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Modulating the Mechanical Properties of Self-Assembled Peptide Hydrogels via Native Chemical Ligation

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Abstract

Hydrogels produced from self-assembling peptides and peptide derivatives are being investigated as synthetic extracellular matrices for defined cell culture substrates and scaffolds for regenerative medicine. In many cases, however, they are less stiff than the tissues and extracellular matrices they are intended to mimic, and they are prone to cohesive failure. We employed native chemical ligation to produce peptide bonds between the termini of fibrillized β -sheet peptides to increase gel stiffness in a chemically specific manner while maintaining the morphology of the self-assembled fibrils. Polymerization, fibril structure, and mechanical properties were measured by SDS-PAGE, mass spectrometry, TEM, circular dichroism, and oscillating rheometry; and cellular responses to matrix stiffening were investigated in cultures of human umbilical vein endothelial cells (HUVECs). Ligation led to a fivefold increase in storage modulus and a significant enhancement of HUVEC proliferation and expression of CD31 on the surface of the gels. The approach was also orthogonal to the inclusion of unprotected RGD-functionalized self-assembling peptides, which further increased proliferation. This strategy broadens the utility of self-assembled peptide materials for applications that require enhancement or modulation of matrix mechanical properties by providing a chemoselective means for doing so without significantly disrupting the gels' fibrillar structure.

INTRODUCTION

Hydrogels constructed from fibril-forming peptides, peptidomimetics, and peptide derivatives are useful as chemically defined extracellular matrices [1–5], but their relatively modest storage moduli of up to about 10 kPa and their tendency to fail cohesively present challenges for their application in biomedicine and biotechnology. The work reported here provides a route for increasing the stiffness of β -sheet fibrillar hydrogels in a manner that is chemoselective, that maintains fibril geometry, and that produces native peptide bonds between self-assembled peptides.

The viscoelasticity of any matrix, synthetic or natural, profoundly affects cell behavior, both in culture and in vivo (for recent reviews, see [6,7]). In soft tissues, matrix elastic moduli span several orders of magnitude, from around 100 Pa for the softest tissues such as lymph node [8] and brain [9] to around 100 kPa for more collagenous tissues such as skin [10]. Matrix stiffness significantly affects cell spreading, motility, focal adhesion assembly, and differentiation [11,12], so controlling matrix stiffness is important for applying these materials

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as 3-D cell culture matrices or as biomaterials for regenerative medicine. In addition, practical considerations inherent to these applications dictate that 3-D culture substrates must withstand medium changes and handling, and implanted biomaterial scaffolds or coatings must withstand the forces generated by placement and normal functioning of such devices.

Motivated by both the biological importance of matrix stiffness and the practical issues surrounding the translation of peptide hydrogels into biomedical technologies, efforts to improve these materials' mechanical properties have included increasing the peptides' concentration or modifying the ionic strength of the gelation environment [13–16]. Stiffening can also be achieved over days to weeks through natural matrix secretion by cells in culture [2]. Here we investigated a novel method for rapidly increasing the stiffness of self-assembled β -sheet fibrillar peptide hydrogels using native chemical ligation (NCL), a chemoselective approach that has been widely utilized for joining unprotected peptides in solution, one with an N-terminal Cys residue and one with a C-terminal thioester [17]. For several chemical and biological reasons, it was hypothesized that this reaction would be advantageous for stabilizing peptide hydrogels for use as 3-D culture matrices or as scaffolds for regenerative medicine. First, because NCL is carried out in aqueous conditions at neutral pH with unprotected peptides, cross-linking would be able to take place directly in water-swollen gels. Second, cross-linking would be achieved through the formation of native peptide bonds between the termini of adjacent peptides, which would not be expected to significantly disrupt the self-assembled fibrils and which would avoid non-native structures that could potentially increase the material's immunogenicity. Third, the reaction is chemically specific, allowing for the incorporation of any additional amino acid sequences within the matrix (e.g. cell-binding sequences, substrates for proteolysis, growth factor binding sequences, etc.). Although NCL has almost exclusively been used to join two different soluble peptides possessing either an N-terminal Cys residue or a C-terminal thioester, Hartgerink and coworkers recently employed it to polymerize short collagen-mimetic peptides containing both functionalities into long soluble fibers [18]. In the work reported here, we designed and investigated a gel-forming system of peptide α -thioesters with N-terminal Cys residues based on an amino acid sequence that forms β -sheet fibrillar networks, Q11 (Ac-QQKFQFQFEQQ-Am) [19,20]. We describe significant matrix stiffening through NCL, a dramatic improvement in endothelial cell proliferation and CD31 expression on the matrices' surfaces, and the orthogonality of this approach to the inclusion of RGDS-functionalized peptides.

MATERIALS AND METHODS

Synthesis of peptides and peptide thioesters

Peptide synthesis reagents were purchased from NovaBiochem. Peptides Q11 (Ac-QQKFQFQFEQQ-Am, m/z calcd: 1527.7; found: 1527.7), RGDS-Q11 (Ac-GGRGDSGGG-(Q11)-Am, m/z calcd: 2227.3; found: 2227.6), and RDGS-Q11 (Ac-GGRRDSGGG-(Q11)-Am, m/z calcd: 2227.3; found: 2227.0) were synthesized on a 0.25 mmol scale using a CS Bio 136 automated peptide synthesizer on Rink amide AM resin using standard Fmoc protocols and activation with HBTU/HOBt. All peptides were double-coupled and cleaved/deprotected with 95:2.5:2.5 TFA:triisopropylsilane (TIS):H₂O. Peptides were precipitated and washed several times with cold diethyl ether, dissolved in water, lyophilized on a Labconco freeze-drying system, and stored as lyophilized powders below -20°C . CQ11G-thioester (Cys-(Q11)-Gly-COSR, R=CH₂CH₂CO₂C₂H₅) was synthesized on a 0.1 mmol scale with standard Fmoc protocols using H-Gly-sulfamylbutyryl NovaSyn® TG resin (NovaBiochem Cat# 04-12-3714) and Boc-Cys(Trt)-OH. Following synthesis, the peptidyl resin was activated with 1 M trimethylsilyldiazomethane in 1:1 THF:hexane for 2 h, washed with THF, and cleaved using ethyl-3-mercaptopropionate (50 equiv) and sodium thiophenolate (0.5 equiv) in DMF. The resin was removed by filtration and washed three times with DMF, and the DMF was removed

by rotary evaporation. The fully protected peptide ($[M+K]^+$ calcd: 3753.5; found 3756.5, see Supporting Information for mass spectrum) was precipitated in cold diethyl ether and stored as a dry powder below -20°C until deprotection and experimentation.

Hydrogel formation, ligation reactions, and disulfide formation

Q11 hydrogels were formed by dissolving 30–35 mM peptide in water and layering Dulbecco's phosphate buffered saline (PBS, 0.2 g/L KCl, 0.2 g/L KH_2PO_4 , 8 g/L NaCl, 1.15 g/L Na_2HPO_4) over the peptide solution. With careful pipetting, mixing of the layers could be avoided, allowing the peptide layer to gel under the PBS layer by diffusion of buffer constituents into the peptide layer. For CQ11G-thioester, ligation polymerization was prevented until experimentation by storing it as a fully protected peptide. Immediately prior to experimentation, CQ11G-thioester was deprotected for 1 h with 95:2.5:2.5 TFA:TIS: H_2O , precipitated in cold diethyl ether, and washed 5–6 times with ether. The fully deprotected peptide thioester (m/z calcd: 1763.0; found: 1762.2, see supplementary information for chromatogram) was soluble in water up to concentrations of at least 35 mM. In aqueous solutions of the peptide thioester, ligation was significantly inhibited by low pH (pH 2–3), which most likely arose from residual TFA. To initiate rapid ligation, ligation buffer (20 mM tris (2-carboxyethyl) phosphine (TCEP), 200 mM mercaptophenylacetic acid (MPAA) in PBS, pH 7.4) was layered on top of the peptide thioester solutions, and ligation reactions were monitored for various time points. To induce disulfide bond formation after self-assembly and ligation, the gels were additionally incubated under 100 mM iodine in PBS, pH 7.4. Penetration of the colored iodine solution into the gel was assessed visually and took between 40 min and 1 h, depending on gel geometry. The gels were then washed in PBS until the iodine diffused from the gels by visual inspection, about 1 h, and complete oxidation was verified using Ellman's reagent.

SDS-PAGE and mass spectrometry

After ligation of CQ11G-thioester gels for time periods between 5 seconds and 2 hours, the ligation buffer was removed and reactions were terminated by adding 1 M iodoacetamide and vortexing vigorously to fragment the gel and allow rapid iodoacetamide penetration. Samples were diluted to 0.4 mM in SDS-PAGE sample buffer (2% SDS, 40% glycerol, 0.25 mg/mL bromophenol blue, 8 M urea in 200 mM Tris buffer, pH 6.8), in which the presence of 8 M urea prevents non-covalent peptide oligomerization [21]. Samples were electrophoresed on 10–20% Tris-tricine gels (Biorad), which were then fixed with 40% methanol and stained with Coomassie G250. Polymerization products were also analyzed by MALDI-TOF mass spectrometry on a Bruker Biflex III instrument using α -cyano-4-hydroxycinnamic acid as the matrix. A pipette tip was used to remove small pieces of gel, which were mixed vigorously with matrix and spotted onto the MALDI plate. Dehydration of the spot on the plate effectively stopped ligation reactions, so iodoacetamide was not used in MALDI analyses.

Oscillating Rheometry

A Bohlin Gemini rheometer (Malvern Instruments, Worcestershire, UK) was utilized to measure gel viscoelasticity. To produce gels of consistent geometry, a glass fiber filter paper template with an 8 mm hole was centered on the bottom rheometer plate. This template was pre-saturated with buffer (PBS, PBS supplemented with 500 mM NaCl, or ligation buffer), and 80 μL of peptide solution was pipetted into the template's central hole. Additional buffer was then gently layered over the peptide solution in excess, and gelation was allowed to progress for 40 min. After gelation, the filter paper template was removed, leaving a cylindrical gel on the lower plate. For oxidized gels, an additional step of incubation under a layer of 100 mM iodine in PBS for 45 min was added. Complete oxidation of gels under similar conditions was verified with Ellman's reagent. To prevent evaporation, these steps were performed in a

humidified chamber. After gelation and removal of the template, the upper plate (parallel plate, 8mm diameter) was lowered until it was in conformal contact with the top surface of the hydrogel, corresponding to gap distances of 1.0–1.5 mm. Standard deviations for the gap settings of each group were less than 100 μm . Storage modulus and loss modulus were measured at 0.1% strain, the temperature was maintained at 25°C, and at least three independent gels were measured for each formulation.

Transmission Electron Microscopy

Stock solutions of 0.5 mM peptide in water were prepared and mixed 1:2 with PBS (Q11) or ligation buffer (CQ11G-thioester), vortexed, sonicated, and incubated overnight at room temperature. Oxidation of peptide fibrils was achieved by adding 100 mM iodine in PBS, pH 7.4, for 1 h. Samples were then applied to 200 mesh lacey carbon grids (Electron Microscopy Sciences), stained with 1% uranyl acetate for 2 min, and analyzed immediately using a JEOL 1230 TEM (JEOL, Ltd., Tokyo, Japan).

Circular Dichroism Spectroscopy

An AVIV 215 circular dichroism spectropolarimeter (Aviv Biomedical, Lakewood, NJ) was used with 0.1 cm path length quartz cells. To eliminate any aggregates formed during storage, Q11 was solubilized and disaggregated in a small amount of TFA for 15 min immediately before CD experiments. CQ11G-thioester was deprotected immediately prior to experimentation. Peptides were then precipitated with cold diethyl ether, collected via centrifugation, washed an additional eight times with ether, and dried under a stream of nitrogen. Stock solutions of 1 mM peptide at pH 7 were prepared in degassed water, and peptide concentrations were determined by Phe absorbance at 257 nm. Owing to the strong UV absorbance of MPAA, it could not be used in buffers for CD analysis, and chloride-containing buffers were avoided to minimize CD signal diminishment. Instead, samples were diluted to a working concentration of 0.3 mM and 0.5 mM in 4.3 mM KH_2PO_4 , 1.4 mM Na_2HPO_4 , 140 mM KF, pH 7, in which ligated dimers and small amounts of trimers were verified by MALDI-TOF MS after overnight incubation. Triplicate scans were averaged at 25°C. In these conditions, we observed adequate signal strength and PMT dynode voltage values less than 500 V at wavelengths greater than 185 nm.

Endothelial Cell Culture

Primary human umbilical vein endothelial cells (HUVECs), EGM-2 medium, and subculture reagents were purchased from Lonza. Prior to seeding onto peptide gels, HUVECs were maintained in EGM-2 medium at 37°C/95% relative humidity/5% CO_2 in T-75 flasks coated with 5% gelatin (Fisher Cat# G7-500) and subcultured according to the supplier's protocols. Gels were produced by pipetting 60 μL of 32 mM peptide in water into 24-well culture inserts (1.13 cm^2 area, Fisher Cat# PICM-01250). The plates were sealed and these solutions were maintained at 4°C overnight. This incubation step increased the viscosity of the peptide solution such that subsequent buffer addition produced extremely smooth gel surfaces for cell culture. We then gently added PBS into the outer and inner chambers, inducing further assembly and producing a uniform, translucent gel about 500 μm thick. For CQ11G-thioester gels, we added ligation buffer instead of PBS, aspirated it after 40 min, and washed the ligated gel with sterile PBS at least 15 times over a 24-hour period to completely remove TCEP and MPAA. All gels were stable at 4°C for at least three days. To seed cells, the PBS was replaced with EGM-2 medium, and HUVECs were seeded on top of the gels at a density of 8,850 cells cm^{-2} . After 3 days of incubation, MTS assay (Promega Cat# G3582) was performed according to the manufacturer's protocol and fit to a standard curve constructed with known quantities of HUVECs. For immunofluorescence staining, cultures were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum for one hour. The

primary antibody was anti-human CD31 mouse IgG₁(R&D Cat# BBA7, 1:100 dilution), and the secondary antibody was AlexaFluor 488 goat anti-mouse IgG₁(γ 1) (Invitrogen cat# A21121 1:200 dilution). Cultures were counterstained with 1 μ g/mL DAPI.

Statistical Analysis

Proliferation data were analyzed by one-way ANOVA with Tukey's HSD post hoc comparisons. Results are reported as means $\pm$ standard deviations. p-values < 0.01 were considered significant.

RESULTS AND DISCUSSION

Peptide gel stiffening through NCL

Both Q11 and CQ11G-thioester were synthesized in good yield and purity (see supplemental information for mass spectra and HPLC chromatograms). The peptide Q11 formed gels as previously described when dissolved in water and incubated under a layer of PBS [19, 20]. The storage modulus (G') of 30 mM (4.6% w/v) Q11 gels prepared in this manner was 10.5 kPa at 1 Hz (Figure 1a), and the storage modulus of 5 mM (0.76% w/v) Q11 gels was 1.2 kPa (Figure 1b). For clarity, all moduli are reported in the text at 1 Hz unless otherwise specified. The G' values observed for Q11 were consistent across replicate samples and were comparable to gels formed by other self-assembling peptides [14, 22, 23] and peptide amphiphiles [15] at similar concentrations and in similar conditions. In contrast, 30 mM CQ11G-thioester gels ligated for 40 min under ligation buffer had a storage modulus of 48.5 kPa, a nearly fivefold increase over 30 mM Q11 (Figure 1a), and ligated gels of 5 mM CQ11G-thioester had a storage modulus of 9.1 kPa (Figure 1b), a sixfold increase over 5 mM Q11. All peptides displayed rheological properties consistent with hydrogels, including frequency insensitivity, loss tangent values ($\tan\delta = G''/G'$) near 0.1, and no crossing of G' and G'' at any measured frequency.

Loss tangent values were also relatively independent from concentration or ligation ($\tan\delta$ was 0.135 for 30 mM Q11 gels, 0.105 for ligated 30 mM gels, 0.125 for 5 mM Q11 gels, and 0.113 for ligated 5 mM gels). These similar $\tan\delta$ values indicated that network connectivity did not likely change significantly upon ligation. It is possible that the slight decrease in $\tan\delta$ for the ligated samples arose from a slightly higher cross-link density [24]; however, changes in other aspects such as fibril morphology, fibril stiffness, or lateral aggregation could also have produced this effect. In prior work, adjustment of peptide concentration has been a significant means for modulating the stiffness of self-assembled peptide matrices [25]. By employing NCL, G' could be adjusted independently of peptide concentration, a property that is advantageous for studying how matrix stiffness affects cell behavior without confounding stiffness with other experimental variables. Most importantly, however, the use of NCL to stiffen peptide matrices enables the production of gels with G' in the range of 10–50 kPa. This stiffness is reflective of highly collagenous soft tissues but has not previously been accessible in fibril-forming peptide matrices, so the present strategy broadens the utility of self-assembled peptide biomaterials towards the engineering of such tissues.

Monitoring the course of ligation polymerization

We utilized SDS-PAGE and mass spectrometry to investigate the molecular species responsible for gel stiffening. To prevent premature ligation of CQ11G-thioester, it was stored in its fully protected state and deprotected immediately before experimentation (see methods). Both Q11 and CQ11G-thioester were soluble in water up to at least 50 mM, and the initial pH of these solutions ranged from pH 2 to pH 4, presumably a result of residual TFA. Owing to the pH-dependence of NCL [17,26,27], ligation was almost completely inhibited in initial solutions of CQ11G-thioester. When CQ11G-thioester peptide solutions were pipetted into flip-top tubes and overlaid with PBS containing 200 mM MPAA and 20 mM TCEP, peptide

trimers formed within seconds and polymerization proceeded to at least hexamers (78 amino acids) by 48 min, as observed with SDS-PAGE (Figure 2a). Identically treated Q11 control samples did not demonstrate these higher molecular weight bands, indicating that the polymerization bands observed for CQ11G-thioester likely represented covalent polymers as opposed to non-covalent aggregates. Moreover, ligation reactions were performed in the presence of 20 mM TCEP, and polymerizations were terminated with iodoacetamide, both of which should eliminate disulfide bond formation during the reaction and analysis, respectively. For both Q11 and CQ11G-thioester, the smallest peptide in each lane ran with an apparent molecular weight of about 3.5 kDa, which is larger than the true molecular weight of the monomeric peptides (1.5 kDa for Q11 and 1.7 kDa for CQ11G-thioester). Such deviations in electrophoretic mobility are common for short peptides [28]. A number of reports have indicated that 8 M urea prevents multimerization of similar fibrillizing peptides such as A β fragments [21,29,30], but these peptides migrate anomalously owing to variations in SDS binding [21] or peptide conformation [30]. Given the similar conditions used here and the absence of a multimeric ladder for Q11, it is likely that the slightly slower migration of these peptides is likewise caused by variable SDS binding or by conformational effects. During the first few minutes of the reaction, the monomer predominated, but by 12 min the monomer band began to diminish and the dimer band became the predominant species. This dimer continued to predominate through 48 min as trimers through hexamers were also formed. We hypothesized that the persistent dimer was attributable to the formation of a 26-amino acid cyclic peptide composed of two CQ11G peptides, which would be favored by the anti-parallel arrangement of Q11 strands suggested by FTIR data in previous studies [19] and would terminate polymerization. To investigate this aspect, we utilized MALDI-TOF mass spectrometry (Figure 2b), which at 2 h of reaction time showed the presence of monomer ($[M+H]^+$ calcd: 1763.0; found: 1764.4), dimer ($[M+H]^+$ calcd: 3390.8; found: 3394.7), cyclic dimer ($[M+H]^+$ calcd: 3256.6; found: 3254.4), trimer ($[M+H]^+$ calcd: 5018.6; found: 5022.5), and cyclic trimer ($[M+H]^+$ calcd: 4884.4; found: 4881.0). Polymerization products and cyclic peptides were distinguishable from non-covalent aggregates because peptides polymerized via NCL would possess only one thioester of ethyl-3-mercaptopropionate (134.2 Da), whereas non-covalent aggregates or disulfides of unpolymerized peptides would be expected to possess more than one of these groups. Similarly, the m/z ratios of peaks attributed to cyclic peptides did not reflect the presence of any thioesters. However, peaks identified as cyclic species could possibly represent disulfides, for example symmetric disulfides of cyclic monomers ($[M+H]^+$ calcd: 3256.6) or mixed disulfides between a cyclic monomer and a cyclic dimer ($[M+H]^+$ calcd: 4882.2). The presence of these species is possible but not likely given the absence of any cyclic monomer peak ($[M+H]^+$ calcd: 1627.8) and the presence of 20 mM TCEP in the reaction buffer. Smaller amounts of tetramer, pentamer, and hexamer were also observed when detection of lower molecular weight species was suppressed (Supplementary Information, Figure S2). A detailed measurement of molecular weight distribution (e.g. by GPC-MALS) is challenging for self-assembling peptides owing to their propensity to aggregate in standard mobile phases, but by using SDS-PAGE and MALDI-TOF mass spectrometry, it can be concluded that polymerization proceeds to at least hexamers (78 amino acids) and that the extent of polymerization is modulated by the formation of cyclic species. In this way, polymerization proceeds to an extent that increases the stiffness of the gels (Figure 1), but not to such a degree that the peptides precipitate.

Contribution of ionic strength and disulfide bonds to matrix viscoelasticity

Experiments aimed at studying the effect of ionic strength and disulfide bond formation were performed to determine the extent to which these aspects contribute to matrix stiffening in Q11-based gels. To assess the impact of ionic strength, we compared the viscoelastic properties of Q11 gels incubated under the same buffer as used for ligation (PBS containing 200 mM MPAA and 20 mM TCEP) and under PBS supplemented with NaCl (500 mM total). These

buffers stiffened the matrix to 23 kPa and 25 kPa, respectively (Figure 3a), indicating that increased ionic strength can account for as much as 35% of the total increase in G' observed for NCL-stiffened gels. Similar degrees of stiffening by increased ionic strength have been reported for other β -sheet forming peptides as well [13, 14]. However, a significantly smaller degree of stiffening is achievable with buffers of high ionic strength alone compared to NCL, and high ionic strength cannot be maintained in cell culture or for in vivo applications. In addition to investigating ionic strength, we investigated whether oxidation of the N-terminal Cys residues on CQ11G-thioester impacted the gel's mechanical properties. This aspect is important given that some degree of disulfide bond formation is expected in cell culture conditions. Cysteine oxidation had no effect on the viscoelasticity of ligated gels, even with complete oxidation of all Cys residues to disulfides as verified with Ellman's reagent (Figure 3b). Collectively, these experiments indicated that the oligomerized peptide species produced via NCL contribute to most of the observed increase in G' and that the accumulation of disulfide bonds over time in culture is not likely to contribute to changes in the viscoelastic properties of the matrix.

The effect of ligation on fibril structure and folding

TEM with negative staining indicated that ligated CQ11G-thioester peptides formed fibrils (Figure 4). CQ11G-thioester peptides that had been assembled, ligated, and oxidized also formed fibrils, and both oxidized and unoxidized ligated fibrils shared a number of morphological features with Q11 fibrils. Fibrils of all groups were between 11–13 nm wide, and individual fibrils several hundred nanometers long were observed for each group. In addition, uranyl acetate preferentially stained the fibril periphery in all groups, producing dark edges and leaving less electron-dense fibril cores. The most significant difference between the morphologies of unligated and ligated fibrils was that Q11 fibrils appeared somewhat more laterally aggregated than ligated fibrils (Figure 4a); however, it is difficult to assess network connectivity or lateral aggregation in gels from negative-stained TEM samples because they have been adsorbed and dried onto the TEM grid. A more detailed measurement of the spatial arrangement of fibrils in the hydrogels could be provided with additional techniques such as cryo-TEM, but it is nevertheless clear from negative-stained TEM samples that the peptides retained the ability to fibrillize after ligation and oxidation, and the major morphological features of the fibrils remained similar.

Circular dichroism spectroscopy was utilized to evaluate secondary structural changes induced by ligation. Q11 displayed a minimum at 224 nm and a maximum around 205 nm, attributable to β -turn structure (Figure 5) [31,32] and consistent with the behavior of Q11 reported previously [19]. CD spectra of Q11 were independent of concentration in the range tested (0.3–0.5 mM). Upon ligation, CD spectra displayed small differences compared to Q11. The long-wavelength minimum shifted slightly to 222 nm and became more pronounced, particularly at 0.5 mM peptide concentration. The maximum at 205 remained, but at lower intensity. The most significant change induced by ligation was the appearance of a new maximum at 190 nm. This band may be attributed to β -sheet structure and is similar to the dichroic maxima exhibited by the “all- β ” proteins superoxide dismutase and prealbumin [32]. This assignment is not unambiguous in the absence of additional analyses such as FTIR, but the bands at 205 nm and 222 nm and the fibrillization behavior observed by TEM make it likely that this peak arises from β -sheet structure. This additional β -sheet content by CD may be contributed by the cyclic peptide observed in SDS-PAGE and mass spectrometric analyses, or it may arise from longer β -sheet segments between turns that would be produced by the ligation of adjacent β -sheet strands. Collectively, TEM and CD indicated that fibril morphology is subtly but not significantly altered by ligation polymerization, and the structure remains predominantly β -sheet.

The effect of ligation on endothelial cell growth and proliferation

Growth of HUVECs was slow on Q11 gels, and less than half of the originally seeded cells remained attached to the gels by the third day in culture. This poor ability of Q11 to support HUVECs on its gels' surfaces was possibly a result of cohesive failure in the gel leading to cell detachment, or it may have arisen from a lack of engagement of integrins or other cell attachment proteins. Despite Q11's otherwise useful property of forming chemically defined self-assembled gels in culture medium, this aspect has previously hindered its use as a defined nanostructured coating for supporting cells. In contrast, ligated CQ11G-thioester gels supported significantly higher rates of HUVEC growth and proliferation. By 72 h, HUVECs on ligated gels were nearly confluent and expressed significantly higher levels of the cell-cell adhesion protein CD31 (PECAM) (Figure 6). Cells on ligated gels adopted a cobblestone morphology by phase microscopy (Figure 6c) and expressed CD31 at cell-cell contacts (Figure 6a). CD31 is an important mediator of wound healing, angiogenesis, and inflammatory processes, and its expression at cell-cell contacts is expected in normally functional endothelial cells [33]. Cells on unligated Q11 gels were in contrast more spindle-shaped, significantly less confluent, and did not consistently express CD31 at cell-cell contacts (Figure 6b, d).

Quantification of cell growth and proliferation by MTS assay indicated that HUVECs on ligated gels proliferated to more than 150% of their original seeding density by 64 h, whereas fewer than 50% of the originally seeded cells remained on unligated Q11 gels (Figure 7). In addition to this enhancement in proliferation produced from ligation polymerization, the chemoselectivity of this approach was demonstrated by incorporating an RGDS-functionalized Q11 peptide into the ligated gels. Enhancement of HUVEC proliferation significantly above the level induced by ligation alone and abrogation of this response with scrambled RDGS-functionalized Q11 indicated that the RGD sequence was functionally presented by the ligated gels and was not compromised by the ligation chemistry (Figure 7). This result is important for the development of Q11-based gels with additional functionality. Further work is needed to determine the origin of the significant increase in proliferation supported by ligated gels, as we are not certain whether this phenomenon arises from specific engagement of cell-matrix mechanosensing pathways [6,7,34] or whether this is simply attributable to a reduction in cohesive failure of the material that would otherwise lead to cell detachment.

Recent work has indicated the importance of the 30–40kPa regime of matrix elastic modulus for favoring certain cellular behaviors such as a commitment to osteogenic phenotypes [12]. The approach reported here enables chemically defined β -sheet fibrillar peptide matrices to achieve these stiffness values, opening up opportunities to investigate such materials for cell and tissue types that require stiffer matrices. In addition, increasing the stiffness of these scaffolds is likely to improve their practical applicability as coatings for biomaterials. In the example demonstrated here, endothelial growth, proliferation, and function was enhanced by gel ligation, a result that will facilitate the development of these materials as biomaterials coatings for blood-contacting biomedical devices such as vascular prostheses.

CONCLUSIONS

This work provides a means for stiffening self-assembled peptide hydrogel culture substrates in a way that maintains the advantageous chemical definition and modularity inherent in self-assembling systems. Reactions were aqueous and chemically specific, and polymerization was self-limited by the formation of cyclic oligomers, so the peptides did not grow to such molecular weights as to become insoluble. In addition, stiffening by NCL could be achieved at multiple peptide concentrations, effectively decoupling matrix elasticity from peptide concentration. Ligation of the gels significantly improved HUVEC growth and proliferation, and the chemoselectivity of NCL was capitalized upon to produce gels with added biofunctionality, as

illustrated through the incorporation of unprotected RGDS-Q11 and its subsequent enhancement of cell proliferation even further.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Viscoelastic behavior of ligated CQ11G-thioester (G' ●; G'' ○) and Q11 (G' ■; G'' □). Peptide concentration in the gels was 30 mM (a) or 5 mM (b). $n=3$; means $\pm$ SD.

Figure 2
 . SDS-PAGE (a) and MALDI-TOF mass spectrometry (b) indicated that trimers formed by 5 s, with species up to hexamers produced by 36 min. SDS-PAGE showed the persistence of dimer, and MALDI indicated that this dimer band was a mixture of 26-amino acid linear thioesters and 26-amino acid cyclic peptides.

Figure 3.

Contribution of ionic strength (a) and Cys oxidation (b) to matrix viscoelasticity. Ligated CQ11G-thioester gels (G' ●; G'' ○), Q11 gels in ligation buffer (G' ▼; G'' ▽) and Q11 gels in PBS with 500 mM NaCl (G' ■; G'' □) (a). Ligated CQ11G-thioester (G' ●; G'' ○) and ligated/oxidized CQ11G-thioester (G' ▼; G'' ▽) (b). The concentration of all gels was 30 mM. $n=3$; means $\pm$ SD.

Figure 4. Transmission Electronic Microscopy of Q11 (a), ligated CQ11G-thioester (b), and ligated/oxidized CQ11G-thioester (c).

Figure 5. Circular Dichroism of ligated CQ11G-thioester (0.3 mM ■; 0.5 mM □) and Q11 (0.3 mM ▼; 0.5 mM ▽).

Figure 6. CD31 expression was greater at 72 h in culture for HUVECs on ligated CQ11G-thioester (a) than on Q11 (b); green, CD31; blue, DAPI. HUVECs were nearly confluent on CQ11G-thioester (c), but spindle-shaped and sparse on Q11 (d).

Figure 7.

HUVEC proliferation at 64 h. * $p < 0.01$, ANOVA with Tukey HSD post-hoc test compared to Q11; ** $p < 0.01$, ANOVA with Tukey HSD post-hoc test compared to all others ($n = 4$; means $\pm$ SD).