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Control of self-assembling oligopeptide matrix formation through systematic variation of amino acid sequence

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Abstract

In order to elucidate design principles for biocompatible materials that can be created by in situ transformation from self-assembling oligopeptides, we investigate a class of oligopeptides that can self-assemble in salt solutions to form three-dimensional matrices. This class of peptides possesses a repeated sequence of amino acid residues with the type: hydrophobic/negatively-charged/hydrophobic/positively-charged. We systematically vary three chief aspects of this sequence type: (1) the hydrophobic side chains; (2) the charged side-chains; and (3) the number of repeats. Employing a rheometric assay to judge matrix formation, we determine the critical concentration of NaCl salt solution required to drive transformation from viscous state to gel state. We find that increasing side-chain hydrophobicity decreases the critical salt concentration in accord with our previous validation of DLVO theory for explaining this self-assembly phenomenon Caplan et al. (Biomacromolecules 1 (2000) 627). Further, we find that increasing the number of repeats yields a biphasic dependence—first decreasing, then increasing, the critical salt concentration. We believe that this result is likely due to an unequal competition between a greater hydrophobic (favorable) effect and a greater entropic (unfavorable) effect as the peptide length is increased. Finally, we find that we can use this understanding to rationally alter the charged side-chains to create a self-assembling oligopeptide sequence that at pH 7 remains viscous in the absence of salt but gels in the presence of physiological salt concentrations, a highly useful property for technological applications. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Oligopeptide; Biomaterial; Self-assembly; Amino acid sequence

1. Introduction

An especially desirable feature of a biomaterial useful for applications in drug delivery, wound healing, and tissue engineering is the capability for self-assembly into a three-dimensional matrix scaffold in situ under physiological conditions [1]. It is also desirable to have an oligopeptide material because they have potential for specific interactions with cell surface receptors such as integrin receptors and they will resorb into biocompatible degradation products [2]. The class of oligopeptides originally discovered by Zhang et al. [3] exhibits these properties, along with others such as favorable cell interactions [4,5]. The key characteristic of this family is that the sequence has alternating hydrophobic side chains and charged side chains [3]. In the case of one sequence of the family, named KFE12 (sequence shown in Table 1, structure shown in Fig. 1a(i)), the hydrophobic side chains are phenylalanine, and the charged side chains alternate between glutamic acid and lysine. Upon addition of a sufficient concentration of salt, these peptides form a network of filaments that behaves as an elastic gel [6]. Our aim is to produce a material that would undergo a transition from being soluble (forming a viscous solution) outside of the human body to a gel once it is injected into the human body. Oligopeptides with this property have been designed previously, but so far they require significant changes in temperature or

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pH to achieve the transition [7]; these changes may be harmful to cells and some therapeutic molecules. Through theoretical understanding concerning fundamental self-assembly principles, it is possible to alter the underlying amino acid sequence to obtain a material suitable for specific application—not only in terms of self-assembly conditions but also of mechanical and cell-interaction properties.

Table 1
Amino acid sequences studied

Name	Repeat	Sequence
KFE12	$(FKFE)_3$	FKFEFKFEFKFE
KIE12	$(IKIE)_3$	IKIEIKIEIKIE
KVE12	$(VKVE)_3$	VKVEVKVEVKVE
KFE8	$(FKFE)_2$	FKFEFKFE
KFE16	$(FKFE)_4$	FKFEFKFEFKFEFKFE
KFQ12	$(FKFQ)_3$	FKFQFKFQFKFQ

Our previous work has elucidated the reason why addition of salt drives hydrogel formation [8]. We demonstrated that self-assembly of KFE12 is regulated by the superposition of van der Waals attraction and electrical double-layer repulsion as quantified by Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. Qualitatively this theory indicates that electrostatic repulsion between the charged side chains creates a kinetic barrier to self-assembly. If the amplitude of the barrier is decreased to near kT, the oligopeptides will approach to within several angstroms and become non-covalently

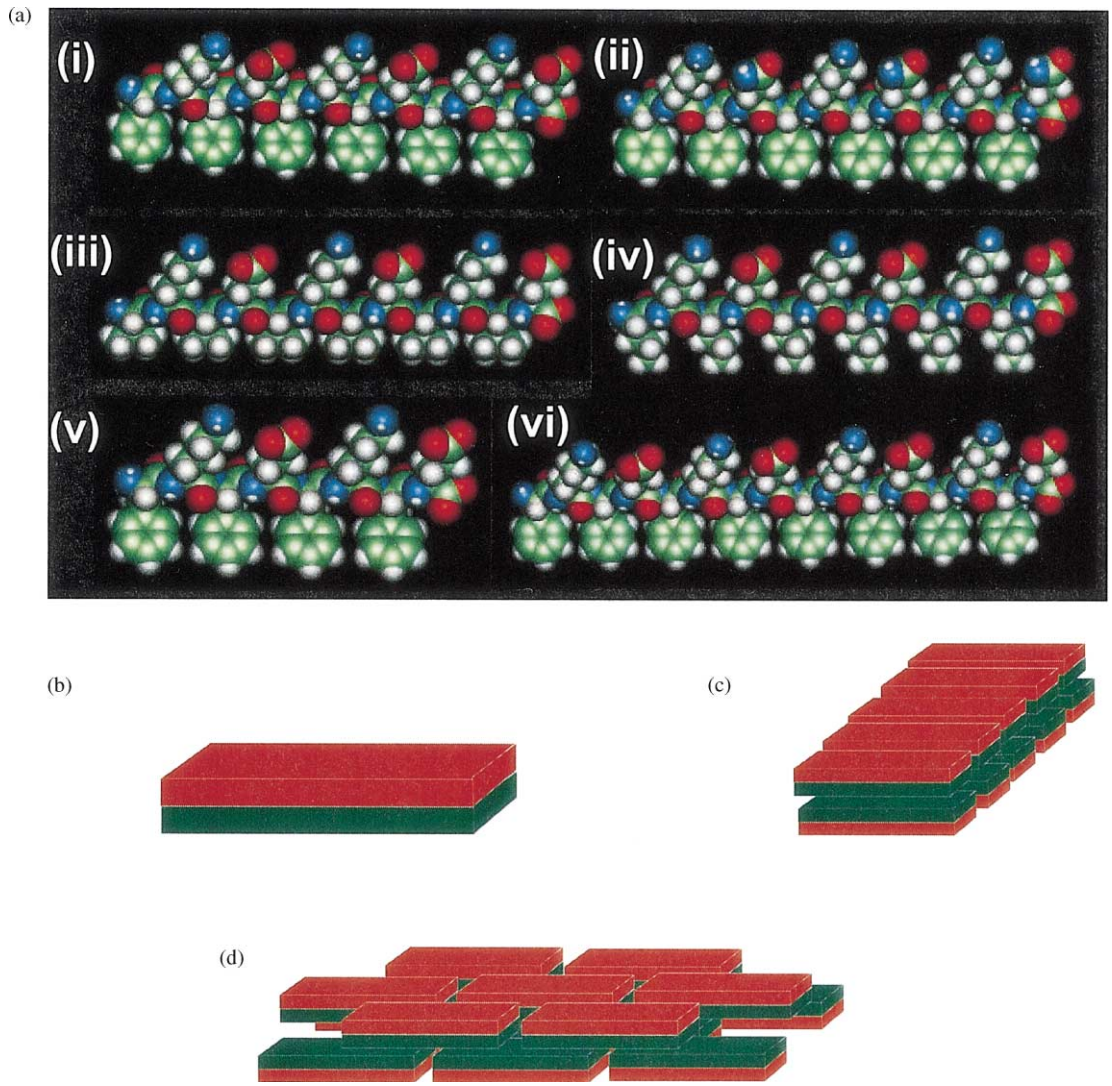

Fig. 1. β-sheet structure formed by EAK family repeats: (a) molecular models of the six sequences studied (carbon = green, nitrogen = blue, oxygen = red, hydrogen = white): (i) KFE12, (ii) KFQ12, (iii) KVE12, (iv) KIE12, (v) KFE8, (vi) KFE16; (b) schematic representation showing hydrophilic side chains as a red block and hydrophobic side chains as a green block; (c) hypothetical structure with unstaggered arrangement of monomers forming filament; (d) hypothetical structure with staggered arrangement of monomers forming filament. Previous circular dichroism studies have shown that this family oligopeptides forms β-sheets [3].

bound to each other by van der Waals forces and the hydrophobic effect [9]. Quantitatively, DLVO theory predicts that addition of salts should decrease the Debye length of the solvent by screening charged ions from each other thus decreasing the amplitude of the kinetic barrier [10]. Once the salt concentration necessary to decrease the kinetic barrier to near kT is known for one sequence, we can predict the salt concentrations necessary for other sequence based on how the variations in sequence affect the other major parameters in the DLVO calculation. These include the Hamaker constant that represents the attraction due to van der Waals forces, the geometry of the surfaces that are self-assembling, and the electrical potential of the surfaces [11].

In this study, we attempt to understand how to control the conditions under which these oligopeptides self-assemble by variation of oligopeptide sequence. Each oligopeptide consists of several repeats of a four amino acid sequence: hydrophobic–positively charged–hydrophobic–negatively charged. These oligopeptides are suited to this type of variation due to their short and repetitive nature. Their short sequence allows us to synthesize the molecules using solid-state synthesis, thus making it easier to synthesize large quantities of new sequences that we wish to study. Their repetitiveness allows us to vary systematically the three main characteristic attributes: the hydrophobic side chain structure, charged side chain structure, and the length of the molecule.

As shown in Fig. 1b, these oligopeptides have amphipathic character due to forming β-strands. In β-strands each side chain is rotated approximately 180° from its adjacent side chains so that, in the case of the alternating sequences used in this study, all of the hydrophobic side chains face one side of the molecule and all charged side chains face the other side [4,12]. Hence we can vary the hydrophobic nature of the molecule by varying the amino acids used to incorporate the hydrophobic side chains, for example by using valine or isoleucine instead of phenylalanine (Figs. 1a(iii) and (iv), respectively). Likewise, on the other side of the molecule, we can vary the number or character of the charged side chains by varying the amino acids used to incorporate the charged side chains. For example, we could use glutamines instead of glutamic acids in order to remove the negatively charged glutamic acid side chains (Fig. 1-a(ii)). Finally, we can vary the length of the molecules by choosing how many four amino acid repeats to include (Figs. 1a(v) and (vi)). The sequence variants studied are listed in Table 1. We are currently studying the structure into which these monomers assemble when they form the filaments that connect together to form the gel. Our hypothesis, not discussed further in this paper but included to clarify discussion, is that the hydrophobic side of the peptide amphiphiles orient toward the inside of the filament thus excluding water from the insides of the filaments. The oligopeptides could either orient perpendicular to the filament axis (Fig. 1c) or parallel to the filament axis (Fig. 1d), and in both cases the charged side chains must pack within several angstroms of each other in order for these filaments to form. Thus, we can see how the hydrophobic effect would actually drive filament formation but that the proximity of like charges could inhibit self-assembly at high Debye lengths.

Since we aim to create materials that can undergo an in situ transformation from a viscous solution to a gel, the property that we examine in this study is the concentration of salt necessary to drive gelation. Here we report that, through systematically varying each of the three attributes of the repeating sequence and measuring the effect, the salt concentration necessary to drive gelation can be controlled by sequence changes and can be understood using DLVO theory. We also show that, through understanding that salt causes gelation by screening electrostatic repulsion, we can rationally design an oligopeptide sequence that is viscous at pH $\sim$ 7 in the absence of salt but that gels in the presence of salt.

2. Materials and methods

2.1. Materials

All chemicals were used as received unless otherwise specified. Biosynthesis grade *N*,*N*-dimethyl formamide (DMF) and anhydrous ethyl ether were obtained from EM Science (Darmstadt, Germany). Piperidine 99%, methylmorpholine, and thioanisole 99% were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). TFA was ordered from Pierce (Rockford, IL). 1,2-ethanedithiol was supplied by ICN Biomedicals, Inc. (Aurora, OH). Acetic anhydride, dichloromethane, methyl alcohol, sodium hydroxide, and sodium chloride were obtained from Mallinckrodt Chemical (Paris, KY).

2.2. Oligopeptide synthesis

All oligopeptides were synthesized using Fmoc chemistry on a Protein Technologies PS-3 peptide synthesizer (Ranin Instrument Co., Inc., Woburn, MA) using approximately 0.25 mmol rink amide glutamic acid or glutamine resin (AnaSpec, Inc., San Jose, CA) and 1.0 mmol *t*-Boc protected amino acids with 1.0 mmol HBTU activator (AnaSpec, Inc.). Two 20 min deprotection steps in 20% piperidine in DMF were followed by activation of the next amino acid in 0.40 M *n*-methylmorpholine in DMF and a 1 h coupling step as in Wellings and Atherton [13]. The final step consisted of two deprotection steps followed by 20 min of reaction with an excess of acetic anhydride. After drying the resin

by washing with dichloromethane and methyl alcohol, cleavage from the resin and protecting groups was performed using a solution of 8.3 ml trifluoro-acetic acid (TFA), 270 μl 1,2-ethanedithiol, and 450 μl thioanisole for 3 h followed by three precipitations in nearly 50 ml of ethyl ether [13]. After reconstituting in roughly 20 ml of ultra-filtered water (Millipore, Bedford, MA) at 18.2 mΩ, the solution was freeze-dried for several days.

2.3. Rheometric assay for gelation

Roughly 3 mg oligopeptide powder was mixed with ultra-filtered water to bring the concentration to 1 wt%. Immediately after vortexing for about 2 min and sonicating until the oligopeptide was in solution (~20 min), a 200 μl aliquot was placed on the plate of a rheometer (model AR1000, TA Instruments, Inc., New Castle, DE). A 2 cm diameter, 4° stainless steel cone with truncation at 101 μm was lowered, after removal of excess solution, so that the tip was 101 μm above the plate, and a solvent trap was placed around the cone with vacuum grease applied to obtain a liquid tight seal. A test at applied torque of 10 μN m was performed between 50 and 2.0 rad/s at 25°C to provide a baseline. The cone was then stopped and, while maintaining zero velocity on the cone, ~30 ml of the appropriate bath solution was added through a port in the top of the solvent trap, and nearly 1 ml of vegetable oil was laid over the bath to prevent evaporation. After equilibrating for roughly 15 h at 25°C, the sample was tested over a range of frequencies from 10 to 1.0 rad/s at 2.0 μN m oscillatory torque.

These experiments were performed near pH 3 because residual TFA, from synthesis, causes the solution to be at low pH unless neutralized. Although it is possible to remove the TFA either by dialyzing against a different anion or by neutralization to pH ~7, we did not do so because (as will be shown in Fig. 6) all sequences except KFQ12 gel at pH ~7 in the absence of salt. If these oligopeptides were neutralized prior to addition of the salt bath, they would immediately assemble upon mixing in water, and vortexing would destroy filament–filament connections. Thus, unconnected filaments would be pipetted onto the rheometer plate and no further assembly or healing would occur upon addition of the salt bath. For the experiments shown in Fig. 6, the vegetable oil was removed with a micropipette, and then 10 ml of the bath was placed into a centrifuge tube. The pH of this liquid was measured using a pH probe (Orion Research, Inc., Boston, MA) calibrated between pH 4.01 and pH 7.00 using standard buffers (VWR Scientific Products, West Chester, PA).

3. Results & discussion

3.1. Variation of hydrophobic groups

Since this family of oligopeptides forms β-sheets, all of the hydrophobic residues face one side of the molecule [3]. By changing the character of the hydrophobic side chains, we can affect a change in the intermolecular forces with which this side of the molecule interacts with other oligopeptides. We have held the length of the molecule constant, kept the charged side chains the same, and only varied the hydrophobic side chains in the repeating unit. The three sequences of this series studied are: FKFEFK-FEFKFE (KFE12), IKIEIKIEIKIE (KIE12), and VKVEVKVEVKVE (KVE12)—see Figs. 1a(i, iii, iv) and Table 1. Thus, we varied the repeat $(FKFE)_3$ to $(IKIE)_3$ and $(VKVE)_3$. The different side chains impart different characteristics partly due to the size of the side chains and partly due to the interactions they have with solvent molecules, in this case water. One important interaction each side chain has with water is often referred to as "hydrophobicity" (literally "water fearing") because, being carbon chains and rings, they cannot form hydrogen bonds with water molecules so it is energetically unfavorable for water to solvate these side chains. Thus, by variation of the amino acid sequence, we are varying the hydrophobicity of one side of the oligopeptide molecule.

To study this, we carried out rheological analyses on these oligopeptide sequences varying the concentration of sodium chloride (NaCl) in the solvent trap surrounding the sample. Experimentally, we found KFE12 at 1 wt% in water (approximately 5.90 mM) to be a viscous solution at low NaCl concentrations (Fig. 2a); but a gel at high NaCl concentration (Fig. 2b). This sol–gel transition was examined using a rheometer that measures the complex modulus. For a viscous solution, the viscous component of the complex modulus, the loss modulus (G″), decreases with decreasing oscillatory frequency, and the elastic component of the complex modulus, the storage modulus (G′), is low. For gels, G′ and G″ are relatively constant with oscillatory frequency, and G′ is much greater than zero [14]. These experiments were performed near pH 3; we present the reasons for this in Section 2.3. The critical coagulation concentration (CCC) is defined as the salt concentration below which the oligopeptide does not form a gel and above which the oligopeptide does form a gel. For KFE12, we can see that the CCC is between 0.1 and 1.0 mM NaCl.

We surmised that variation of these hydrophobic side chains would affect the salt concentration at which the oligopeptides assembled because increasing the side chain hydrophobicity would increase the driving force for assembly by increasing the energetic penalty for

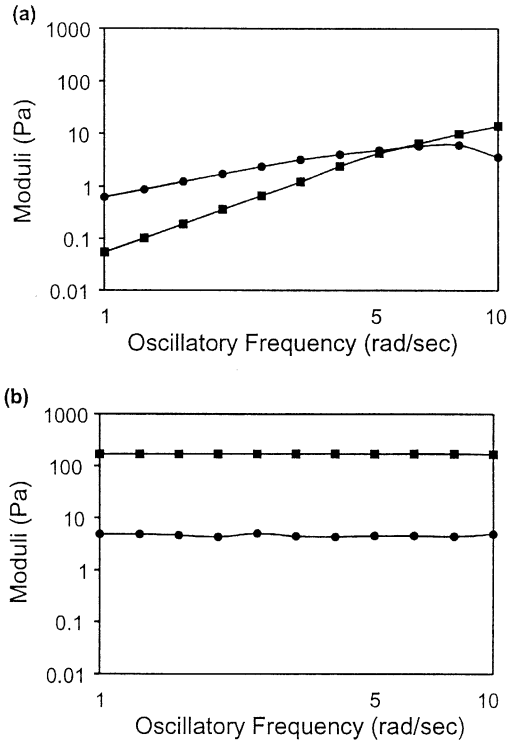

Fig. 2. Primary data for gel formation of 1 wt% KFE12 equilibrated with (a) 0.1 mM NaCl and (b) 1.0 mM NaCl. The storage modulus (squares), G′, and loss modulus (circles), G″, are plotted against oscillatory frequency on log–log scales.

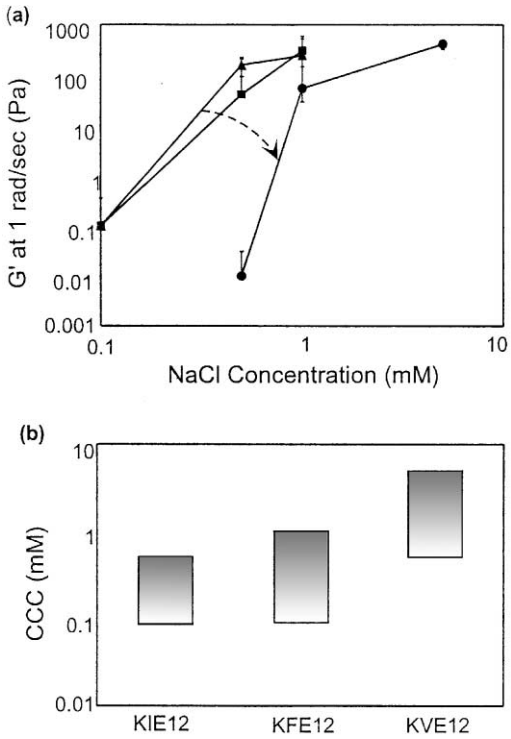

Fig. 3. Trends for variation of hydrophobic side chains. (a) G′ plotted against NaCl concentration for averages of several experiments with 95% confidence intervals on log–log scales: triangles—KIE12, squares—KFE12, circles—KVE12. Points lower than 1.0 Pa are viscous and points with no lower error bar (due to it intersecting zero) show points that are not reproducibly gelled. Arrows show increasing CCC. (b) Bars representing the possible range for the CCC for each sequence are shown white at the bottom representing samples known to be viscous and black at the top representing samples known to be gelled.

being unassembled. Based on our previous corroboration that DLVO theory governs assembly, we have shown that hydrogel formation occurs when the intermolecular attraction due to the hydrophobic effect dominates over the intermolecular repulsion due to positive charges on the lysine side chains [8]. It is useful to think of the structure proposed in Figs. 1c and d to understand this phenomenon. The hydrophobic attraction, that causes the hydrophobic side of the peptides to orient toward the filament interior, is the driving force for assembly, but electrostatic repulsion, between side chains forced to within several angstroms in the assembled filaments, inhibits assembly until the Debye length is lowered by addition of salt. This suggests that increasing side chain hydrophobicity would decrease the CCC by increasing the driving force for assembly relative to the electrostatic inhibition. To test this prediction, rheology experiments for KIE12 and KVE12 were carried out. From each of these tests, average values of G′ at 1.0 rad/s with 95% confidence intervals are plotted in Fig. 3a. As can be seen in this figure, low frequency G′ near zero (noted by the absence of a lower error bar because the error bar would pass through zero) is indicative of a viscous solution and low frequency G′ much greater than zero is indicative of gels. From this we can see that the CCC for KIE12 is between 0.1 and 0.5 mM NaCl, and the CCC for KVE12 is between 0.5 and 5.0 mM NaCl. These data are replotted in Fig. 3b to elucidate the variation of CCC with variation of hydrophobic side chains.

These data are consistent with the CCC being inversely proportional to the extent of hydrophobicity because the CCC decreases as we change the hydrophobic side chains from V to I. Hydrophobicity scales of the natural amino acids verify that I (which has four carbons) is more hydrophobic than V (which has three carbons). One scale, that measured the free energy of transfer of amino acids from water to ethanol, reports $\Delta f_{t,\text{leucine}} = 1800\,\text{cal/mol}$ and $\Delta f_{t,\text{valine}} = 1500\,\text{cal/mol}$ [15]. Three other methods of measuring hydrophobicity confirm this relative ordering (numerical values reported in Table 2): Bull and Breese [16] by measuring surface tension, Vorob'ev [17] by using absorption millimeter spectroscopy to determine extent of hydration, and Urry by finding the minimum temperature at which an entropically driven assembly process occurred [18]. Although these are only two data points, they do not contradict our hypothesis that increasing hydrophobicity will decrease the CCC required to drive gel formation.

Table 2
Hydrophobicity scales

	Phenylalanine	Isoleucine	Valine
Nozaki and Tanford [15]	2500 cal/mol	1800 cal/mol (Leu)	1500 cal/mol
Bull and Breese [16]	−2300 cal/mol	−2260 cal/mol	−1560 cal/mol
Urryc [18]	−30°C	10°C	24°C
Vorobev [17]	31 ± 1	37 ± 1	30 ± 1

By this hypothesis phenylalanine, which has a CCC between isoleucine and valine, should have a hydrophobicity between isoleucine and valine. Since phenylalanine has an aromatic hydrocarbon side chain (rather than an aliphatic one like I and V), having more carbons will not necessarily make it more hydrophobic. One reason for this is that the π electrons of the aromatic ring lead to stronger van der Waals attraction with surrounding water molecules thus decreasing the energetic penalty for hydration [19]. This uncertainty is also found in the published hydrophobicity scales in that they do not agree on how the hydrophobicity of F compares to those of I and V. Three of the scales in Table 2 report F being more hydrophobic than both V and I, but Vorob'ev (measuring hydration by absorption millimeter spectroscopy) reports F as having a hydrophobicity greater than V but less than I (consistent with our hypothesis). Since these hydrophobicity scales are merely empirical representations of different ways of measuring the energy of interactions between amino acids and water, our experimental method may give a more applicable indication of the driving force for folding (or in this case self-assembly) than the other hydrophobicity scales. Self-assembly includes the loss of entropy associated with fixing the side chain inside a hydrophobic core as well as any enthalpic energy gain associated with side chain–side chain interactions (e.g., π electron–π electron interactions between F side chains in the filament interior). Although the scale developed by Urry does include some of these interactions, the formation of hydrophobic cores has been shown to be cooperative so the sequence surrounding the amino acid of interest is important to its behavior. Thus, the sequence that we report in this paper may be more applicable to β-sheet assembly processes and may also allow room to measure combinations of different hydrophobic side chains to probe the cooperativity.

3.2. Variation of oligopeptide length

We next varied length of KFE12 by using two, three, and four FKFE repeats to form FKFEFKFE (KFE8), FKFEFKFEFKFE (KFE12), and FKFEFKFEFKFEFKFE (KFE16). Since this variation does not alter the character of each four amino acids repeat, this allows us to determine what effect alteration of molecular weight has upon self-assembly in the absence of other changes. Once again the rheometric technique was used to determine the CCC for each sequence at 1 wt% oligopeptide concentration in water.

Results for KFE12 in NaCl are presented above in Fig. 2. Fig. 4a represents averages and 95% confidence intervals for each of the solvent trap conditions tested with each oligopeptide sequence. The absence of a low error bar denotes a point where the confidence interval includes zero; thus, these points denote non-gelled samples whereas those with G′ values much greater than zero denote gelled samples. These data show that the CCC for KFE8 is between 1.0 and 5.0 mM NaCl, the CCC for KFE12 is between 0.1 and 1.0 mM NaCl, and the CCC for KFE16 is between 1.0 and 5.0 mM NaCl. This is replotted in Fig. 4b to show the biphasic trend as oligopeptide length is increased.

We can offer one possible hypothesis for these data that suggest that a biphasic trend should exist if more lengths were tested. Competition between an increase in intermolecular attraction with increasing length and an increase in chain entropy with increasing length could cause a minimum CCC at intermediate length. The former can best be conceived as a ratio of the

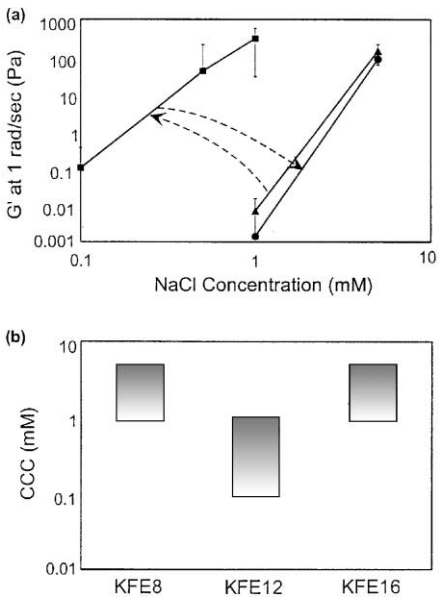

Fig. 4. Trends for variation of oligopeptide length. (a) G′ plotted against NaCl concentration for averages of several experiments with 95% confidence intervals on log–log scales: triangles—KFE8, squares—KFE12, circles—KFE16. Points lower than 1.0 Pa are viscous and points with no lower error bar (due to it intersecting zero) show points that are not reproducibly gelled. Arrows show increasing oligopeptide length. (b) Bars representing the possible range for the CCC for each sequence are shown white at the bottom representing samples known to be viscous and black at the top representing samples known to be gelled.

hydrophobicity over the charge potential on the molecule. If we simplify the problem by assuming that these oligopeptides are cylinders that have charge smeared uniformly over their surfaces the surface potential would be described by

$$\psi_o = \frac{q}{2\pi\varepsilon\kappa R_c(2R_c + L)K_1(\kappa R_c)/K_0(\kappa R_c)},$$

where q is the charge per molecule, κ is the inverse Debye length, R_c is the radius of the oligopeptide, L is the length of the oligopeptide, and K_1 and K_0 are Bessel functions [11]. If we assume that the hydrophobic effect is additive, we can just multiply the energy of transfer from Nozaki and Tanford by the number of hydrophobic side chains. In this way, the difference between KFE12 and KFE8 could be determined as

$$\frac{\left[\dfrac{3e}{2\pi\varepsilon\kappa R_c(2R_c + 2.1\,\text{nm})K_1(\kappa R_c)/K_0(\kappa R_c)}\right]}{6(2500\,\text{cal/mol})} :$$

$$\frac{\left[\dfrac{2e}{2\pi\varepsilon\kappa R_c(2R_c + 1.4\,\text{nm})K_1(\kappa R_c)/K_0(\kappa R_c)}\right]}{4(2500\,\text{cal/mol})},$$

where e is the charge of an electron and reasonable values have been substituted for L for each oligopeptide. Everything will cancel except for the $(2R_c + L)$ terms so the charge/hydrophobicity ratio shows that charge is more important for KFE8 than it is for KFE12, and likewise that charge becomes less important as length increases. In the proposed hypothesis, this decreasing importance of charge is in competition with the increasing entropy of the backbone as the length increases. In order to pack effectively into filaments, each molecule must maintain itself as a β-strand that packs together with other oligopeptides to form a β-sheet. As the length increases, more bond angles become less flexible to maintain the β-strand. Such an increase in order correlates to a loss of entropy that decreases the energetic driving force for assembly. This hypothesis is consistent with the data shown in Fig. 4b, but further research is needed to confirm whether this trend is truly biphasic.

3.3. Variation of charged groups

Alternating with the hydrophobic side chains in KFE12 are the charged groups lysine (K) and glutamic acid (E). Since self-assembly occurs when intermolecular attraction dominates over charge repulsion, variation of these charged side chains should affect the self-assembly behavior of the oligopeptides by affecting the charge repulsion. We can show this by varying the glutamic acid side chains to glutamine (Q) side chains to form FKFQFKFQFKFQ (KFQ12). The glutamic acid side chain consists of two carbons with a carboxylic acid group on the end; glutamine differs only by an NH_2 group that replaces the OH of the carboxylic acid. We expect that this variation will effect the CCC due to altering the hydration properties of the side chains. Figs. 5a and b show data, acquired as described in Sections 3.1 and 3.2, for these two sequences. Whereas the CCC for KFE12 is between 0.1 and 1.0 mM NaCl, the CCC for KFQ12 is between 10 and 50 mM NaCl. This marked difference can only be due to the change from the OH on glutamic acid to NH_2 on glutamine, but there is no obvious explanation for how this change could affect the CCC. One possible explanation is that hydrogen bonding, between the carbonyl oxygen (C=O) on one glutamic acid side chain and the hydroxyl group (OH) on the glutamic acid side chain of a different oligopeptide molecule, could be increasing the attractive interaction between KFE12 molecules by allowing the formation of H-bonded dimers. This configuration of hydrogen bonding is known to increase the boiling points of many compounds with carboxylic acid groups and may be increasing the attractive interactions

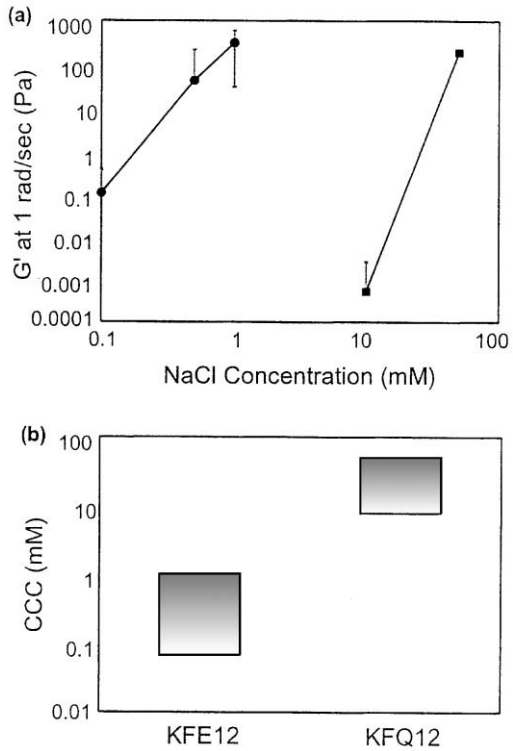

Fig. 5. Trends for variation of charged side chains. (a) G′ plotted against NaCl concentration for averages of several experiments with 95% confidence intervals on log–log scales: squares—KFQ12, circles—KFE12. Points lower than 1.0 Pa are viscous and points with no lower error bar (due to it intersecting zero) show points that are not reproducibly gelled; the KFQ12 sample at 50 mM has error bars too narrow to appear separate from the data point. (b) Bars representing the possible range for the CCC for each sequence are shown white at the bottom representing samples known to be viscous and black at the top representing samples known to be gelled.

between KFE12, whereas KFQ12 is not capable of this type of interaction.

Because the glutamine side chain will not become charged at any pH, but the glutamic acid becomes negatively charged at pH > 4.5 [20], this variation should have a dramatic effect on the conditions necessary for self-assembly in the absence of exogenous salt. To examine the behavior with pH, we used NaOH instead of NaCl in the solvent trap. We added various concentrations of NaOH and measured the pH of the bath at equilibrium. Since in our previous work [8] we have demonstrated that the cation was not sufficient to cause gelation, addition of Na^+ ions should not cause gelation. However, OH^- is still a negatively charged ion and shields the positive charges on the lysine side chains in the same way that the Cl^- ions do; but the OH^- concentration does not reach 1.0 mM (the CCC for Cl^- with KFE12) until the solution reaches pH > 11. Thus, if gelation occurs at pH < 11, decreasing Debye length cannot be the cause for disappearance of the kinetic barrier so there must be an alternative cause. In our previous work, we demonstrated that KFE12, in the absence of exogenous salt, assembles when the net charge on the molecule is near zero. Fig. 6a shows the equilibrium bath pH versus G′ at 1 rad/s for KFE12 at 1 wt%. The behavior shown in Fig. 6a is consistent with this hypothesis because, due to the pK of glutamic acid being 4.5 and the pK of lysine being 10.5 [20], KFE12 should be positively charged at pH < 4.5, negatively charged at pH > 10.5, and have a net charge near zero at pH between 4.5 and 10.5. It is possible that not all lysine and glutamic acid side chains become ionized. In order to see the phenomena shown in Fig. 6 many of these side chains must be disassociated from their counter-ions, but it is possible that some remain bound.

In designing a biomaterial for in situ transformation, however, it is more desirable to have an oligopeptide sequence that is viscous at pH ~ 7 in the absence of salt but that gels when sufficient salt is added. Based upon the prediction that, in the absence of exogenous salt, the oligopeptide will form a gel when the molecular net charge is near zero, we predict that KFQ12 will remain viscous at pH ~ 7 at low salt concentrations. We make this prediction because KFQ12 will be positively charged at pH < 10.5 because the lysine side chains will be positively charged at pH < 10.5 but the glutamine side chains will not become charged at any of the pHs studied (between 3 and 12) [20]. In order to test this prediction we repeated the experiments performed above but studied KFQ12 instead. The results, shown in Fig. 6b, demonstrate that KFQ12 remains viscous at pH < 10.5, thus confirming our prediction. Further, addition of 150 mM NaCl to a bath already containing sufficient NaOH to bring the bath to pH ~ 7 causes gel formation as shown in the squares in Fig. 6b. Therefore, a 1 wt% solution of KFQ12 in water can be brought to pH ~ 7 without initiating gelation, injected into the body, and by diffusion of salt from physiologic saline gelation will occur.

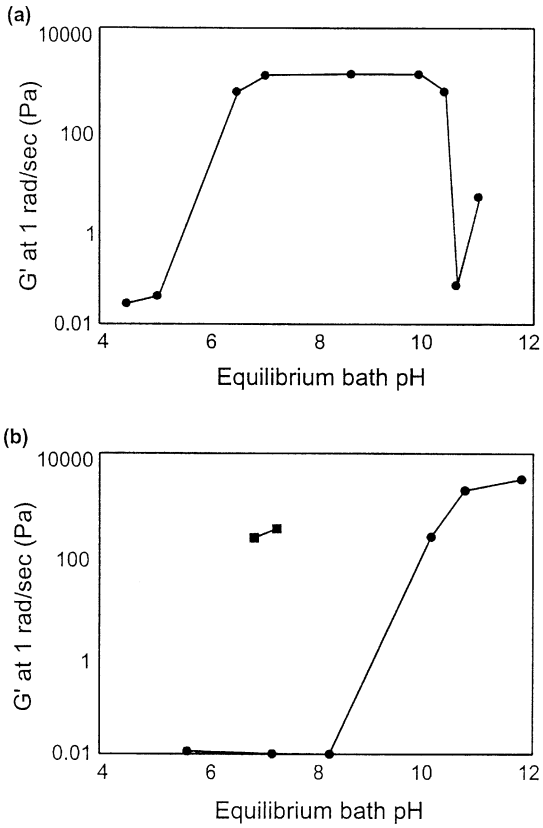

Fig. 6. Rheology results for addition of NaOH. Data for G′ at 1 rad/s for (a) KFE12 and (b) KFQ12 equilibrated in various concentrations of NaOH. The pH of the bath at equilibrium was measured after each experiment and is plotted on the x-axis. The squares in (b) represent experiments on KFQ12 to which NaOH and 150 mM NaCl were added; the equilibrium bath pH was measured after the experiment.

4. Conclusions

We systematically varied each of the three attributes of the repeating sequence and measured the effect that each variation had on the CCC. Variation of the hydrophobic side chains showed that an increase in hydrophobicity decreased the CCC; however, it is not always unambiguous whether one amino acid is more hydrophobic than another due to differences in how hydrophobicity scales are determined. Variation of oligopeptide length shows a minimum for which we propose a hypothesis that this is due to two competing energetic phenomena. When the molecule is small, additional repeats increase the molecule's hydrophobicity faster than they increase the electrostatic repulsion so the CCC decreases with increasing length. In large oligopeptides, additional repeats increase the entropy loss upon self-assembly so the CCC increases with

increasing length. In variation of the charged side chains we showed that, through knowledge of DLVO theory, we could rationally alter KFE12 to KFQ12 to create a sequence that is viscous at pH ~ 7 in the absence of salt but that gels in the presence of salt.

This demonstrates that changing the amino acid sequence of an oligopeptide is analogous to changing the monomer or polymerization method for synthesis of a traditional hydrocarbon polymer. Through many years of research, engineers have gained the ability to rationally design polymers to have desirable properties. Ultimately we intend to show that knowledge of how oligopeptides form materials, in this case explained by DLVO theory, and how variation of amino acid sequence affects properties will allow us to rationally design oligopeptides with desired properties. In this study we set the parameters as a material that would be viscous at pH ~ 7 in the absence of salt and gelled in the presence of physiologic salt concentrations. We were able to use DLVO theory and systematic changes in amino acid sequence to design an oligopeptide with this desired property.

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