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Repeated Rapid Shear-Responsiveness of Peptide Hydrogels with Tunable Shear Modulus

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Abstract

A pair of mutually-attractive but self-repulsive decapeptides, with alternating charged/neutral amino acid sequence patterns, was found to co-assemble into a viscoelastic material upon mixing at a low total peptide concentration of 0.25 wt%. Circular dichroism spectroscopy of individual decapeptide solutions revealed their random coil conformation. Transmission electron microscopy images showed the nanofibrillar network structure of the hydrogel. Dynamic rheological characterization revealed its high elasticity and shear-thinning nature. Furthermore, the co-assembled hydrogel was capable of rapid recoveries from repeated shear-induced breakdowns (compliance), a property desirable for designing injectable biomaterials for controlled drug delivery and tissue engineering applications. A systematic variation of the neutral amino acids in the sequence revealed some of the critical design principles involved in this novel class of biomaterials. Lowering the hydrophobicity of the neutral amino acids lowered the elastic modulus and the resilience of the assembled hydrogel, thereby providing a means to fine-tune material property. Replacement of neutral amino acids in the sequence with proline (a β -sheet breaker) impaired the ability of the peptides to co-assemble into a hydrogel.

Introduction

Hydrogels are a class of viscoelastic materials that possess numerous biomedical application potentials, for instance, as encapsulation matrices for controlled drug release or as scaffolds for tissue growth and repair.^{1–4} Hydrogels are typically made of high molecular weight polymers of synthetic origin like derivatives of acrylic acid, ethylene oxide, vinyl alcohol etc., or from natural sources like gelatin, collagen, fibrin and polysaccharide-derived polymers like agarose, chitosan etc. Hydrogels obtained from natural sources are particularly appealing since they are more likely to be biodegradable and biocompatible for *in vivo* applications. The gelation of protein- or polypeptide-based polymers that are chemically synthesized⁵ or genetically produced^{2,6} have been extensively studied. Recent studies have focused on small peptides^{7–10} or its derivatives¹¹ that can form hydrogels by self-assembly phenomenon which eliminates the need for chemical cross-linking. These peptides can be synthesized monodisperse with precise control over molecular weight, sequence and chirality which enables programmability and reproducibility in terms of materials property. In addition, peptide-based hydrogels are bioresorbable. A desirable property for hydrogels to have is the

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Supporting Information Available. 30 cycles of repeated recovery of shear-induced breakdown (fig. S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

ability to respond rapidly to mechanical stresses,^{5,12} particularly shears, in the human body. Physical hydrogels have limited inherent mechanical strength due to the non-covalent nature of molecular entanglements and hence will break under shear stresses. However, if the hydrogel can recover quickly after the cessation of stresses, then the limitation in mechanical strength is circumvented. Hence, we intend to design a peptide-based hydrogel that can recover rapidly after repeated mechanical breakdowns. This property would be beneficial for designing injectable scaffolds for controlled drug release⁶ and for various tissue engineering applications.^{13,14} Currently, Matrigel®, a cocktail extract from extra cellular matrix (ECM) of mouse tumor,¹⁵ is the most commonly used scaffold for the majority of artificial tissue repair constructs. However, there is a need for *de novo* designed scaffold that is highly elastic and compliant,^{16,17} which would enhance cell motility and cell-cell interactions during their proliferation. Furthermore, the scaffolds should be tunable for the needs of specific tissue engineering application. For instance, developing a cardiac tissue construct involves applying radial cyclic strain (mechanical conditioning) to the developing tissue construct. This cyclic strain helps the growing cells to stretch and orient circumferentially. This process imparts certain amount of shear force on to the surrounding gel scaffold causing them to remodel into fibrillar structures that mimics the extra-cellular matrix.^{14,18} A tunable peptide-based scaffold that has a fibrillar structure (could act as morphogenetic guide to cells) and is dynamically responsive to repeated strains caused either by cell motility, orientation or proliferation, might foster the growth of the developing tissue constructs.¹⁹⁻²¹

The key to rapid recovery is a fast and reversible assembly-disassembly-reassembly process. Previous design of peptide based hydrogels rested on the principle of self-complementarity, i.e., spontaneous assembly of a single peptide chain complementary to itself.^{5,7,10,22} We choose a modular design strategy based on mutual-attraction but self-repulsion, analogous to the design of heterodimeric coiled-coils.²³ This design principle is implemented through the following pair of decapeptide modules:

Positively-charged peptide module	Negatively-charged peptide module
Acetyl-WK(VK) ₄ -amide KVW10	Acetyl-EW(EV) ₄ -amide EVW10

Valine was chosen as neutral amino acid since it is known to have higher propensity to form β -sheet.²⁴⁻²⁶ Further, valine is also found in natural elastic biopolymers like elastin and the self-complementary, hydrogel forming sequences reported earlier adopted β -sheet conformation.²⁷ The *N*- and *C*-termini of the peptides were acetylated and amidated to eliminate terminal charges that might interfere with the co-assembly. This *N*- and *C*-termini modification might also enhance their resistance against endopeptidase degradation (which recognizes the free amino- and carboxy- terminal) for future *in vivo* applications. The alternating charge/apolar sequence pattern originates from the discovery that self-complementary oligopeptides with such a sequence pattern can form hydrogels.^{7,28-31}

Separating positively (K) and negatively (E) charged residues into two modules enables mutual-attraction but self-repulsion, which drives gelation upon mixing. High net charge density (50 % of the side chains are charged in each peptide) should significantly enhance the long-range attraction between the two oppositely charged modules (which also enhances the solubility of these peptides). Further, due to the repetitiveness of each module, exact sequence matching is not necessary for association. These features should lead to faster assembly rate and hence quicker recovery. Rapid recovery should also benefit from the small size of the peptides. Additionally, self-repulsion of each module prevents uncontrolled spontaneous self-assembly and allows one to control the initiation of gelation via mixing. Mixing induced

physical gelation can preserve the pH and ionic strength of the sample and hence will benefit *in vivo* applications.

Experimental Section

Sample Preparation

The decapeptides were synthesized using standard Fmoc Chemistry³² on Rink Amide MBHA resin (which gives a C-terminal amide). The N-terminal of each peptide was acetylated by acetic anhydride. Each peptide was then cleaved off the resin using 90 % trifluoroacetic acid with 2.5 % each of ethylene dithiol, tri-isopropyl silane, water (as scavengers) and dichloromethane. The cleavage product was rotavaped and washed with ether multiple times. The product was then dissolved in H₂O/CH₃CN (60 : 40 v/v) mixture and lyophilized. The lyophilized crude peptide was purified using reverse-phase (ZORBAX 300SB-C18, 9.4 × 250 mm, 5 μm) and/or ion-exchange (ZORBAX 300-SCX, 9.4 × 250 mm, 5 μm) liquid chromatography (Agilent technologies, HP1100 chromatograph system, Wilmington, DE, USA). The molecular weight and purity of each peptide was verified by MALDI mass spectrometry and analytical HPLC, respectively. All amino acids were purchased from Novabiochem in their protected forms and used directly for solid-phase synthesis without further purification. A Trp (W) residue was incorporated in each peptide as a spectroscopic probe. Each peptide sample was dissolved in 50 mM phosphate or acetate buffer at appropriate pH and dialyzed at room temperature for 2-4 hrs using a dialysis membrane with a molecular weight cutoff of 100 Da. KVV10 peptide's solubility was not high enough at pH 7.0, so lower pH's like 5.5 and 6.0 were used to characterize this peptide pair. A plausible explanation for this could be a pKa shift of lysines towards lower pH that could be attributed to their hydrophobicity (lysine has an additional methylene group when compared to glutamic acid). The concentration of each peptide sample was determined based on the UV absorption of the Trp residue in each peptide, using an extinction co-efficient of 5690 M⁻¹·cm⁻¹ at 280 nm,³³ with light scattering corrected.³⁴

Circular Dichroism Spectroscopy

Circular dichroic spectra were obtained using an AVIV 62DS circular dichroism spectropolarimeter equipped with a water bath. Cylindrical cell of 0.1 mm path length was used for the measurements. The instrument was calibrated using 0.06 % (w/v) ammonium *d*-10-camphor sulfonate before use and flushed with nitrogen during operation. Ellipticity measurement was normalized to mean residue ellipticity (θ), expressed in units of deg·cm²·dmole⁻¹.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) images were acquired from Hitachi H7100 electron microscope operated at 75KV accelerating voltage. Each sample (thin section of gel or solution) was placed on a 200 mesh copper grid and negatively stained with uranyl acetate for an hour. The samples were left to dry in a desiccator before acquiring TEM image.

Rheological Characterization

Rheological studies of the hydrogel were conducted by loading the just mixed decapeptide-pair into a 50 mm cone-and-plate module of a strain-controlled, software-operated rheometer (ARES-100; TA instrument, Piscataway, NJ), and immediately followed by a series of rheometrical tests at 25 °C.

For viscoelastic property determination, the hydrogel sample was sequentially subjected to an 8-hr of time sweep test, a frequency sweep test (frequency from 0.01 to 10 rad/sec) and a series of stress relaxation tests with a defined step-strain (with amplitude at 0.1, 0.2, 0.3, 0.5, 0.6, 0.8,

1, 1.2, 1.5, 2, 5, 10, 20, 50 and 100 %, respectively). In time sweep tests, the applied strain had a 0.2 %-amplitude at a 1-rad/sec frequency. Frequency sweep test was conducted at 0.2 % amplitude and data were acquired at a log mode with 4 data points per frequency decade for 10 min. A shear modulus G' versus strain profile was analyzed according to previously published procedures.³⁵ G' was acquired from the stress relaxation tests (see above for the corresponding strain amplitudes) at elapsed time = 0.1, 1, 10, and 100 sec, respectively.

To characterize the responsiveness of the hydrogel from repeated shear-induced breakdowns, the peptide mixture was allowed to gel for 4 hrs (under 0.2 % strain amplitude and 1 rad/sec frequency) and consequently subjected to a routine of recovery cycles for more than 15 hrs (fig. S1). Each cycle consists of a 2-min of breakdown phase with a continuous 200 %-amplitude sine-wave shear, and a 30-min of recovery phase with a constant 0.2 % strain at 1 rad/sec frequency.

Congo Red Dye Binding Experiment

Concentration of congo red dye (CR) stock solution in 50 mM phosphate or ammonium acetate buffer at pH 6 was obtained using an extinction co-efficient value of $59300 \text{ M}^{-1}\cdot\text{cm}^{-1}$ at 505 nm.³⁶ Peptide samples dialyzed in appropriate buffers were mixed with CR stock solution such that in each experiment the total concentration of peptide was 0.25 wt% and that of CR was 25 μM .

Results & Discussions

Circular dichroism (CD) spectrum is characteristic for different peptide/protein secondary structures:³⁷ β -sheets are characterized by a negative band near 215 nm and a strong positive band between 195 and 200 nm; α -helices are characterized by a double minima at 208 and 222 nm and a positive band at 190 nm; type I β -turn resembles α -helix with its positive band being weaker whereas type II β -turn resembles β -sheet spectrum which is red shifted by 5 to 10 nm. Since, CD spectra (fig. 1) of these individual peptide solutions has a strong minima near 205 nm and lacks characteristics of any standard secondary structures they can be inferred to be in the random coil conformation. However, poly(pro)II type conformation³⁸ cannot be ruled out for KSW10 due to the presence of a weak positive band at 220 nm.

Mixing of oppositely charged peptide solutions induced the formation of a viscoelastic material. Transmission electron microscopy (TEM) was used to study the morphology of the co-assembled hydrogel. TEM images of hydrogel (both 0.25 wt% and 0.5 wt%) showed a network of nanofibrillar structures extending over several micrometers. On the other hand, individual peptide solutions did not have any fibrillar structures (fig. 2).

A series of rheological measurements were conducted to evaluate the viscoelastic properties of the hydrogel formed by the decapeptide pair (fig. 3). Most importantly, we tested the repeated recoverability of the hydrogel from shear-induced breakdowns (fig. 3a) based on an initial assessment of its shear responsiveness (fig. 3b). After being subjected to 200 % shear, the recovery of the elastic modulus, G' , was almost identical to initial gelation (fig. 3b), indicating that the shear-induced breakdown is reversible. To monitor repeated recoveries of G' , the hydrogel underwent 30-cycles of break-and-recovery with a 2-minute strain break period followed by a 30-minute recovery period. Based on preliminary experiments, a 2-minute breakdown period was chosen to achieve complete breakdown of the hydrogel network and a 30-minute recovery period was chosen in order to achieve the equilibrium modulus. Figure 3a shows that G' recovered back to 90 % of its original value after 12 break-recovery cycles. Even after 30 cycles, G' recovered to more than 70 % of its original value (fig. S1). This decrease in original G' could probably be due to evaporation of water from the sample.¹⁰ Rapid

recoveries from shear-induced breakdowns were observed at both pH 5.5 (fig. 3a) and pH 6.0 (fig. 3b).

Other aspects of the viscoelastic properties of the hydrogel were also probed. At 1 rad/sec frequency, G' reached a plateau above 1000 dynes/cm² and the viscous modulus was in the range of 100 dynes/cm² (fig. 3c), revealing a stiff, solid-like hydrogel at 0.25 wt% concentration. Also, G' was relatively independent of frequency (ω) within the frequency range of 0.01-10 rad/sec (fig. 3c), suggesting that the hydrogel has little mobility up to 600s ($t = 2\pi/\omega$). To test its resilience, well-spaced strains were applied 10 minutes apart in a step-incremental manner to observe how the hydrogel relaxes strains.³⁵ Shear moduli plotted against strain amplitude at several elapsed times (refer experimental section) indicated the hydrogel cannot resist a strain over 2 % and beyond this yield strain it exhibited a shear-thinning property (fig. 3d). At 100 % strain, G' dropped to 4 dynes/cm², suggesting complete disruption of the hydrogel network. Nevertheless, the hydrogel was able to regain its elastic strength rapidly (50-60% instantaneously << if possible exact seconds >>) after such disruptions (fig. 3a & b). Shear-thinning nature and responsiveness would be particularly beneficial in designing injectable biomaterials for tissue engineering and controlled drug delivery applications. Biomaterials which are dynamically responsive to the external strain might foster the growth of tissue scaffolds by increasing the cell motility and cell-cell interactions. Further, these scaffolds could incorporate pendent cell adhesive motifs (like RGD from integrin or YIGSR from laminin) to enhance their biofunctionality.

In order to comprehend further design principles involved in this novel class of co-assembling biomaterials, we systematically varied the neutral amino acids in the sequence. Substitution of valine in the sequence with alanine or serine (less hydrophobic neutral amino acids) resulted in a weaker gel with lower G' values (fig. 4a) and resilience (fig. 4b). Even though the G' values for valine and alanine pair were not significantly different, the valine pair had higher resilience (2%) compared to the alanine pair (0.4%) and that of 1wt% serine gel (0.2%). On the other hand, substitution of valine with proline, another important component of natural elastomer – elastin³⁹ and a dominant component in collagen, did not form a hydrogel (fig. 4a). To investigate the basis of this behavior, congo red dye (CR) binding experiment was performed with valine and proline decapeptide pairs. CR is a histological stain for β -sheet type amyloid aggregates.^{40,41} Binding of CR to the fibrillar aggregates causes a hyperchromic red shift in the absorption spectrum of CR.^{36,42} Individual peptide solutions did not bind to CR (fig. 5), in agreement with their random coil conformation as seen in CD spectra. The proline decapeptide pair did not change the absorption spectrum of CR when compared with the CR spectrum with individual peptide solutions (fig 5a), whereas the valine pair caused a considerable hyperchromic shift in the absorption spectra of CR in reference to individual peptide solutions (fig. 5b). The increased light scattering at higher wavelengths in the valine pair is due to the increased size of the aggregates corresponding to hydrogel formation. These results emphasize that the propensity of the sequence to adopt a β -sheet kind of structure is more important for the co-assembly of these biomaterials than the hydrophobicity of the neutral amino acids.

Even though proline has similar hydrophobicity like valine (proline have same number of saturated carbon atoms in the side chains like valine), it cannot adopt a β -sheet type of structure due to constraints in its backbone dihedral angles^{43,44} and hence is not able to form a hydrogel.

Conclusions

A pair of mutually-attractive but self-repulsive peptide modules were found to form viscoelastic materials when mixed with each other at very low concentrations (0.25 wt%). The CD spectra of individual peptide solutions showed that they were in random coil conformation.

TEM images of the gel revealed its nano-fibrillar morphology. Systematic substitution of neutral amino acids in the sequence with less hydrophobic amino acids lowered the elastic modulus and resilience of the hydrogel indicating the possibility of tuning materials property.⁴⁵ Whereas substitution of neutral amino acids with proline (a β -sheet breaker) hindered hydrogel formation. Hence, β -sheet propensity of these sequences is a vital parameter for the assembly of this class of novel biomaterials. Modular design strategy implemented through mutually-attractive, self-repulsive oligopeptides enables one to assemble materials with novel tunable properties. KVV10:EVV10 pair was able to regain its mechanical strength rapidly after repeated shear-induced breakdowns. This is the first time that repeated, rapid recoveries of elastic modulus after shear-induced breakdowns have been observed in peptide hydrogels. This property of the biomaterial along with its high elasticity and shear-thinning nature would be highly desirable for different biomedical applications, for instance, as an injectable delivery vehicle for drugs and cells or as a scaffold for tissue engineering.¹⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Far UV circular dichroism spectra of 1 wt% peptide solutions (KSW10, ESW10, KPW10, EPW10) in 50 mM phosphate buffer, pH 6.0, 25 °C and 1 wt% KVV10 and EVW10 peptide solutions in 30 mM ammonium acetate buffer pH 6.0, 25 °C.

Figure 2. Transmission Electron microscopic images of peptide samples (solution and gel) prepared in pH 6.0 buffer. The scale bar in each figure represents 1 μm .

Figure 3.
Viscoelastic properties of a hydrogel assembled from KVV10:EVW10 decapeptide pair. (a) 12 cycles of hydrogel recovery from shear-induced breakdowns. The dashed line denotes 90 % of the original elasticity value (G_0'). (b) Comparison of the original gelation curve with the first recovery curve after the hydrogel was subjected to 200 % shear. (c) Elastic (G') and viscous (G'') moduli versus frequency (ω) at an applied strain γ of 0.2 %. (d) Shear Modulus G' versus strain γ . The arrow points to the yielding strain at which the hydrogel started to breakdown. The total peptide concentration was 0.25 wt% for all the measurements.

Figure 4.

(a) Time sweep measurement of different peptide pairs prepared in 50 mM phosphate buffer. Filled symbols represent G' and open symbols represent G'' . $\blacklozenge/\lozenge$ represent KKW10:EVW10 pair (pH 6.0), $\blacksquare/\square$ represent KAW10:EAW10 pair (pH 7.0), $\blacktriangledown/\triangledown$ represent KSW10:ESW10 pair (pH 7.0) and $\circ/\circ$ represent KPW10:EPW10 pair (pH 6.0). The total peptide concentration was 0.25wt% for all the measurements. **(b) Strain sweep measurement** of different peptide pairs in 50mM phosphate buffer - EVW10:KKW10 (0.25% wt, pH 6.0, $\blacklozenge$), EAW10:KAW10 (0.25% wt, pH 7.0, $\blacksquare$), ESW10:KSW10 (1% wt, pH 7.0, $\blacktriangledown$), arrows point to the yield value of the peptide pairs.

Figure 5.

Congo Red dye binding experiment (a) KPW10:EPW10 pair with CR in 50 mM phosphate buffer pH 6.0 (b) KVV10:EVW10 pair with CR in 50mM ammonium acetate buffer pH 6.0. The total peptide concentration was 0.25 wt% and CR concentration was 25 μ M for all the measurements. Hyperchromic shift is seen in KVV10:EVW10 pair with CR spectrum.