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Assembly Properties of an Alanine-Rich, Lysine-Containing Peptide and the Formation of Peptide/Polymer Hybrid Hydrogels

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Abstract

We are interested in developing peptide/polymer hybrid hydrogels that are chemically diverse and structurally complex. Towards this end, an alanine-based peptide doped with charged lysines with a sequence of (AKA₃KA)₂ (AK2) was selected from the crosslinking regions of the natural elastin. Pluronic® F127, known to self-assemble into defined micellar structures, was employed as the synthetic building blocks. Fundamental investigations on the environmental effects on the secondary structure and assembly properties of AK2 peptide were carried out with or without the F-127 micelles. At a relatively low peptide concentration (~0.5 mg/mL), the F127 micelles are capable of not only increasing the peptide helicity but also stabilizing it against thermal

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denaturation. At a higher peptide concentration in basic media, the AK2 peptide developed a substantial amount of β -sheet structure that is conducive to the formation of nanofibrils. The fibril formation was confirmed collectively by atomic force microscopy (AFM), small angle neutron scattering (SANS) and transmission electron microscopy (TEM). The assembly kinetics is strongly dependent on solution temperature and pH; an increased temperature and a more basic environment led to faster fibril assembly. The self-assembled nanoscale structures were covalently interlocked via the Michael-type addition reaction between vinyl sulfone-decorated F127 micelles and the lysine amines exposed at the surface of the nanofibers. The crosslinked hybrid hydrogels were viscoelastic, exhibiting an elastic modulus of approximately 17 kPa and a loss tangent of 0.2.

Keywords

Hydrogels; Micelles; Peptides; Self-Assembly; Crosslinking

Introduction

The natural extracellular matrix (ECM) provides ample inspiration for the design of artificial scaffolds for tissue engineering applications. Many structural proteins in the natural ECM are hierarchically assembled, functionally diverse and mechanically robust. Strategic incorporation of unique biological motifs, such as oligomeric peptide or nucleic acid aptamer, into synthetic polymers has resulted in a class of hybrid, biomimetic materials that are promising for tissue engineering applications.[1] A variety of hybrid hydrogels have been engineered to mimic the natural proteins in terms of their hierarchical structures,[2] dynamic responsiveness[3] and defined cellular functions,[4,5] with the added attributes of tunability and processibility provided by the synthetic polymer constituents.

We are interested in developing peptide/polymer hybrid hydrogels that mimic the molecular architecture and mechanical properties of natural elastin. Elastin is one of the most important structural proteins found in many mechanically active tissues, providing elasticity that is required for the proper function of these tissues. Elastin achieves its excellent mechanical properties through a multiblock copolypeptide structure composed largely of two types of short segments that alternate along the polypeptide chain: highly flexible hydrophobic segments composed of VPGVG repeats, with many transient structures that can easily change their conformation when stretched; and alanine- and lysine-rich α -helical segments, which form crosslinks between adjacent molecules via the action of lysyl oxidase in the ECM.[6] Taking advantage of the recent advances in peptide synthesis and polymer engineering, we have successfully synthesized multiblock hybrid polymers consisting of flexible poly(ethylene glycol) (PEG) alternating with alanine-rich, lysine-containing peptides with a sequence of (AKA₃KA)₂ (AK2) that are the structural component of the crosslinking domains of natural elastin. Covalent crosslinking of the resulting multiblock copolymers by hexamethylene diisocyanate through the lysine side chains in the peptidic blocks gave rise to hybrid hydrogels with comparable mechanical properties to that of natural elastin.[7]

While the propargyl glycine terminated-AK2 peptide exhibited 13% helical content at pH 12, the fractional helicity was significantly reduced when the peptide was intercepted with PEG regularly in the multiblock copolymer. Such reduction was attributed to the combined effects of steric hindrance and the hydrophilicity of the PEG block.[7] In natural elastin, the VPGVG-rich, elastic domains are capable of undergoing coacervation, creating a highly hydrophobic microenvironment that is conducive to helix formation in the crosslinking domains. Consequently, the lysine residues are pre-registered on the same face of the helices, facilitating subsequent zero-length covalent crosslinking by lysyl oxidase.[8,9] In

our original design, PEG was used in place of the hydrophobic segments because of its conformational flexibility and its widespread use as a biomaterial substrate, although it lacks the hydrophobicity and the inverse temperature responsiveness of the VPGVG-rich domains in natural elastin.

In this study, PEG was replaced with an amphiphilic, ABA-block copolymer consisting of PEG end blocks and a poly(propylene oxide) (PPO) center block. Commonly known as Pluronics[®], this type of copolymers self-assembles into micelles with a water-incompatible compartment that is segregated from the aqueous exterior by the PEO shell.[10] Rather than employing step growth polymerization for the construction of peptide/PEG alternating copolymers prior to the initiation of the gelation reaction,[7] a more straight forward strategy was adopted for the synthesis of hybrid, hierarchical hydrogels through the covalent coupling between the self-assembled peptide nanofibrils and the block copolymer micelles. While the association characteristics of Pluronics are well understood, little is known about the assembly properties of the AK2 peptide. A fundamental question is how the alteration of the environmental parameters, such as the peptide concentration and the hydrophobicity of its surrounding media, as well as the solution temperature and pH, affect the conformation and the assembly properties of the AK2 peptide; and more importantly, under what conditions, the fibril assembly can be induced. The utility of the biological building blocks for translating information from the monomer level to materials with pre-determined organization, combined with the diverse nanostructures afforded by synthetic copolymers, should afford significant tunability of the structure and properties of the peptide/polymer hybrid hydrogels. [1]

Herein, we report conformation and assembly characteristics of the AK2 peptide under various environmental conditions. The peptide conformation was analyzed by circular dichroism (CD), the assembly potential was interrogated by attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR), small angle neutron scattering (SANS) and dynamic light scattering (DLS). The morphology of the assembled structures was visualized by atomic force microscopy (AFM) and transmission electron microscopy (TEM). Finally, peptide/F-127 hybrid hydrogels were synthesized via a Michael-type addition reaction between the peptide and vinyl sulfone-modified Pluronic[®] polymer. The viscoelastic properties of the hybrid hydrogels were evaluated using a custom-built, torsional wave apparatus (TWA) at frequencies close to those of human phonation.

Experimental Section

Materials

Appropriately side-chain-protected Fmoc-amino acids, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT), and Rink Amide MBHA resin were purchased from Novabiochem (EMD Biosciences, San Diego, CA). Pluronic[®] F127 ($M_w = 12,600$ g/mol) was a gift from BASF Polymers (Florham Park, NJ). Divinyl sulfone (DVS), sodium hydride (NaH), tri-isopropylsilane (TIS), trifluoroacetic acid (TFA), piperidine and all organic solvents were purchased from Sigma-Aldrich (St. Louis, MO). Deuterium oxide (D_2O) and deuterated dimethylsulfoxide ($DMSO-d_6$) were purchased from Cambridge Isotopes (Andover, MA).

Chemical modification of F127 with DVS (DVS-F127)

The terminal hydroxyl groups of Pluronic[®] F127 were modified with DVS (Figure 1) following a previously reported procedure with minor revisions.[11] Prior to the reaction, F127 (4.0 g) was azeotropically dried in 50 mL of toluene. To a round-bottom flask containing F127 in toluene was added NaH (5 molar excess relative to the OH groups) and DVS (15 molar excess). The reaction mixture was stirred in the dark at room temperature

under nitrogen for 5 days. The solution was subsequently filtered, washed with toluene, and concentrated by rotary evaporation. The solution was precipitated into cold diethyl ether three times and a white solid was collected by centrifugation. The yield was 37% and the degree of end group derivatization of 81% was confirmed by ^1H NMR. ^1H NMR (CDCl_3): 1.1 ppm (m, PPO CH_3), 3.4 ppm (m, PPG CH), 3.5 ppm (m, PPO CH_2), 3.6 ppm (m, PEO CH_2), 6.1 and 6.4 ppm (d, 2H, $\text{CH}_2=\text{CHSO}_2$), 6.8 ppm (m, 1H, $\text{CH}_2=\text{CHSO}_2$).

Synthesis of AK2 peptide

The peptide with a sequence of $\text{Ac}-(\text{AKA}_3\text{KA})_2\text{-NH}_2$ (AK2, Figure 1), where A = Fmoc-L-alanine and K = Fmoc-L-lysine (Boc), was prepared on Rink Amide MBHA resin using standard Fmoc solid-phase peptide protocols on a PS3 solid-phase peptide synthesizer (Protein Technologies, Inc., Tucson, AZ). The N-terminus was acetylated by capping with acetic anhydride. The peptide was cleaved from the resin using TFA/water/TIS (95:2.5:2.5, v/v), precipitated in cold diethyl ether two times, and collected by centrifugation. The crude peptide was purified via reverse-phase chromatography on a Delta 600 high-performance liquid chromatography (Waters Delta 600, Waters Corporation, Milford, MA) equipped with a preparative Symmetry 300 C18 column (Waters; 5 μm particle size, 3.9×150 mm). The purity of the peptide was 90%, as determined by HPLC. ESI mass spectrometry confirmed the mass of the peptide as (m/z) 1282.5 [$(\text{M}+\text{H})^+$, calculated 1282.6], 1304.6 [$(\text{M}+\text{Na})^+$, calculated 1304.6].

Compositional characterization

^1H NMR spectra were acquired on a Bruker AV400 NMR spectrometer (400 MHz) under standard quantitative conditions and were analyzed with Bruker Topspin software. All chemical shift values were calibrated using solvent peaks (from proton impurities, 4.8 ppm for D_2O , 7.24 ppm for CDCl_3). For ESI-MS analyses, samples were dissolved in methanol at a concentration of 0.1–0.2 mg/mL and analyzed in positive ion mode on a Thermo Finnegan LCQ Advantage mass spectrometer with a Surveyor MS pump.

CD spectroscopy

The CD spectra were collected on a Jasco J-810 spectropolarimeter (Jasco Inc, Easton, MD) equipped with a Jasco PTC-424S temperature controller. Background scans of phosphate-buffered saline (PBS, pH 12) were recorded and subtracted from the sample scans automatically using the Spectra Manager software. Samples were equilibrated at the desired temperature for 30 min prior to data collection. Equilibration was indicated by the absence of further changes in the CD signal at longer equilibration times. Samples were prepared at a peptide concentration of 0.5 mg/mL and the solution pH was adjusted using NaOH (1 N in DI H_2O). The F127 polymer concentration was maintained at 10 wt%. All CD spectra were recorded in a quartz cuvette with 1 mm path length at wavelengths varying from 200 to 260 nm. CD melting curves were acquired at 5 to 85 $^\circ\text{C}$ with a 5 $^\circ\text{C}$ increment. The molar ellipticity, $[\theta]_{\text{MRW}}$ ($\text{deg cm}^2 \text{dmol}^{-1}$), was calculated using the molecular weight of the peptide and cell path length. [12]

ATR FT-IR measurements

ATR FT-IR spectra were collected on a Nexus 670 FT-IR, (Thermo Nicolet, Waltham, MA). Prior to the measurements, a predetermined amount of peptide was dissolved in PBS/ D_2O at a concentration of 10 mg/mL and peptide assembly was initiated by adjusting the solution pH to ~ 12 using NaOH (1 N in D_2O). For all measurements, the peptide solution was dropped on the silicon ATR crystal after purging with nitrogen. Spectra were taken at a resolution of 2 cm^{-1} and an average of 512 scans was reported. A background of PBS/ D_2O solution was subtracted.

Small angle neutron scattering (SANS)

SANS experiments were performed on a 30 m SANS instrument (NG7) at the National Center for Neutron Research (NCNR) at National Institute of Standards and Technology (NIST). [13] The neutrons were monochromated to a wavelength of 6 Å with a wavelength spread $\Delta\lambda/\lambda = 0.12$. The three adopted sample-to-detector distances were 1, 4 and 15 m, covering a scattering vector range of $0.003 \text{ Å}^{-1} < q < 0.4 \text{ Å}^{-1}$ [$q = (4\pi/\lambda)\sin(\theta/2)$]. For sample preparation, 20 mg of AK2 peptide was dissolved in 500 µL deuterated PBS, forming a 4 wt% peptide stock solution at pH 7.4. Then another 500 µL deuterated PBS of pH 14 was added to the stock solution, leading to a 20 mg/mL peptide solution at pH of ~13. After mixing, the sample was incubated overnight at room temperature in titanium cells before scattering was performed. SANS data were reduced and analyzed according to published protocols. [14]

Dynamic light scattering (DLS)

[15] DLS measurements were conducted with a Malvern (Westborough, MA) Zetasizer Nano ZS equipped with 4 mW He-Ne, 633 nm laser at an angle of 173°. Separate peptide solutions were freshly prepared at a concentration of 1 mg/mL at pH 7.4 and 12, respectively. All peptide solutions were filtered (0.22 µm) into a low-volume quartz cuvette and the samples were tested at 25 °C or 60 °C. The high temperature samples were maintained at 60 °C in a water bath after removal from the instrument. The count rate was recorded for each sample as the average of 12 measurements per run, and the data reported is the average of 3 runs.

Atomic force microscopy (AFM)

The AK2 peptide was dissolved in DI H₂O at a concentration of 1 mg/mL. After the pH was adjusted to 12, the solution was deposited onto freshly cleaved mica and the samples were allowed to air-dry. Analyses were performed using a Veeco Dimension V atomic force microscope (Veeco Instruments, California, USA) in tapping mode using an aluminum-coated silicon tip with a cantilever of spring constant 40 Nm⁻¹ (tip radius < 10 nm). The height and diameter of the peptide fibrils were measured using NanoScope AFM image analysis software (Veeco Instruments, version 6).

Hydrogel synthesis

F127 was added to ice-cold PBS (100 µL) at pH 12 at a concentration of 150 mg/mL. The mixture was vigorously vortexed to ensure complete dissolution. The peptide was subsequently added to a final concentration of 20 mg/mL and the solution was vortexed again. A cylindrical gel disk was obtained after the polymer/peptide mixture was incubated at 60 °C for 48 h inside a 1 cc syringe.

Transmission electron microscopy (TEM)

TEM was employed to assess the morphologies of the self-assembled peptide and the peptide/F127 hybrid. The peptide samples were prepared following the procedure described above for AFM. Peptide/F127 hybrids were generated by incubating peptide (1 mg/mL) with DVS-F127 (10 mg/mL) at pH 12 at 60 °C for 2 days. The above solutions were separately applied onto a carbon thin film supported on a 300 mesh copper grid. The salt deposits were removed by gentle washing. Excess solvent was removed by blotting with solvent-absorbing filter paper after 1 min. For negative staining, aqueous uranyl acetate (15 µL, 1 wt% in water) solution was pipetted onto the samples and was allowed to equilibrate for 1 min before water was removed with filter paper. The stained samples were examined using a JEOL 2000-FX TEM at 200 kV accelerating voltage on a CCD camera.

Torsional wave analysis (TWA)

Hybrid hydrogels were prepared as described above. Small cylindrical samples were cut from the gel disks and the dry weight was measured. Samples were subsequently soaked in PBS at pH 7.4 for 24 h and the wet weight was obtained. The equilibrium swelling ratio was calculated as the wet weight divided by the dry weight from an average of 3 samples. The hydrated samples, sandwiched between two hexagonal plates, were then placed in an environmental chamber at ~35 °C and ~95% relative humidity for mechanical testing using a custom-built torsional wave apparatus (TWA). After the top plate was positioned and the laser was aligned, digital pictures were taken for data reduction purposes. A frequency sweep over a specified range was conducted with a galvanometer. At each frequency, measured rotations of the top and bottom plates were used to determine the ratio of the amplitudes of the rotations of the two plates. Comparisons of the frequency dependence of this ratio with that predicted for torsional waves in a linear viscoelastic material allows the storage modulus and the loss angle, in shear, to be calculated by a best-fit procedure.[16–19] Three separate samples were tested under the same conditions, each analyzed by three consecutive measurements. The experimental data reported are an average of three separate tests.

Results and Discussion

While alanine-rich peptides generally exhibit helical propensity, their secondary structures and assembly properties are strongly dependent on environmental factors, such as temperature, pH, concentration and the polarity of the solvent. The previously synthesized PEG/AK2 multiblock copolymers do not permit facile alteration of the peptide structures. On the other hand, the introduction of amphiphilic block copolymers to the peptide solution effectively altered the hydrophobic microenvironment surrounding the peptide without generating unnecessary spatial restrictions to the peptide, allowing the peptide assembly to be systematically investigated. The hierarchical structures assembled from peptide and synthetic polymer can be locked in by covalent coupling between the constituent building blocks.

Peptide conformation

Due to the electrostatic repulsion between the charged lysine residues, the AK2 peptide adopts a random coil conformation in aqueous solution at neutral pH.[7] Deprotonation of the lysine amines at pH 12 resulted in the development of partial α -helical conformation as evidenced by the characteristic minima at 208 and 222 nm in the CD spectrum (Figure 2A). The fractional helicity was calculated by relating the average mean residue ellipticity of the peptide at 222 nm, $[\theta]_{222}$, to that of an idealized 100% helical peptide.[12] A 4.5% helicity implies the presence of a significant amount of unstructured peptide population under the conditions employed. The addition of F127 at low concentrations of 1×10^{-5} wt% up to 1 wt% had no effect on the peptide conformation (data not shown). At these concentrations under ambient temperature, the F127 polymer exists as unimers. [20] However, the peptide underwent a moderate increase in helicity (fractional helicity of 8.2%) when the F127 concentration was increased to 10 wt% (Figure 2A), a concentration at which F127 is known to assemble into micelles.[10,20]

Figure 2B summarizes the results from thermal melting experiments of the AK2 peptide with or without F127 micelles. In both cases, the values of $[\theta]_{222}$ decrease as the temperature is elevated from 5 to 85 °C, demonstrating the loss of α -helical structure. The loss of helical structure at high temperature can be attributed to the helix-coil transition due to the disruption of intrahelical H-bonding. The peptide helicity was significantly higher in the presence of F127 compared to the peptide alone at all temperatures up to 65 °C. Due to

the lack of a low-temperature baseline in the thermal unfolding experiments, the melting temperature of the helix could not be estimated from commonly employed two state models, thus the temperature at which the helix-to-nonhelix conformation is complete was used as an estimate of helix stability in these experiments. The melting of the peptide with or without F127 was estimated to be complete at 30 °C and 65 °C, respectively (Figure 2B). Collectively, our CD results suggest that at a relatively low peptide concentration (~0.5 mg/mL), the F127 micelles are capable of not only increasing the peptide helicity but also stabilizing it against thermal denaturation.

The stabilization effect provided by F127 micelles is not surprising since peptide conformation is sensitive to the composition and the polarity of the medium in which it is dispersed.[21–23] Two potential scenarios exist for the peptide/F127 pair: the peptide could potentially complex with the block copolymer micelles externally or be sequestered in the hydrophobic core. If associated with the micelle corona, the peptide has to arrange itself in the most favorable conformation to interact with the PEO shell through H-bonding. Computational studies[24] indicated that the peptide/micelle association is extremely intermittent, frequently breaking and reforming due to the competition from the surrounding water molecules. Therefore, external micelle/peptide association alone would not lead to the observed increase in peptide helicity and thermal stability. Alternatively, the Pluronic micelles could sequester the soluble peptide into the hydrophobic interior. When fully extended, the (AKA₃KA)₂ peptide is approximately 65 Å long; if 100% helical, the peptide would be approximately 20 Å long. Thus, given that F127 micelles exhibit a hydrodynamic radius of ~110 Å at 25 °C at a polymer concentration of 10 wt%, [25,26] the F127 micelles could accommodate the peptide molecules without causing thermodynamic instability of the micelles. Although the helical conformation could be stabilized through intramolecular H-bonds within the micelle core,[21] such effect alone would not be particularly strong considering the relative “polar” nature of the Pluronic core.[27] Collectively, we believe that the micelle/peptide interaction, whether through the hydrophilic corona or the hydrophobic core, increased the helical content of the peptide, as well as their thermal stability.

The secondary structure of the AK2 peptide at a higher concentration (10 mg/mL) at pH 12 in the absence of F127 was investigated by ATR FTIR. Figure 3 shows the conformationally sensitive amide I absorption band at approximately 1600–1700 cm⁻¹. This band is largely due to stretching vibrations of the carbonyl groups in the peptide amide bonds and is sensitive to various structural determinants.[28–30] In D₂O solution, the amide I band is broadened and the characteristic peaks for α -helix, β -sheet and random coils shift to lower wavenumbers due to hydrogen bonding interactions between D₂O and the peptide.[31,32] Thus, caution must be exercised in comparing the absolute wavenumber values between solid state and solution FTIR spectra. As illustrated in Figure 3, the amide I band of the AK2 peptide in D₂O can be deconvoluted into four components. The components centered near 1630 and 1650 cm⁻¹ are attributable to the amide I absorption band of α -helical and random coil domains of the peptide, respectively.[28,31,33] The components centered near 1620 cm⁻¹ (strong) and 1682 (weak) cm⁻¹ can be attributed to $\nu_{\perp}$ and $\nu_{\parallel}$ modes of the anti-parallel β -sheet conformation, and the absorption band at 1671 cm⁻¹ may result from the carbonyl vibration of the residual TFA in the peptide.[34]

Our ATR FTIR result shows that a significant β -sheet structure was developed at a peptide concentration of 10 mg/mL under alkaline conditions. Such conformation, however, is absent from the peptide solutions at low concentrations used for the CD analysis. It is worth mentioning that the CD spectra were acquired on freshly prepared peptide solutions whereas the ATR FTIR results were obtained from aged (1 h) peptide solutions at a higher concentration. While alanine-rich peptides exhibit a strong propensity for α -helical structure, the formation of β -sheet is observed under appropriate conditions. In general, peptide

aggregation is sensitive to peptide concentration, temperature, solution pH and ionic strength. Increasing concentration has been shown to result in a transition from α -helix to β -sheet aggregates.[35] In addition, short peptides with the sequence (AAKA)₄ have been demonstrated to have more β -strand structure than peptides composed solely of alanine residues.[36] While the (AAKA)₄ peptide had substantial helical fraction at low concentrations, hydrogels formed at higher peptide concentration in the presence of counter ions. In our case, the lysine amines were mostly deprotonated and were regularly spaced by short stretches of alanine sequences. The hydrophobic interaction between the alanine side chains could give rise to multistrand sheet layers that are further reinforced by the hydrogen bonding between the amide bonds from the adjacent peptide chains.

Fibril assembly and stability

The presence of β -sheet conformation motivated us to investigate whether the peptide can be driven into fibril assemblies under basic conditions. AFM analysis (Figure 4) of the AK2 peptide deposited on mica revealed the presence of fibrils of varying sizes. Fibrils of ~2 nm in height and ~14 nm in width were detected, although larger aggregates were also present. The local structure of peptide fibrils was assessed by SANS. In Figure 5, scattering intensity was plotted against the scattering vector q for peptide fibrils formed in deuterated solution after overnight incubation at ambient temperature. The result of curve fitting with the summed model is also included. The summed model is the linear combination of the parallelepiped form factor and the power-law model. [14] The parallelepiped form factor describes scattering from a rectangular solid with A as height, B as width and C as length, which takes the form of

$$P(q) = \frac{scale}{\sqrt{V_p}} \int_0^1 \varphi_Q(\mu \sqrt{1-\sigma^2}, a) [S(\frac{\mu c \sigma}{2})]^2 d\sigma$$

and $\varphi_Q(\mu, a) = \int_0^1 [S(\frac{\mu}{2} \cos(\frac{\pi}{2} u)) S(\frac{\mu}{2} \sin(\frac{\pi}{2} u))]^2 du,$

in which $S(x) = \frac{\sin x}{x}, a = \frac{A}{B}, c = \frac{C}{B} \mu = qB.$ (1)

Here it was used to quantify fibril morphology which corresponds to mid- q and high q scattering. The power-law model has the function of

$$I(q) = I_0 q^{-m}, \quad (2)$$

which was applied to describe scattering in the low q regime. In Figure 5B, it was justified that the resulting summed model fit curve in Figure 5A was the linear combination of the power law model and parallelepiped form factor, as in the form of

$$I_{sum} = n_1 P(q) + n_2 I(q) + \text{incoherent background}. \quad (3)$$

The resulting fit agreed well with scattering data in Figure 5A, indicating that the geometry of self-assembled peptide fibrils can be approximated as a rectangular cuboid. Fitting results demonstrate that the fibril height is 3.5 nm and width is 12.7 nm. Fibrils of such dimensions have been detected by AFM. A close inspection of the AFM profiles reveals the presence of fibrils of varying thickness and diameter (Figure 4). We note that because of the convolution effect of the AFM tip with the surface nanoscale structures, the fibrils may be narrower than they appear in the AFM images. In addition, the solvent evaporation necessary for the preparation of the AFM samples may have facilitated the formation of compact structures as

well as further assembly due to interaction between hydrophobic alanine residues. In the low q regime, the power-law exponent, m , was approximated to be 3.5, which can be attributed to either inter-fibril scattering or higher-order structures (>100 nm). For further clarification, future scattering experiments will be conducted in a broader q -range.

The temperature-dependent fibril assembly was monitored at pH 7.4 and 12. At 25°C, a low level of scattering was detected at both neutral and basic pH, indicative of the absence of fibril formation. After the initial count rate was recorded, the solutions were immediately heated to and maintained at 60 °C. The DLS kinetic curves (Figure 6) revealed minimal peptide assembly at pH 7.4 at 60 °C over a period of 20 h. At pH 12, on the other hand, the count rate increased immediately upon heating and the assembly process continued for about 6 h before reaching equilibrium. The increase in the count rate may reflect the increase in the number of nanoassemblies or the length of nanoassemblies.[15] The large variation in the count rate after the initiation of the assembly at 60 °C under basic conditions reflects the heterogeneity of the assembled structures. In general, self-assembly is initiated by a nucleation and growth process that leads to the thickening or elongation of the fibril or branching into clusters.[37] Interestingly, at ambient temperature, the assembly took approximately 6 h to initiate, reaching a steady level after 24 h of incubation (data not shown). Our results confirmed the faster assembly kinetics for the peptide at higher temperature and that the deprotonation of the lysine amines is a prerequisite for fibril assembly.

Alanine-rich peptides are known to adopt α -helical structures and can undergo further aggregation due to the hydrophobic character of the alanine residues.[12,38] Alanine-rich peptide or protein materials have been shown to assemble into fibril-like structures.[39,40] Similar short (16-residue) alanine-based peptides with evenly spaced lysine residues have been shown to form stable α -helices at concentrations up to 80 μ M, and the helical content increased upon raising the pH to 12.[38] Peptides with the sequence Ac-(AAKA) $_n$ -NH $_2$ ($n = 3$ or 4) display polyproline II (PPII) or α -helical conformations, respectively, at low concentrations similar to those employed here, but undergo transitions to antiparallel β -sheets when the concentration is increased to about 10 mM, resulting in hydrogel formation. [36]

Although the underlying mechanism for the fibril assembly is beyond the scope of the current investigation, we speculate that the AK2 peptide aggregates through similar transformation processes as the Ac-(AAKA) $_4$ -NH $_2$ peptide, which differs slightly in its peptide composition. Under the experimental conditions involved, it is possible that the AK2 peptide aggregates via an antiparallel arrangement of the β -sheets through the hydrophobic interactions between the alanine residues laterally stabilized by hydrogen-bonding between lysine and peptide carbonyls of adjacent polypeptide chains. Recall that although the ATR FTIR spectrum revealed the presence of a predominantly β -sheet conformation, other conformers, including α -helical and random coil, do exist. Thus, the fibrillar aggregates may contain a substantial amount of disordered structures.

Hydrogel formation

We are interested in synthesizing peptide/polymer hybrid hydrogels via covalent interlocking of the nanofibrils and spherical micelles. F127 was end-functionalized with vinyl sulfone to allow for its covalent coupling with the peptide through the lysine amines via a Michael-type addition reaction. The amines in lysine exhibited high reactivity towards the vinyl sulfone at basic conditions in which the lysine sidechain was deprotonated.[41–43] Heating (60 °C) not only accelerated the reaction but also enhanced the fibril assembly (see above), resulting in the formation of stable, viscoelastic hydrogels.

To facilitate TEM observation, ultrathin films were deposited on the copper grid from dilute solutions of peptide alone or peptide/F-127 mixture after heating at 60 °C for two days. Under the gelation conditions, the F127 polymer readily assembles into spherical, micellar structures with diameters of ~10.8 nm and aggregation numbers of about 108.[44] Transmission electron micrographs of the peptide alone (Figure 7A) revealed the presence of fragmented fibrils, reminiscent of the structures observed in AFM. No particulate matter was found in the peptide samples, suggesting that the peptide alone does not aggregate to form particles or beads under the experimental conditions employed. Notably, both nanofibrils and micellar particles are present in the TEM image of the peptide/F127 hybrid (Figure 7B), confirming that the crosslinking condition employed is conducive to fibril assembly and does not dissociate the micelle structures. A close inspection of the electron micrograph of the peptide/F127 hybrid shows that the majority of the spherical particles are associated with the fibrils rather than being randomly dispersed in the interstitial space, as would be consistent with the partial covalent coupling between them

The experimental evidence collected so far allows us to gain a global understanding of peptide assembly in the presence of F127. The freshly prepared peptide at low concentrations can be readily solubilized by the F127 micelles. Under such conditions, the peptide was mostly disorganized with a low α -helical content. At a peptide concentration of 10 mg/mL, a strong β -sheet component was developed. Fibril formation via the stacking of the β -sheet motifs under basic conditions occurred readily since lysine amines are deprotonated. At the elevated temperature, the peptide preferentially assembled into β -sheet rich structures that are beyond the sequestration capacity of the micelles. Hydrophobically driven assemblies, once initiated, are difficult to reverse.[45] The coupling reaction between the lysine amines on the surfaces of the nanofibrils and the vinyl groups on the micelle corona then essentially locked in and stabilized the respective assembled structures.

To assess the applicability of the hybrid hydrogels for vocal fold tissue engineering, the viscoelasticity of the hybrid hydrogels was evaluated using a custom-designed torsional wave apparatus (TWA) at human phonation frequencies.[17,18] The as-synthesized hydrogel disks were allowed to swell at pH 7.4 for 24 h prior to the measurement and the estimated swelling ratio was 16.8 ± 3.4 . The presence of a distinct resonance peak (Figure 8) with a peak amplification factor well above 1 suggests that the hydrogels are stably crosslinked. The insert in Figure 8 shows that the sample was properly mounted and the swollen gel supported the weight of the top plate. In torsional wave analysis, the location of the resonance peak reflects the stiffness of the hydrogels and the height of the peak corresponds to the loss angle of the gels. Good agreement between the predicted linear viscoelastic response and experimental data (Figure 8) was observed for all samples tested. Two important parameters that can be deduced from the TWA experiments are the elastic modulus (G') and the loss tangent ($\tan \delta$: ratio between the loss modulus, G'' , and the elastic modulus, G'). The average storage modulus for the hybrid hydrogels was measured as $16,981 \pm 3,775$ Pa with a $\tan \delta$ was approximately 0.19 ± 0.03 . Perfectly elastic gels exhibit $\tan \delta$ values close to zero. Physical entanglement of the peptide fibrils may have contributed to the overall mechanical integrity of the hybrid hydrogels.

The resultant hydrogels are hybrid and contain hierarchically assembled structures. In particular, the peptide fibrils could provide physical support for cell attachment, whereas the micellar particles could be used as release depots for therapeutic molecules. The combination of fibrous objects with spherical entities allows for the cellular functions to be mediated synergistically. The major drawback of the current hydrogel system is the incompatibility of the crosslinking process with mammalian cells. This limitation can be overcome by slight modification of the peptide sequence. For example, cysteine-bearing oligopeptide with a higher alanine/lysine ratio is likely to permit the peptide assembly[46]

and the subsequent covalent crosslinking [47] to occur under physiological conditions. Given the potential of this type of hybrid, hierarchical hydrogels for tissue engineering applications, further in-depth studies employing peptides with various compositions and assembly potential are warranted.

Conclusion

The conformational behaviors and the assembly characteristics of an alanine-rich, lysine-containing peptide [(AKA₃KA)₂, AK2] that is prevalent in the crosslinking regions of natural elastin were systematically investigated employing various spectroscopic and scattering techniques under different environmental conditions. We show that when associated with F127 micelles, the peptide exhibited increased fractional helicity and improved thermal stability as compared to the free peptide. When the solution concentration was increased, the free peptide developed substantial β -sheet structure when the lysine amines were deprotonated. When incubated at 60 °C under basic conditions, the peptide rapidly assembled into nanofibers. Once the aggregation process was initiated, the fibrillar objects could not be solubilized by the micelles. The self-assembled nanoscale structures were covalently interlocked via a Michael-type addition reaction between the vinyl sulfone decorated F127 micelles and the lysine amines exposed at the surface of the nanofibers. The crosslinked hybrid hydrogels were viscoelastic, exhibiting an elastic modulus of approximately 17 kPa and a loss tangent of 0.2. Future investigations include slight modification of the peptide composition and the crosslinking chemistry so as to allow for the formation of hierarchically structured hybrid hydrogels under physiologically relevant conditions.

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Figure 1. Chemical structure of alanine-rich, lysine-containing peptide with a sequence of Ac-(AKA₃KA)₂-NH₂ (AK2, top) and vinyl sulfone-terminated Pluronic[®] F127 (DVS-F127, bottom, m=100, n=65).

Figure 2.

Characterization of peptide conformation by circular dichroism (CD). (A): CD spectra of AK2 with (filled circle) or without (open circle) F127. (B): Change of molar ellipticity at 222 nm as a function of temperature for the AK2 peptide with (filled circle) or without (open circle) F127.

Figure 3.

ATR FT-IR spectrum of amide I region for AK2 in D₂O. Peptide assembly was induced by the addition of 1 N NaOH to a peptide/D₂O solution at a concentration of 10 mg/mL. The broad peak was deconvoluted into four components. 1620 and 1682 cm⁻¹ anti-parallel β sheet; 1630 cm⁻¹: α -helical; 1650 cm⁻¹: random coil; 1671 cm⁻¹: carbonyl peak from TFA.

Figure 4. AFM height image of AK2 assembled from 1 mg/mL solution at pH 12. The height section profile shows the presence of fibrils with a width of ~14 nm and a height of ~2 nm.

Figure 5.

(A): SANS scattering intensity as a function of scattering vector q for 2 wt% AK2 peptide in deuterated PBS buffer solution at pH ~13. The solid line is summed model fit for peptide solution. (B): the resulting summed model fit (solid line) shown in A was decomposed into corresponding power-law model fit (circle) and parallelepiped form factor fit (square).

Figure 6. Temperature-dependent assembly kinetics of AK2 at pH 7.4 (open circles) and 12 (filled circles). Immediately after the average counts were recorded at 25 °C at time 0, samples were heated to 60 °C and the assembly was characterized by dynamic light scattering (DLS). Peptide concentration was maintained to be 1 mg/mL at both pH conditions.

Figure 7. Transmission electron micrographs of peptide fibrils (A) and peptide/F127 hybrid (B). Samples were negatively stained by uranyl acetate.

Figure 8.

Frequency dependent amplification factor for a representative hybrid hydrogel. The insert shows the sample sandwiched between the top and the bottom plates. Open circles were experimental results and the solid line was the model prediction. Three consecutive measurements were conducted on the same sample and the experimental data reported are an average of three tests.