)-21 693 18 76 (M.P.L.).

† University Hospital Zurich.

§ Ecole Polytechnique Fédérale de Lausanne (EPFL).

‡ These authors contributed equally.

|| Present address: Institute of Health and Biomedical Innovation (IHBI), Queensland University of Technology, Brisbane, Australia.

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enous cysteine residue for site-specific conjugation with functional PEG.[15] This turned out to be somewhat successful, but there are several complications with this strategy, such as incorrect protein folding and side-reactions with other precursor functional groups.

Enzymatic reactions have become increasingly attractive targets for the fabrication of advanced materials[16] and can overcome both of the above challenges in aECM engineering. Most enzymes catalyze chemical reactions at low temperature, neutral pH, and in buffered aqueous solutions, mild conditions under which many conventional chemical reactions fail. Enzymes can also be exceptionally selective for their substrates, allowing for sophisticated, biologically inspired aECM designs without the complication of side-reactions and cellular toxicity. Indeed, recent development efforts in this area have produced a number of remarkable examples for such smart enzyme-responsive materials.[16] Two types of catalytic reactions have mainly been exploited: (i) synthetic hydrogels have been engineered to degrade in response to the action of proteolytic enzymes[17−24] and (ii) molecular building blocks have been designed to form hydrogels via the mild catalytic action of cross-linking enzymes.[25−28] With these advances, enzyme-responsive materials begin to capture the complex biological functionality of ECM-derived, proteinaceous biomaterials such as collagen or fibrin. However, in order to truly rival the complexity found in natural biomaterials, several hurdles need to be overcome. These include the use of enzymatic reactions to functionalize enzyme-responsive materials with the necessary biomolecular signals to elicit a desired cellular response,[3−5] and/or the combination of multiple enzymatic reactions in the same enzyme-responsive material, just as it occurs in some biopolymer gels that are formed *and* degraded by different enzymatic reactions.

We present a scheme that aims to overcome these hurdles. Our group has previously described approaches to enzymatically incorporate protein growth factors within fibrin matrices by endowing them with substrate domains for the coagulation transglutaminase factor XIIIa.[29] On the other hand, we have described approaches to chemically cross-link reactive PEGs with peptides designed to degrade in the presence of MMPs.[20] Here, we describe a fusion of these approaches: factor XIIIa is utilized to cross-link peptide-derivatized PEGs and simultaneously incorporate bioactive peptides, all within a context that leads to enzymatically triggered degradation of the matrix. In this fusion, no nonenzymatic chemical reactions are employed, and thus the potential for nonspecific reactions on the bioactive protein are minimized.

Experimental Section

PEG and Peptides. Eight-arm PEG, mol wt 40 000, was purchased from Nektar (Huntsville, AL). Divinyl sulfone was purchased from Aldrich (Buchs, Switzerland). PEG vinylsulfone (PEG-VS) was produced and characterized as described elsewhere.[30] The factor XIIIa substrate peptides Ac-FKGGGPQGIWGQ-ERCG-NH_2 (named *TG-MMP-Lys*, mol wt 1717.9 g/mol), H-NQEQVSPL-ERCGNH_2 (*TG-Gln*, mol wt 1358.5 g/mol), and the adhesion ligand H-NQEQVSPL-RGDSPG-NH_2 (*TG-Gln-RGD*, mol wt 1539.6 g/mol) were obtained from NeoMPS (Strasbourg, France) reactive cysteine, in a molecular environment that was optimized as described elsewhere.[14] Two types of control building blocks were chosen: the peptide Ac-FKGG-GDQGIAGF-ERCG-NH_2 as an MMP-insensitive linker[33] and the adhesion peptide Ac-GCGYG*RGDSP*G-NH_2 lacking a factor XIIIa substrate. Both peptides were obtained from NeoMPS.

PEG−Peptide Conjugate Design: Coupling of *TG-MMP-Lys* or *TG-Gln* to Eight-Arm PEG-VS via Michael-Type Addition. *TG-MMP-Lys* and *TG-Gln* were added to PEG-VS in a 1.2-fold molar excess over VS groups in 0.3 M triethanolamine (pH 8.0) at 37 °C for 2 h. The reaction solution was subsequently dialyzed (Slide-A-Lyzer 7K, MWCO: 7; or Snake Skin, MWCO 10K, PIERCE, Rockford, IL) against ultrapure water for 3 days at 4 °C. After dialysis, the product (*8-PEG-MMP-Lys* and *8-PEG-Gln*, respectively) was lyophilized to obtain a white powder. ^{1}H NMR (D_2O) was used to confirm peptide−PEG conjugation via Michael-type addition. Characteristic VS peaks at 6.3 ppm (d, 1H, =C*H*2), 6.4 ppm (d, 1H, =C*H*2), and 6.8 ppm (dd, 1H, −SO2C*H*=) are completely absent in both products *8-PEG-MMP-Lys* and *8-PEG-Gln*, respectively (Figure S1A−C in Supporting Information).

Activation of FXIIIa by Thrombin. A 100 μL portion of factor XIIIa (200 U/mL, a generous gift of Baxter BioSurgery, Vienna, Austria) was activated with 10 μL of thrombin (20 U/mL, Sigma-Aldrich, Switzerland) for 30 min at 37 °C. Small aliquots of activated factor XIIIa were stored at −80 °C for further use.

Formation of PEG−Based Hydrogels. Hydrogel networks were formed by factor XIIIa cross-linking of *n-PEG-Gln* with *n-PEG-MMP-Lys*. For example, to prepare 100 μL of stoichiometrically balanced ([*Lys*]/[*Gln*] = 1) 10% w/v gel, 4.87 mg of *8-PEG-Gln* and 5.13 mg of *8-PEG-MMP-Lys* were needed. A spare volume of 16 μL of the total reaction volume was used for the potential incorporation of the target biologically active component (e.g., *TG-Gln-RGD*) or cells. The cross-linking reaction was performed in Tris buffered saline (TBS; 50 mM, pH 7.6) containing 50 mM calcium chloride and 10 U/mL thrombin-activated factor XIIIa (if not stated otherwise). For the formation of hydrogel disks, drops of a yet liquid reaction mixture (20−40 μL) were sandwiched between sterile hydrophobic glass microscopy slides (obtained by treatment with SigmaCote, Sigma-Aldrich, Switzerland) separated by spacers (ca. 1 mm thickness) and clamped with binder clips. Prior to gelation, the drop of reaction mixture contacting only hydrophobic surfaces spread spontaneously to form a disc. Although gelation occurred within a few minutes at 37 °C in a humidified incubator, the cross-linking reaction was allowed to proceed for about 1 h to achieve complete cross-linking.

In Situ Rheometry. Gelation kinetics was studied by performing small strain oscillatory shear experiments on a Bohlin CVO 120 high-resolution rheometer with plate−plate geometry at 37 °C and under humidified atmosphere. After adding factor XIIIa (concentration stated in each individual case), the reaction mixture (32 μL), containing precursors in stoichiometrically balanced amounts (*8-PEG-Gln* and *8-PEG-MMP-Lys*, 5% w/v), was quickly vortexed and poured onto the center of the bottom plate. The upper plate (2 cm in diameter) was immediately lowered to a measuring gap size of 0.1 mm, and the dynamic oscillating measurement was started. The evolution of storage (G') and loss (G'') moduli and phase angle δ (°) at a constant frequency of 0.5 Hz and a constant strain of 0.05 was recorded as a function of time. The gel time (t_G), as a kinetic parameter for network formation, was defined as the time of crossover of G' and G''.[21,30]

Determination of Nonbound *TG-Gln-RGD*. Different amounts of *TG-Gln-RGD* peptide, as well as a negative control RGD peptide not comprising a factor XIIIa cross-linking site (Ac-GCGYG*RGD-SP*GNH2), were incorporated into hydrogel discs (30 μL each) formed from 5% or 10% w/v polymer solutions (n = 4 per *TG-Gln-RGD* concentration, Table 1). After network formation, the hydrogels were swollen to equilibrium in 500 μL phosphate buffered saline (PBS; pH 7.4) for 3 days. Subsequently, the washing buffer was completely removed, and the gels were stored in 1.5 mL PBS prior to rheological and equilibrium swelling measurements (described below). The amount of released peptide in the washing buffer was analyzed by reversed phase chromatography, using a Waters autopurification system equipped with a ZQ MS detector. The samples were run at 1 mL/min on an Atlantis dC18 column (4.6 × 150 mm), eluting with 0.1% trifluoroacetic acid in water/acetonitrile (82:18 v/v). The peptide was detected in the positive ES mode. (For more Information on the analysis, see Figures S2−S8, Supporting Information.)

Table 1. Hydrogel Compositions Used in This Work and Determined Properties

material precursor conditions			hydrogels characteristics after swelling (PBS, pH 7.4)			
concentration (w/v)[a]	*TG-Gln-RGD*/ *PEG-MMP-Lys* (%)[b]	*PEG-MMP-Lys* or *PEG-Gln* (mM)[c]	(w/v)[d]	*G′* (Pa)[e]	*Q*	*TG-Gln-RGD* in gels (μM)[f]
5%	0	4.02	3.1%	1494 ± 55	32.1 ± 0.3	0
	2.2	4.02	3.1%	1308 ± 33	32.8 ± 0.7	48 ± 1 [90.2%][g]
	4.3	4.02	3.0%	1292 ± 34	33.9 ± 0.6	74 ± 3 [72.2%]
	21.7	4.02	2.4%	813 ± 42	41.9 ± 1.2	264 ± 23 [64.0%]
	43.5	4.02	1.7%	237 ± 60	59.9 ± 0.6	351 ± 71 [60.8%]
10%	0	8.03	4.2%	3726 ± 213	23.8 ± 0.3	0
	1.1	8.03	4.0%	ND	24.9 ± 0.7	35 ± 1 [100%][h]
	2.2	8.03	3.9%	3652 ± 410	25.6 ± 0.6	59 ± 5 [86.3%]
	10.8	8.03	3.6%	3146 ± 284	27.7 ± 0.8	250 ± 16 [80.3%]
	21.7	8.03	3.3%	2065 ± 90	30.5 ± 1.0	477 ± 26 [84.2%]

[a] Total solid percent in hydrogels prior to swelling calculated from the amounts of *8-PEG-MMP-Lys* and *8-PEG-Gln* employed. [b] Moles *TG-Gln-RGD* as a percentage of the Lys substrates in *8-PEG-MMP-Lys*. [c] Concentration of Lys or Gln substrates from *8-PEG-MMP-Lys* or *8-PEG-Gln* precursors in hydrogels before swelling. [d] Total solid percent in swollen hydrogels calculated from the amount of *8-PEG-MMP-Lys* and *8-PEG-Gln* used to form the materials. [e] Elastic shear moduli (*G′*) of swollen hydrogel disks. [f] Concentration of effectively incorporated *TG-Gln-RGD* in swollen hydrogels. [g] Incorporation efficiency calculated with the release data. [h] No *TG-Gln-RGD* could be detected in the release buffer (ND: not determined; average of $n = 4 \pm$ sd).

Rheometry on Swollen Networks. Storage and loss moduli (*G′* and *G″*) of swollen gels containing various amounts of immobilized *TG-Gln-RGD* ligand (Table 1) were obtained by small-strain oscillatory shear rheometry by using the above instrument as described elsewhere.[21,30] Briefly, swollen preformed hydrogel disks of 1−1.4 mm thickness produced as described above were sandwiched between the two plates of the rheometer with compression up to a range between 75% and 85% of their original thickness to avoid slipping. Measurements were then conducted in a constant strain (0.05) mode as a function of frequency (from 0.1 to 10 Hz) to obtain dynamic mechanical spectra ($n = 4$ per condition).

Equilibrium Swelling Measurements. Swollen hydrogels were weighted just prior to rheology testing, and the swelling ratio *Q* was determined as the swollen gel mass divided by the gel's dry mass (calculated from the reaction conditions).

Degradation of Gels by MMP-1. The weight of gels ($n = 5$) was measured during incubation (at 37 °C) with MMP-1 (40 nM). Active MMP-1 was a generous gift from Dr. G. B. Fields, Florida Atlantic University, Boca Raton, FL.

Cell Culture. Neonatal normal human dermal fibroblasts (Clonetics, San Diego, CA) were grown under standard cell culture conditions in fibroblast cell culture medium (Dulbecco's modified Eagle's medium, with 10% fetal bovine serum and 1% antibiotic−antimycotic, GIBCO BRL, Life Technologies, Grand Island, NY) at 37 °C and 5% CO_2.

Cell Adhesion on Hydrogel Surfaces. Investigation of cell adhesion on swollen hydrogels (5% w/v prior to swelling, Table 1) containing different concentrations of immobilized *TG-Gln-RGD* ligands (0, 10, 50, and 100 μM) was performed in medium containing 0.1% fetal bovine serum. Cells were cultured at a density of 10 000 cells/cm^2 for 4 h and subsequently fixed and double-stained for nuclei and f-actin as described below. Stained cells on the different gels were imaged using an inverted fluorescence microscope (Zeiss, Axiovert 200) and a 10× objective. Projected cell areas (>35 cells per sample, three independent samples per *TG-Gln-RGD* concentration), delineated by the stained cytoskeletal f-actin, were measured using Image J software. Statistical analysis of cell size data was carried out on SPSS 11 for Macintosh. Initially, Kolmorogov−Smirnoff and Levene tests were carried out to check for normality of distribution and homogeneity of variance. As both tests were significant and therefore not meeting model expectations, nonparametric statistical tests had to be used. The Mann−Whitney test was used to compare cell sizes, and two-tailed *p* values below 0.05 were considered significant in different conditions with correction of the *p* value for multiple testing according to the Bonferroni method.

Cell Encapsulation within Hydrogels. Cells were added to the hydrogel precursor solution (1.5% w/v, 50 μM RGD) through the spare volume just after adding the FXIII enzyme to obtain a final concentration of 10 U/mL gel. Hydrogels were polymerized at 37 °C and 5% CO_2 for 30 min and subsequently immersed in cell culture medium.

Quantification of Cell Viability. Gels (1.5% w/v, 50 μM RGD) were formed in the presence of cells as described above. After 1 day in culture, a live/dead assay consisting of calcein-AM and ethidium homodimer (Molecular Probes, Eugene, OR) was performed. Cell culture medium was supplemented with 1 μM of calcein-AM and 1 μM ethidium homodimer. After extensive washing with PBS, fluorescence images were taken, and the number of live and dead cells were counted.

Staining and Confocal Microscopy. Fibroblasts were stained for f-actin and nuclei. Samples were fixed and permeabilized in 4% paraformaldehyde containing 0.2% Triton X-100 in PBS for 20 min at 4 °C. Samples were incubated for 10 min in 0.1 M glycine followed by a wash step in PBS. For f-actin staining, the gels were incubated protected from light with 0.4 U/mL rhodamine-labeled phalloidin (R415; Molecular Probes, Eugene, OR) in PBS with 1% bovine serum albumin for 1 h at 4 °C. After washing the samples three times for 5 min in PBS, cell nuclei were co-stained with 1 ng/μL 4′,6-diamidino-2 phenylindole (DAPI) (D-1306; Molecular Probes, Eugene, OR) in PBS for 10 min at 4 °C. Z-series of approximately 30 equidistant *x*−*y* scans at 15 μm intervals were acquired and projected onto a single plane using the Imaris software package (Bitplane, Zurich, Switzerland).

Results and Discussion

Gel Precursor Design and Synthesis (Figure 1). Factor XIIIa, an activated transglutaminase cross-linking enzyme that plays a key role in fibrin clot formation upon tissue damage,[34] catalyzes acyl-transfer reactions between the α-carboxamide group of protein-bound glutaminyl (Gln, or Q in the single-letter nomenclature) residues and the ϵ-amino group of lysyl (Lys, or K) residues, resulting in the formation of ϵ-(α-glutamyl)lysine isopeptide side-chain bridges.[35] Similar to the pioneering work on tissue transglutaminase-catalyzed synthetic PEG gel formation presented by the groups of Griffith[25] and Messersmith,[26] we use multiarm PEG macromers functionalized with two different transglutaminase peptide substrates in stoichiometrically equivalent amounts as precursors (Figure 1). These peptidic factor XIIIa substrates were designed here to contain free thiol groups from cysteine residues (Cys, C), allowing their grafting to VS end-groups on multiarm PEG

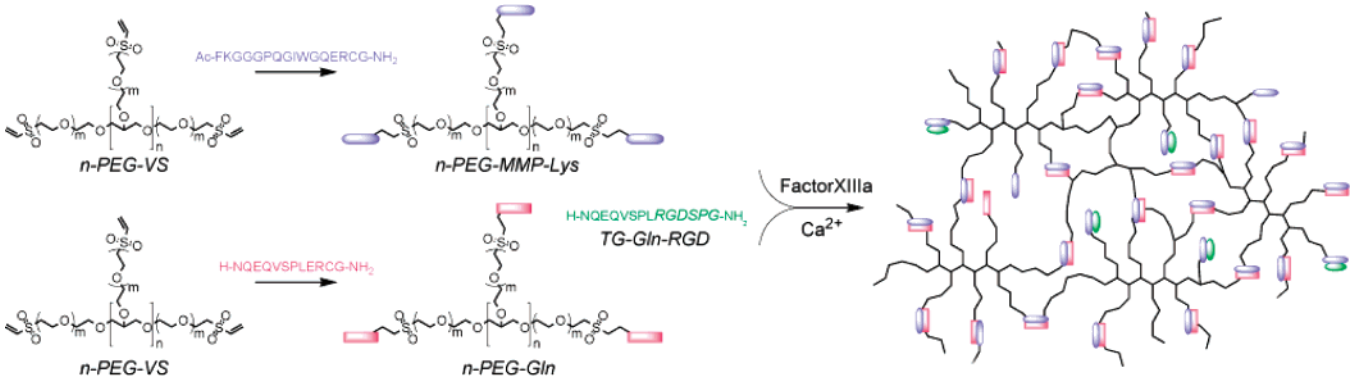

Figure 1. Factor XIIIa-catalyzed PEG hydrogel formation. The transglutaminase enzyme factor XIIIa was used to cross-link two multiarm PEG−peptide conjugates, *n-PEG-MMP-Lys* and *n-PEG-Gln* (here, $n = 8$), in combination with a cell adhesion peptide, *TG-Gln-RGD*, to form multifunctional synthetic hydrogels.

macromers via mild Michael-type addition.[30] ^{1}H NMR analyses were conducted on peptide-conjugated macromers, and we found the reaction to be complete as judged by the absence of characteristic VS peaks after the addition of the peptides (Figure S1, Supporting Information). The transglutaminase factor XIIIa substrates conjugated to the termini of PEG macromers were designed as follows: As the glutamine acceptor substrate, we chose the native sequence NQEQVSPL, derived from the N-terminus of α_2-plasmin inhibitor (α_2PI_{1-8}). Full-length α_2PI and peptides comprising the α_2PI_{1-8} sequence are incorporated into fibrin networks during fibrinogen polymerization.[29,31,32,36] As lysine donor peptide on the complementary PEG macromer, we selected the peptide Ac-FKGG, a sequence that is based on recent work by Hu and Messersmith on optimized transglutaminase substrate peptides for rapid enzymatic formation of hydrogels.[27] Finally, to render the networks susceptible to degradation by proteolytic enzymes, we further designed the lysine donor peptide to contain a substrate for matrix metalloproteases (MMPs), namely GPQG↓IWGQ[20] (with ↓ indicating the cleavage site; the sequence GDQGIAGF was chosen as the MMP-insensitive control linker[33]), in order to target one prototypical family of proteases important in cell migration and tissue regeneration.

Formation of Hydrogels via Factor XIIIa Cross-Linking. Polymer networks were created by factor XIIIa-catalyzed stepwise copolymerization of eight-arm PEG precursors bearing complementary enzyme factor XIIIa peptide substrates: Mixing of the two stoichiometrically balanced PEG−peptide conjugates at 37 °C and neutral pH in a Tris buffer that contains 50 mM calcium chloride and 10 U/mL factor XIIIa results in the formation of gel networks, as evidenced by real-time shear rheometric measurements that indicate the gelation process in situ (Figure 2A). Under these conditions and for 5% w/v precursor concentration, gelation occurs within approximately 5 min. When immersed in PBS, the obtained gels swell considerably and reach an equilibrium polymer concentration of ca. 2−3%, depending on the precursor concentration. Rheometry on gels swollen to equilibrium revealed mechanical spectra that are typical for cross-linked polymer gels,[21,30] with a G' value (1493 ± 55 Pa for 5% and 3726 ± 212 Pa for 10% w/v gels) that is independent of frequency and 1−2 orders of magnitude above G'' (Figure 2B). The dependence of the elastic modulus on precursor concentration indicates the presence of molecular imperfections such as dangling chain ends at lower precursor concentration or chain entanglements or intersecting polymer loops at higher concentration. The physicochemical characteristics of hydrogels obtained from different precursor compositions are summarized in Table 1.

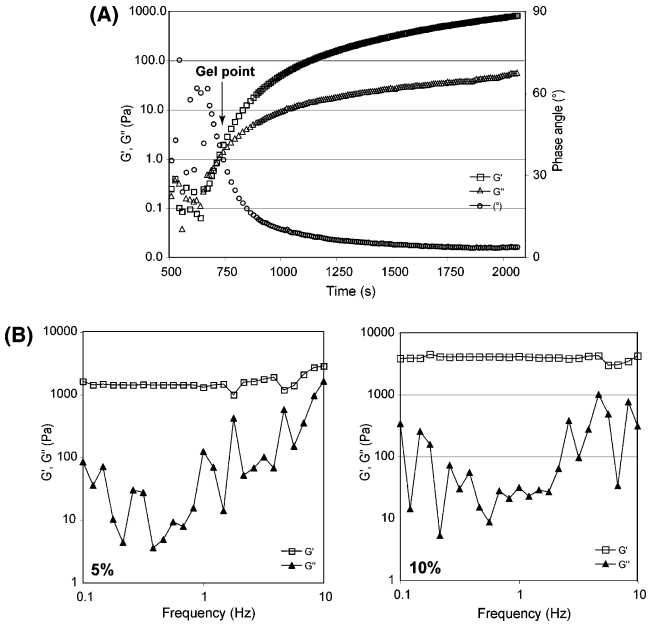

Figure 2. Small-strain oscillatory shear rheometry to determine gelation kinetics in situ (A) and the storage and loss moduli (G' and G'') of swollen gels (B). Cross-linking of precursors at 5% (w/v) solid content occurs within a few minutes at room temperature (A). Mechanical spectra of swollen, elastic polymer networks formed at 5 and 10% (w/v) precursor concentration, respectively (B).

Biomolecule Incorporation via Factor XIIIa and Its Influence on Hydrogel Physicochemical Properties (Figures 3 and 4). In an effort to expand the functionality of existing transglutaminase-cross-linked synthetic hydrogels,[26−28] we explored the possibility of incorporating biomolecules as pendant motifs within the network (Figure 1). The adhesion peptide RGDSP derived from a cell attachment site of fibronectin[37] was chosen as a prototypical adhesion ligand here. In order to render it conducive for factor XIIIa-catalyzed cross-linking, we designed it such that it comprised the above α_2PI_{1-8} sequence NQEQVSPL, allowing its coupling to the Lys-containing PEG-conjugated substrate during network formation. This yielded the sequence H-NQEQVSPL-*RGDSPG*-NH_2, referred to as peptide *TG-Gln-RGD*. Since biological effects of tethered biomolecules are often concentration-dependent, the control of these characteristics in a given aECM system is critical. The efficiency of covalent factor XIIIa-catalyzed *TG-Gln-RGD* incorporation during gel cross-linking was evaluated here by reversed-phase chromatography in conjunction with mass spectroscopy (Figure 3 and Figures S2−S8, Supporting Information). Both 5 and 10%

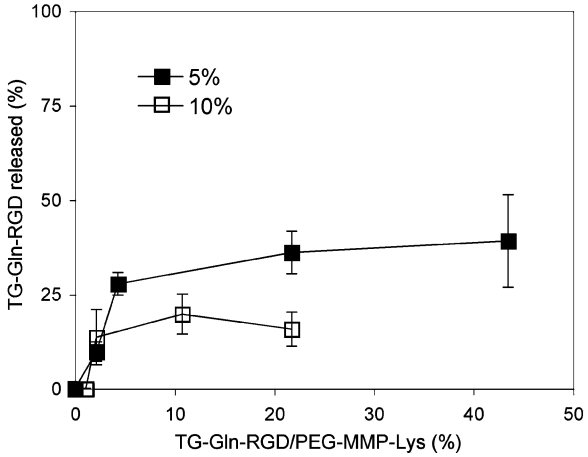

Figure 3. Factor XIIIa-catalyzed biomolecule incorporation. Overall efficiency of biomolecule incorporation into networks was assessed by release studies using reversed-phase chromatography and mass spectroscopy as described in the Supportive Information. Between 60.8% and 80.9% of the *TG-Gln-RGD* ligand (for 5% and 10% gels, respectively) was incorporated at the highest peptide concentration tested.

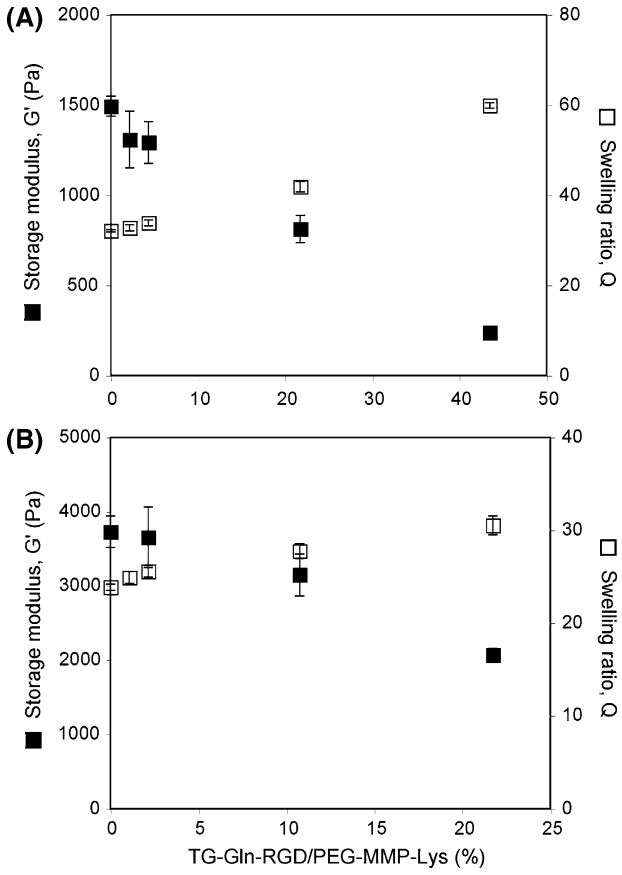

Figure 4. Incorporation of millimolar amounts of exogenous *TG-Gln-RGD* ligands in gels formed at low solid concentration can perturb the network final cross-linking density and hence its physicochemical characteristics as shown here for the elasticity and swelling for 5% gels (A) and 10% gels (B), respectively.

w/v gels were formed with variable molar ratios of *TG-Gln-RGD* over the Lys substrates of the PEG-MMP-Lys precursor as indicated in Table 1, swollen to equilibrium, and their non-incorporated (i.e., freely diffusive) *TG-Gln-RGD* content was measured as described. A negative control peptide lacking a TG-Gln cross-linking site was investigated as well and found to be released from the gel nearly complete (92 ± 7%). On the

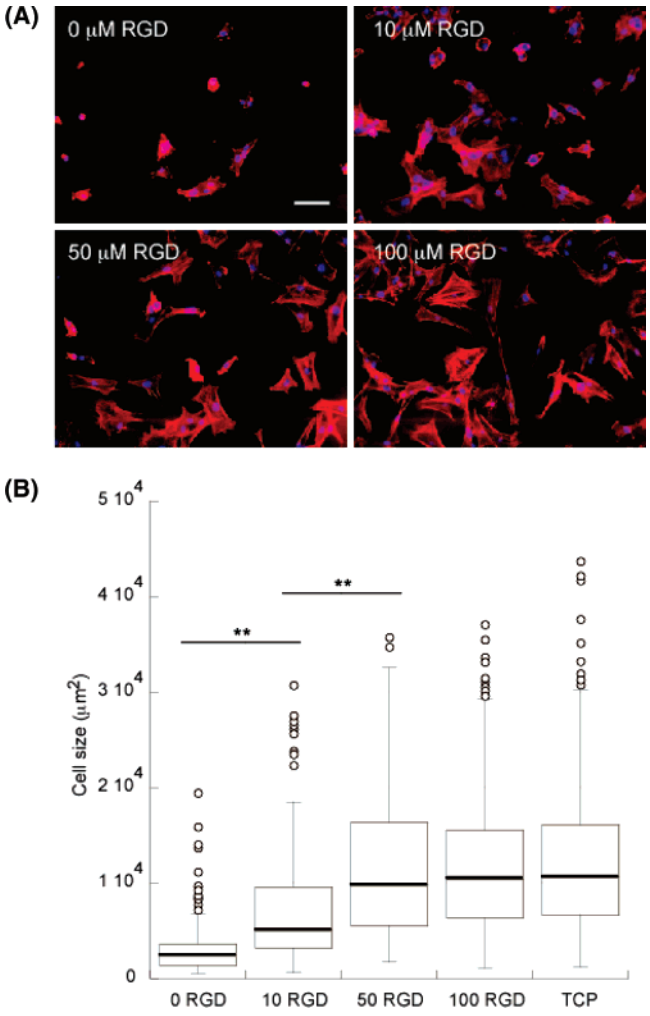

Figure 5. Incorporation of the integrin-binding RGD ligand in the form of *TG-Gln-RGD* renders gel networks adhesive for primary fibroblasts. Spreading is dependent on the ligand concentration as demonstrated by fluorescent microscopy (A, Scale bar: 100 μm) with double staining of cell nuclei (blue) and f-actin cytoskeletal elements (red), and quantified by cell surface areas (B).

contrary, the *TG-Gln-RGD* peptide was readily incorporated within the hydrogel network (Figure 3, Table 1). In general, we detect increasing amounts of incorporated peptide with the concentration of *TG-Gln-RGD* in the reaction mixture. However, at the same time, its incorporation efficiency was observed to decrease. Since the concentrations of TG-Lys and TG-Gln substrates belonging to *8-PEG-MMP-Lys* and *8-PEG-Gln* were stoichiometrically balanced, the addition of *TG-Gln-RGD* in the reaction mixture shifted the stoichiometry substantially off-balance. Consequently, the observed trend may be the result of a more pronounced competition between the incorporation of the biomolecules and the actual polymer cross-linking when the concentration of *TG-Gln-RGD* increased. We reasoned that this might also have a significant impact on the final physicochemical gel properties, as attached pendant biomolecules and dangling nonreacted PEG arms would diminish the number of elastically active chains or the cross-link density of the gel network.

Therefore, the influence of the quantity of incorporated cell-binding sites (*TG-Gln-RGD*) on the equilibrium network properties was assessed by measuring elastic moduli and swelling of hydrogels produced at various *TG-Gln-RGD* concentrations (Table 1). Our results, depicted in Figure 4, clearly showed that mechanical properties decrease and swelling increases with

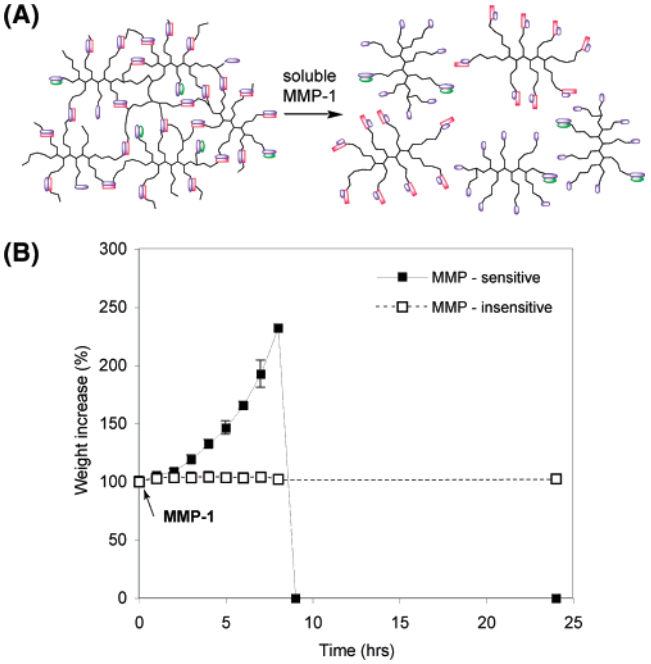

Figure 6. Gel degradation by proteolytic enzymes (A). Incorporation of an MMP-sensitive peptide substrate makes gels susceptible to the action of these enzymes, as shown in the swelling curves in response to incubation with MMP-1 (B).

increasing *TG-Gln-RGD* concentration in the reaction mixture. Although the actual limit was not determined in this study, these changes in macroscopic properties were not considered to be significant at least up to around a 5% molar ratio of *TG-Gln-RGD* over the Lys substrates. At a molar ratio slightly above 20%, the elasticity dropped by a factor of ca. 2 in both 5% and 10% hydrogels. The observed drop in macroscopic structural properties with increasing *TG-Gln-RGD* is not surprising in light of the fact that the stoichiometry is off-balance to this extent, creating large numbers of dangling chains bearing nonreacted glutamine donor sites. This aspect has to be taken into consideration for certain applications requiring very high doses of tethered biomolecule ligands. However, such high concentrations of bound cell adhesion ligands are not necessary to support morphologically normal cell adhesion, as demonstrated in a following set of experiments.

***TG-Gln-RGD*-Modified Networks Support Controlled Cell Adhesion (Figure 5).** The cellular response of *TG-Gln-RGD* network modification was tested in a functional in vitro assay. Factor XIIIa-catalyzed incorporation of the *TG-Gln-RGD* above 10 μM, corresponding to a ca. 0.5% molar ratio of TG-RGD/Lys substrates, makes these novel gels adhesive for primary fibroblasts (Figure 5A,B). Importantly, because of the near-quantitative biomolecule incorporation at low concentrations (Figure 3), the extent of spreading on the gel surfaces is tunable by the concentration of incorporated ligands (Figure 5A), as quantified here by the projected cell spreading area (Figure 5B). At a concentration of ca. 100 μM (molar ratio of 4.3%), we obtain spreading characteristics comparable to the standard tissue culture plastic surface. Fibroblast adhesion is ligand-specific, since the lack of covalently coupled RGD ligand does not permit significant spreading.

Hydrogels are Degradable by Soluble Proteolytic Enzymes (Figure 6). To design these networks with susceptibility to degradation by proteolytic enzymes,[20] GPQG↓IWGQ, a substrate for MMPs, was placed next to the lysine donor peptide Ac-FKGG as described above. Indeed, our otherwise stable aECMs are readily degraded when exposed to active MMP-1 (Figure 6B). Individual cleavage of elastically active chains throughout the entire gel volume (i.e., bulk mechanism as opposed to surface-mediated degradation) leads to an exponential increase of the swollen gel mass over time up to 3 times the original value before complete gel breakdown occurs. Importantly, gels containing the MMP-insensitive sequence GDQGIAGF are stable over prolonged periods of time (up to several weeks, i.e., the longest periods tested) underscoring the specificity in enzyme-responsiveness obtained by molecular design.

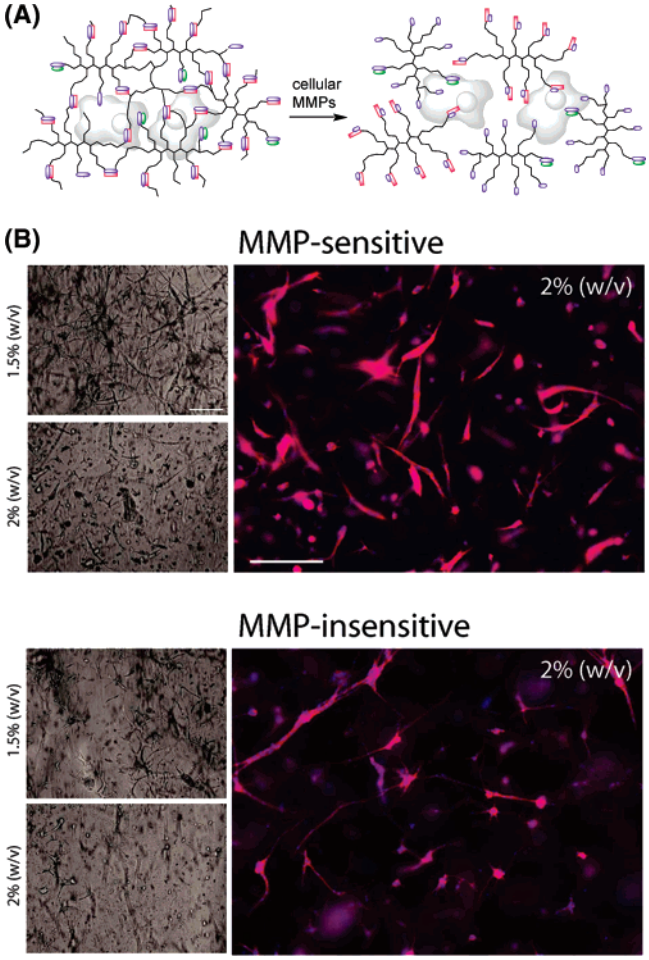

Figure 7. Gel degradation by cell-derived MMPs (A). Single, dispersed cells are capable of locally degrading the surrounding MMP-sensitive gel network via cell-secreted active MMPs (B, Scale bar: 100 μm). Extensive spreading, proliferation, and migration of cells lead to the formation of an interconnected cellular network after one week in culture within 1.5% gels. At 2% precursor content, network formation is substantially reduced, while spindle-shaped cells can still be found. Notably, the absence of proteolytic matrix susceptibility reduces the extent of cell spreading and 3D morphogenetic cellular organization at 2%, while it can still be observed in softer gels (1.5%).

Hydrogels are Degradable by Cell-Derived Proteases (Figure 7). The biological performance of the novel gels was assessed by the growth of individually dispersed cells in the matrix. Primary human fibroblasts known to secrete active MMPs[20] show a viability of higher than 95% when encapsulated three-dimensionally in these gels, suggestive of the physiological character and specificity of the employed enzymatic cross-linking reaction (see Figure S9 in the Supporting Information). Viability after 24 h is independent of proteolytic matrix sensitivity. Within only 1 week in culture in loosely cross-linked matrixes (i.e., formed at $\leq$2% polymer content) containing substrates for MMPs as well as cell adhesion ligands, extensive migration and proliferation leads to the formation of a intercon-

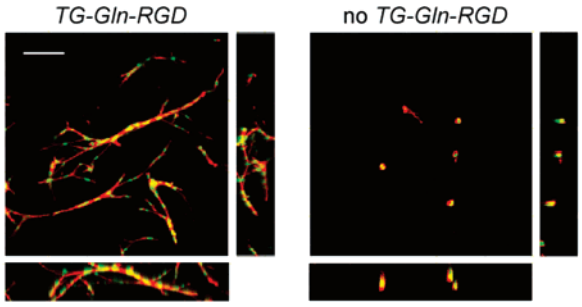

Figure 8. Primary human fibroblasts respond to the presence of adhesion ligands in 3D cell cultures. Extensive cell migration and proteolytic matrix degradation leads to the formation of interconnected cellular networks as described (left), while in the absence of adhesion ligands, cell migration and proliferation are impaired, and cells keep a rounded morphology (right).

nected cellular network (Figure 7A). Such interconnected networks of fibroblasts have been described previously for 3D cultures of fibroblasts in floating collagen gels.[38,39] They could have physiological function during the development of soft connective tissues, serving as an integrated sensory system to monitor and coordinate connective tissue remodeling in response to mechanical signals, similar to the mechanosensory function of the dendritic network of extensions utilized by osteocytes in bone.[40] Not surprisingly, in the absence of incorporated *TG-Gln-RGD*, cells are not able to proliferate and form such supracellular morphogenetic structures (Figure 8). In addition, this process was not observed in gels formed in polymers exceeding 3%. Notably, in gels in which the protease substrate is replaced by an MMP-insensitive linker, the extent of 3D cell spreading and cellular network formation is markedly reduced (Figure 7B, bottom). However, we find that this 3D morphogenetic response is sensitive to the polymer concentration during gel formation: In gels formed at 1.5% precursor, cells are able to form spindle-shaped extensions even within MMP-insensitive matrixes. This small change in precursor concentration and, consequently, matrix cross-linking density thus appears to lead to a transition in 3D migration mechanisms from predominantly proteolytic to non-proteolytic cell migration. This intriguing aspect will be further elucidated in follow-up experiments.

Conclusions

In conclusion, we extend the functional range of existing enzyme-responsive materials for cell-based applications on two fronts. First, we introduce methods to enzymatically attach biomolecules to PEG-based hydrogels with high efficiency, as demonstrated here for the model ligand *TG-Gln-RGD*. Second, we design material building blocks that are responsive to two different enzymatic systems: one for matrix formation and another one for its degradation. Compared to our previous aECM designs based on the chemical cross-linking of reactive PEGs with peptides via Michael-type addition,[20] the use of site-specific enzymatic reactions is an important advance, as it will allow researchers to immobilize desired biomolecules in a more controlled fashion. In order to embrace ongoing challenges in creating next-generation biomaterials, for example, as smart, "living" implants for tissue engineering, the presented approach can be adapted to other material designs and will enable superior control of cell behavior.

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Supporting Information Available. (1) NMR analysis of gel precursors formed via Michael-type addition, (2) determination of nonbound *TG-Gln-RGD* by reversed phase chromatography, and (3) determination of cell viability upon 3D encapsulation. This material is available free of charge via the Internet at http://pubs.acs.org.

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