Macromolecular Research, Vol. 17, No. 8, pp 597-602 (2009)

Investigation on Structure and Properties of a Novel Designed Peptide with Half-Sequence Ionic Complement

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Received October 7, 2008; Revised December 10, 2008; Accepted December 16, 2008

Abstract: Although the existing design principle of full-sequence ionic complement is convenient for the development of peptides, it greatly constrains the exploration of peptides with other possible assembly mechanisms and different yet essential functions. Herein, a novel designed half-sequence ionic complementary peptide (referred to as P9), AC-Pro-Ser-Phe-Asn-Phe-Lys-Phe-Glu-Pro-NH_2, is reported. When transferred from pure water to sodium chloride solution, P9 underwent a dramatic morphological transformation from globular aggregations to nanofibers. Moreover, the rheological experiment showed that the P9 could form a hydrogel with a storage modulus of about 30 Pa even at very low peptide concentration (0.5% (wt/vol)). The P9 hydrogel formed in salt solution could recover in a period of about 1,800 sec, which is faster than that in the pure water. The data suggested that the half-sequence, ionic complementary peptide might be worthy of further research for its special properties.

Keywords: peptide, half-sequence ionic complement, sodium chloride, conformation, fiber, hydrogel.

Introduction

Recently, self-assembly systems present attractive strategy in fabricating novel materials. Many researchers have attempted to exploit the self-assembling properties of proteins and peptides for materials directed towards regenerative medicine, drug delivery, and so forth.[1-8] Typical sequences of designed fibrous proteins and peptides have regular repeats of alternating hydrophobic and hydrophilic residues along the full sequence and the hydrophilic residues possess alternating negative and positive charged amino acid residues. The peptide can form β-sheet structure and further self-assemble spontaneously into three-dimensional nanofiber scaffolds in pure water, in the presence of metal cations or in physiological conditions.[1,4,9-12] One of the typical self-assembling peptides is the KFE12 (n-FKFEFKFEFKFE-c).[10] This peptide contains regular repeats of alternating hydrophobic residues (Phe) and hydrophilic residues (Glu and Lys) along the entire peptide sequence, and the hydrophilic residues are composed of alternating negatively charged residues (Glu) and positively charged residues (Lys) throughout the entire peptide sequence to ensure a full-sequence ionic complement. Although the designing principle of full-sequence ionic complement is convenient for developing peptides, it greatly constrains the exploration of peptides with other possible assembly mechanisms and different yet essential functions. If a new method is adopted without the conventional principle, namely full-sequence ionic complement, it is entirely possible to witness the advent of a new type of peptides with interesting properties.[13] If so, the existing limitation of design will be transcended: meanwhile, the new method will expand the current cognition of biological macromolecular assembly.

In view of this, a 9-amino acid peptide consisting of alternating hydrophobic and hydrophilic residues: AC-Pro-Ser-Phe-Asn-Phe-Lys-Phe-Glu-Pro-NH_2 was designed without full-sequence ionic complement. This paper mainly studied the basic properties of the novel designed peptide P9, including secondary structure, microscopic morphology and rheological properties.

Experimental

Materials. All oligopeptides used in this study were synthesized from Chengdu CP Biochem Co., Ltd., stored at -20 °C, and used without further purification. The amino acid sequence of the peptide was Pro-Ser-Phe-Asn-Phe-Lys-Phe-Glu-Pro, named P9 for short, and the *N*-terminus and C-terminus were protected by acetyl and amino groups, respectively (molecular weight 1,156 Da, purity 95%). Peptide was synthesized by using the standard Fmoc solid-phase chemistry. Peptide homogeneity and composition were analyzed by analytical HPLC and mass spectrometry. The stock solutions of the peptide were prepared at concentration of

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10 mg/mL (1% wt/vol) in milli-Q water (18.2 MΩ, Millipore Milli-Q system) and stored at 4 °C before use.

Circular Dichroism Spectroscopy. CD data were gathered at 25 °C on an AVIV model 400 spectrometer (AVIV Associates, Lakewood, NJ), using a 1-mm path length quartz cuvette. Spectra were collected at 1 nm intervals and 1 nm bandwidth from 190 to 260 nm with 1 s signal averaging time, using 3-times scans for average. Be sure to check the baseline CD signals of the empty cuvette and record a baseline spectrum of the cuvette containing only pure water under identical condition. All spectra were corrected by subtracting the baseline and the data were expressed as mean residue ellipticity, $[\theta]$, which was given the unit of [deg·cm^2·dmol^{-1}]. Peptide from stock solutions (1% wt/vol) was diluted to different concentrations with sodium chloride solution. The concentrations of the peptide solutions for CD measurement were 0.23 mg/mL (200 μM) with different concentration of sodium (0, 0.5, 5 mM). In order to obtain stable signals, all samples were incubated at 4 °C for 48 h.

In order to investigate the effect of salt on secondary structures of peptide P9, a software CDPro (http://lamar.colostate.edu/sreeram/CDPro/main.html) was applied to calculate secondary-structure contents. CDPro is a series of programs for protein CD analysis, containing three popular CD analysis programs, CONTIN, SELCON3, and CDSSTR.[14] The peptide secondary-structure fractions at various environments were calculated by the modified Contin method (CONTINLL program) with comparison to the selected reference proteins set (IBasis10 (SMP56), λ = 240-190 nm). The calculated CD spectral data were compared with the experimental CD spectrum and showed little difference (data not shown), and the values of the root-mean-square deviation (RMSD) and the normalized root-mean-square deviation (NRMSD) are both close to 0.1 and zero (Table I), respectively, suggesting that CONTINLL program and SMP56 fitting are suitable for CD analyses of peptide P9.

Atomic Force Microscopy (AFM). AFM was used to obtain the nanostructure of the peptide. Peptide from the stock solutions (1% wt/vol) was diluted to different concentrations with sodium chloride solutions. The concentrations of the peptide P9 for AFM observation were 5 mg/mL with different concentration of sodium (0, 10, 60, 80 mM). An aliquot of 5 μL peptide solution was evenly placed onto a freshly cleaved mica surface. Each sample was left on the mica surface for about 30 sec. The surface was then rinsed with 200 μL Milli-Q water to remove unattached peptide. The samples were covered with Petri dishes to avoid contamination and air-dried for AFM observation.

AFM was performed at room temperature using the tapping mode on a SPI4000 Probe Station and SPA-400 SPM Unit (Seiko Instruments Inc., Chiba, Japan). All images were obtained by utilizing a 20-μm scanner (400) and an Olympus Si-DF20 cantilever as well as a Si tip of radius 10 nm, spring constant of 12.00 N/m, with resonance frequency of 127.00 kHz. All of the measurements were performed in ambient air. Height images were recorded with 512×512-pixels resolution. For each sample, images were scanned and collected at scales of 5×5 μm^2, 2×2 μm^2, and 1×1 μm^2.

TEM. TEM was performed at room temperature using a HITACHI H-600 electron microscope at 100 kV accelerating voltage. Peptide from the stock solutions (1% wt/vol) was diluted to different concentrations with sodium chloride solutions. The concentrations of the peptide solutions were 5 mg/mL with different concentration of sodium (0, 80 mM). A droplet (about 20 μL) of peptide solution was deposited on a clean surface. Then a copper TEM grid coated with PVFM (polyvinylformal) membrane was immersed into the droplet for 15 sec. The excess liquor on the grid was removed by filter paper. The attached peptide on the grid was then negatively stained by phosphotungstic acid solution (1% wt/vol in water) for about 15 sec and air-dried for TEM observation.

Rheology Properties. Rheological experiments of the gels were performed at 25 °C on a rheometer (HAAKE Rheostress I) with a cone and plate geometry system (cone diameter: 2 cm, angle: 1°, truncation: 46 μm). Peptide from the stock solution (1% wt/vol) was diluted to measurement concentration with sodium chloride solution. The concentrations of the peptide solutions were 5 mg/mL (0.5%) with different concentration of salt (0, 10 mM). An aliquot of 100 μL the peptide solution was placed on the plate. After removal of the excess solution, the cone was lowered and eventually

Table I. The Secondary Structure of P9 at Different Sodium Chloride Concentrations Analyzed Using CDPro

Sodium Chloride Concentration	RMSD/NRMSD	Secondary-Structure Fractions (%)[a]					
		H(r)	H(d)	S(r)	S(d)	Turn	Unrd
Control[b]	0.025/0.018	5.5	10.6	10.7	11.4	24.4	37.3
0.5 mM[c]	0.030/0.020	4.2	10.4	15.3	11.8	23.6	34.7
5 mM[d]	0.136/0.074	4.6	10.6	15.9	12.1	24.0	32.9

[a]Including six types of secondary structures: H(r) is regular α-helix; H(d) is distorted α-helix; S(r) is regular β-strand; S(d) is distorted β-strand (a partial, but far from complete distortion of the regular β-strand because of the lack of some hydrogen bonds); Turn is β-turn structure; Unrd is unordered structure. The total content in one peptide solution: $c_{Helix}+c_{\beta\text{-}Strand}+c_{Turn}+c_{Unordered}=100.0\pm0.1\%$. [b]The peptide in pure water at the concentration of 0.23 mg/mL. [c]The peptide at the concentration of 0.23 mg/mL with 0.5 mM sodium chloride. [d]The peptide at the concentration of 0.23 mg/mL with 5 mM sodium chloride.

the tip was 46 μm above the plate. Stress sweeps were performed from 0.1 to 10 Pa to determine the limit of the samples' linear viscoelastic region. At a constant shear stress of 1 Pa, chosen from the LVE, frequency sweeps ranging from 100 to 0.1 rad/s were performed. Shear recovery experiments were performed after destructing gel network with 1,000% strain at 6 Hz for 180 sec, followed by a time sweep experiment at constant shear stress of 1 Pa and constant frequency of 6 rad/s for 30 min. Temperature control was provided with a temperature regulated circulating water bath (HAAKE Phoenix II).

Results

Expression of the Designed Peptide P9. As is known, KFE8 (n-FKFEFKFE-c) is one of the peptides in the family of full-sequence ionic complementary sequences, which can form fibers.[4] We chose this peptide as a model because shorter peptides show lesser structural and chemical complexity, which facility our study. We selected a part of these sequences (FEFK) to design a non-full-sequence ionic complementary peptide. The designed 9-amino acid peptide P9 consisted of alternating hydrophobic and hydrophilic residues: AC-Pro-Ser-Phe-Asn-Phe-Lys-Phe-Glu-Pro-NH_2. The molecular model of the peptide P9 is shown in Figure 1(A). All of the hydrophobic residues including phenylalanine and proline side chains faced to one direction, and the hydrophilic residues of serine, asparagine, lysine and glutamic acid side chains faced to the other direction to create two distinct faces. The dimensions were about 3.1 nm in length, 1.2 nm in height and 0.4 nm in thickness. Residues Lys and Glu were chosen to supply ionic complement. Owing to the perspective of non-full-sequence ionic complement, only one ionic pair of Lys and Glu was chosen. The rest hydrophilic residues serine and asparagine were not charged amino acid residues. Hence, we named the peptide P9 as a half-sequence ionic complementary peptide. The residues Phe and Pro were supposed to sustain hydrophobic interaction.

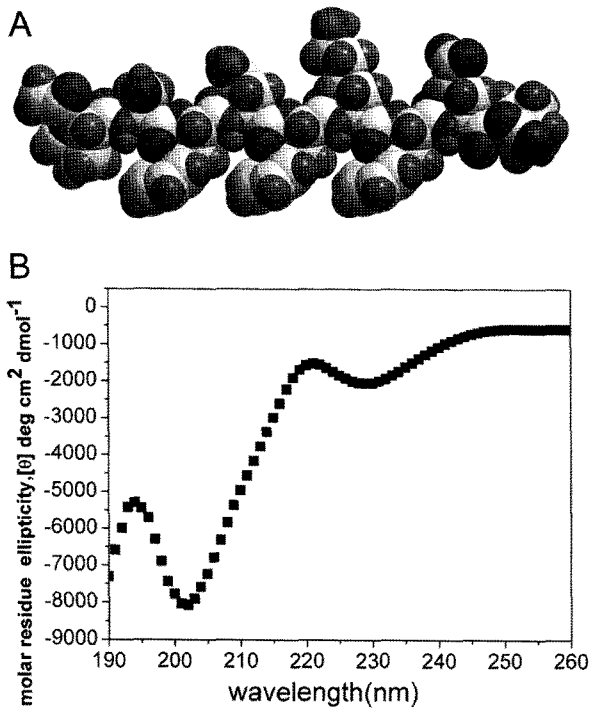

Figure 1. (A) Schematic three-dimensional molecular model of P9: (AC-Pro-Ser-Phe-Asn-Phe-Lys-Phe-Glu-Pro-NH_2). Carbon atoms are white, oxygen atoms are red, nitrogen atoms are blue, and hydrogen atoms are gray. In this conformation, all of the hydrophobic phenylalanine side chains face in one direction, and the residues of serine, asparagine, lysine and glutamic acid side chains face in the other direction to create two distinct faces. The dimensions are about 3.1 nm in length, 1.2 nm in height and 0.4 nm in thickness. (B) The far-UV CD spectrum of the peptide (200 μM) in pure water from 190 to 260 nm is shown.

Secondary Structure of P9. Peptide monomers could interact with each other and self-assemble to form regular secondary structure in special solution environment. The research on secondary structure of the peptide P9 could help us further understand the self-assembling process for the half-sequence ionic complementary peptide. CD spectrum (Figure 1(B)) of P9 (200 μM) in pure water at 25 °C showed negative ellipticities around 202 nm, 230 nm and positive ellipticities near 193 nm, 221 nm. The ellipticity near 193 nm might be resulted from a β-backbone twist. It was not a typical spectrum for either β-strand structure, unordered conformation or α-helix structure. Hence a program CDPro was used to accurately analyze the secondary structure of peptide P9. The contents of α-helix, β-sheet, β-turn and unordered conformation were 16.1, 22.1, 22.4 and 37.3%, respectively, for peptide P9 in pure water. Analysis by CD spectrum revealed that the peptide P9 displayed complex conformation, and the main conformation in pure water was unordered structure.

It is completely different from that of the full-sequence ionic complementary peptide. Generally speaking, the traditional full-sequence ionic complementary peptide possesses a typical β-sheet structure. It is not difficult to infer that it is the half-sequence ionic complement in P9's monomeric structure that might be responsible for the differences in secondary structure.

Microscopic Morphology of P9. The microscopic morphology of a material can determine its possible applications to some extent.[5,15] The full-sequence ionic complementary peptides could form β-sheet structure and further self-assembling into 3D nanofiber scaffolds in pure water, in the presence of metal cations or in physiological conditions. Consequently, the applications of full-sequence ionic complementary peptides had been paid much attention. Herein, the microscopic morphology of P9 was studied. As a result, AFM morphological studies demonstrated that the peptide with the concentration of 5 mg/mL in pure water exhibited

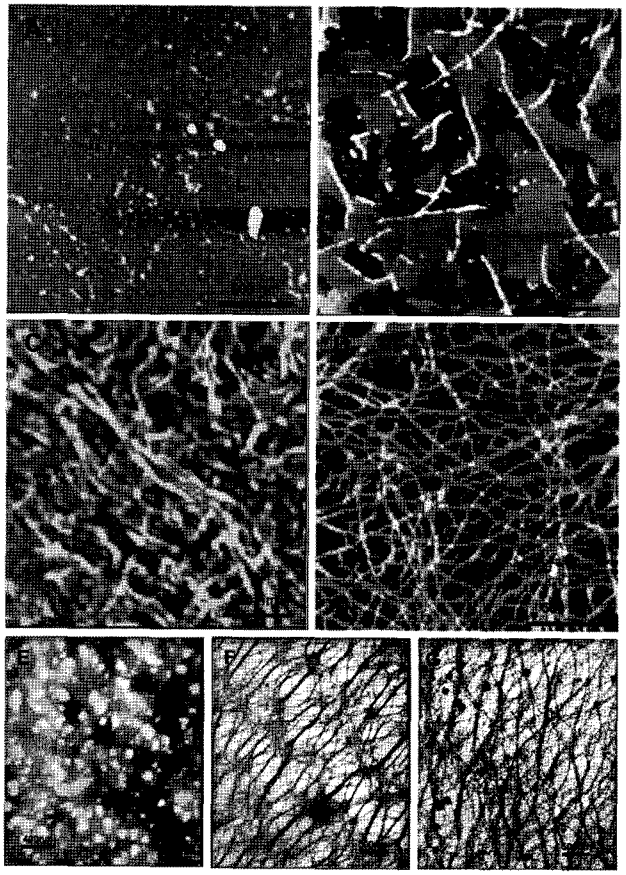

Figure 2. The AFM images of 5 mg/mL (0.5%) P9 at different sodium chloride concentrations: (A) pure water; (B) 10 mM sodium chloride; (C) 60 mM sodium chloride; and (D) 80 mM sodium chloride. TEM images of P9 at the concentration of 5 mg/mL: (E) in pure water; (F) and (G) with 80 mM sodium chloride addition.

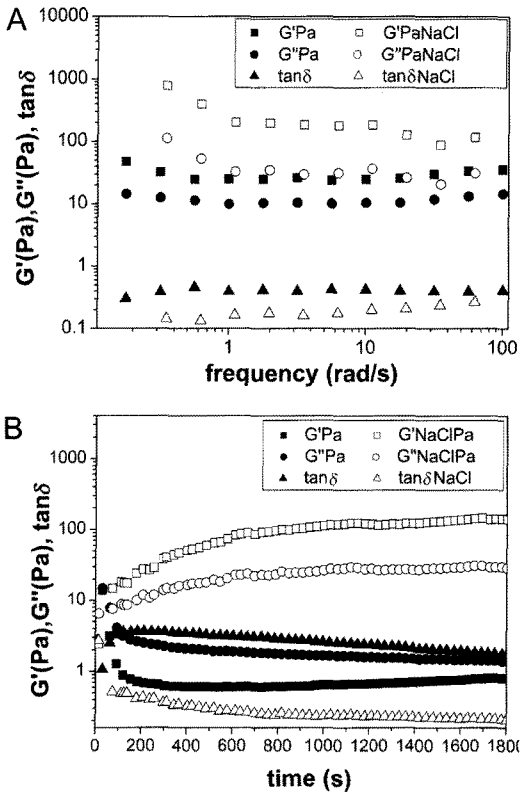

Figure 3. (A) Frequency sweep (1 Pa shear stress) of 0.5% P9 in pure water at 25 °C: storage moduli G' (■), loss moduli G'' (●), tanδ (tanδ=G''/G') (▲); Frequency sweep (1 Pa shear stress) of 0.5% P9 with 10 mM sodium chloride addition at 25 °C: Storage moduli G' (□), loss moduli G'' (○), tanδ (△). (B) Restoration of gel module as a function of time after the cessation of treatment for gel network destruction (1,000% strain at 6 Hz for 180 sec), 0.5% P9 at 25 °C: storage moduli G' (■), loss moduli G'' (●), tanδ (▲); 0.5% P9 with 10 mM sodium chloride addition at 25 °C: Storage moduli G' (□), loss moduli G'' (○), tanδ (△).

many global aggregations (Figure 2(A)).

It is well know that one of the main cations in physiological condition is sodium ion. Hence, sodium chloride solution was selected to investigate the morphology of P9 in salt solution. Surprisingly, the morphology of peptide P9 in aqueous solution of sodium chloride displayed dramatically changes from that in pure water. Sodium chloride solutions with gradient concentration were mixed with the peptide P9. The concentrations of the peptide solutions were 5 mg/mL with different concentration of sodium (10, 60, 80 mM). AFM morphology of P9 in sodium solutions is shown in Figures 2(B-D). The peptide P9 in aqueous solution of 10 mM sodium chloride exhibited short fibers and global aggregations. Interestingly, with the increase of salt concentration, the density of the peptide fibers increased and the length extended. Especially, the peptide in aqueous solution of 80 mM sodium chloride demonstrated excellent fibers with stagger networks, and the maximum of the fiber length extended up to 5 μm.

TEM images also echoed that the peptide P9 at the concentration of 5 mg/mL with 80 mM sodium chloride addition could form fibers (Figures 2(F) and 2(G)), while it formed many global aggregations at the concentration of 5 mg/mL in pure water (Figure 2(E)). The view of the peptide in aqueous solution of 80 mM sodium chloride demonstrated a myriad of dense fibers, which coincided with the AFM images (Figure 2(G)). In addition, the thin fibers were interlaced together into thick ones, parts of which had directional arrangement (Figure 2(F)).

Rheological Property of P9. Rheological measurements were performed for further insight into the self-assembly property of half-sequence ionic complementary peptide P9. The frequency sweep results measured at 25 °C revealed that the storage moduli (G', a measure of the elastic response of the material) of the peptide P9 at the concentration of 5 mg/mL in pure water were larger than their loss moduli (G'', a measure of the viscous response) over all measured frequencies (Figure 3(A)), indicating a gel formation in pure

water. Figure 3(A) also revealed that the peptide P9 formed a gel at the concentration of 5 mg/mL with 10 mM sodium chloride addition. While both peptide solutions were found to form hydrogel, the storage modulus G' (about 150 Pa) of the peptide in aqueous solution of 10 mM sodium chloride was 4 times larger than that (about 30 Pa) in pure water, indicating a more rigid property with introduction salt.

In order to estimate the self-repairing property of the peptide in aqueous solution, a time sweep experiment was performed after the gel had been destroyed by a constant strain of 1,000% at 6 Hz for 180 sec (Figure 3(B)). All chemical and biologic reactions were time-dependent, and the hydrogels' restoration was not an exception. When the gel network had been destroyed, there was no considerable rheological difference between different peptide solutions for about 30 sec. Both peptide solutions exhibited the property of viscous fluid rather than elastic gel. But after about 60 sec, there were the significant differences for the peptide in the different solutions. The peptide at the concentration of 5 mg/mL in pure water still formed a viscous fluid, with a slightly increased elastic modulus G'. Its storage modulus G' was smaller than loss modulus G'' in all measured time after it had been destroyed, indicating a slow recovery process in pure water. In sharp contrast to this, the peptide at the concentration of 5 mg/mL with 10 mM sodium chloride addition could gradually recover its elastic property. The peptide's storage modulus G' (about 18 Pa) was larger than loss modulus G'' (about 9 Pa) in about 60 sec, revealing a gel formation in sodium chloride solution. The storage modulus G' (about 140 Pa) of the restored gel was recovered almost completely in 1,800 sec, rendering that it is a quick recoverable hydrogel. The results revealed that the additional sodium (I) could accelerate the process of gel's recovery. The property of quick recovered hydrogel might suggest a potential utility in tissue engineering, such as a kind of injectable materials.

Discussion

We designed a novel peptide P9 with half-sequence ionic complement, and observed the salt-triggered biophysical and morphological property transition. The morphology of the peptide dramatically underwent an alternation change from globular aggregations to nanofiber formation when it was transferred from the pure water to the sodium chloride solution. The sodium (I) is believed to play a critical role in the half-sequence ionic complementary peptide P9's self-assembly process.

The complexes of peptides with metal ions have been thoroughly studied both experimentally and theoretically.[16-22] Due to various of complex sites in peptide monomer including the nitrogen atom at the N-terminus, the carboxylate group at the C-terminus, and the oxygen or nitrogen atoms presenting in the backbone chain, it is difficult to determine which complex site is for sodium binding. The site is a competitive complexation determined by the particular solvent condition.

CD measurements showed that the peptide was able to undergo conformational change under the influence of additional sodium chloride. The results calculated by CDPro are shown in Table I. The observations in this experiment suggest that the noticeable transition takes place between unordered structure and β-sheet structure. Increasing sodium ions leads to a rise in β-sheet structure content and a decline in unordered structure content. These observations give rise to the proposal of two possible mechanisms for the structural changes (Figure 4). In the first mechanism (Figure 4(A)), with the influence of complexation between sodium (I) and the peptide, a monomeric unit of unordered structure could interact with another unit, which may be a β-sheet unit. Due to intermolecular force, a segment of a peptide molecule begins to convert to β-strand while the other part of it is still unordered. This may form an intermediate that is par-

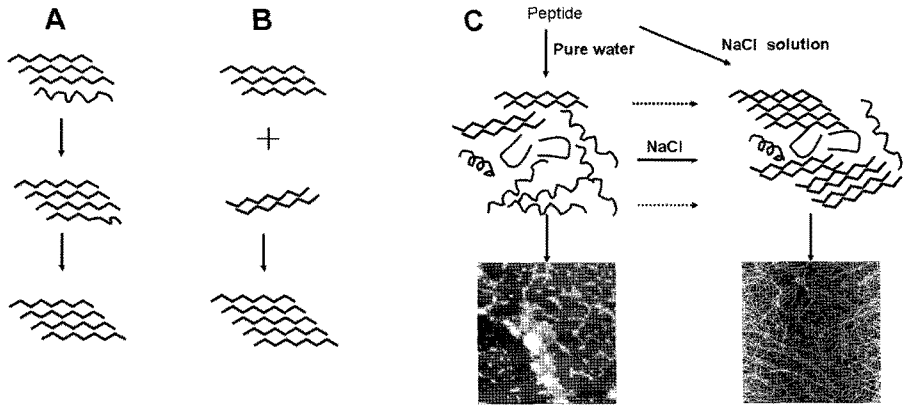

Figure 4. Two possible scenarios for the increased β-sheet content. (A) A peptide molecule begins the β-strand conversion while still be a segment of unordered structure. Eventually, the molecular becomes completely β-strand and be a part of β-sheet. (B) The small piece β-strand domains assemble to bigger ones, which have existed in aqueous solution previously. (C) The proposed self-assembly process for peptide with sodium chloride addition.

tially unordered and partially extended. Eventually, the entire molecule changes to β-strand structure and becomes a part of β-sheet.

In the second model (Figure 4(B)), due to half-sequence ionic complement, there may be a weak electrostatic interaction between peptide molecules, which leads to formation of small pieces of β-strand. The coordination between sodium (I) and the peptide probably strengthens the interactions between molecules and leads to aggregation of the small pieces of β-strand domains to bigger ones, which have existed in aqueous solution previously. It is not possible to determine which model is correct at this moment. However, it is possible that both models work during self-assembly process. Both mechanisms are reversible. Probably, it is a dynamic balance in certain condition. The balance will be transferred with slight solvent changes.

It is believed that secondary structure plays an important role in determining supramolecular structure and macrostructure.[23] It is generally accepted that the most important element for fiber formation is β-strand conformation, but this is not a sufficient condition. Only when the quantity of β-sheet content increases to a critical degree, can fiber be formed. This assumption is reflected in the data. The peptide in pure water contains about 22% β-sheet conformation, which can not bear formation of fiber. Upon the addition of sodium chloride, the content of β-sheet conformation of the peptide increases, and when the content reaches some proportion, the fiber is formed. Such metal ion-responsive structures may provide potential application as novel biomaterials for drug delivery, tissue engineering, and biosensor application.

The dramatic transformation of P9 in biophysical and morphological properties with metal ion addition is patent, indicating that ionic interaction plays an important role in self-assembly process. The designed peptide changing from full-sequence ionic complement to half-sequence ionic complement resulted in a decline in electrostatic interaction to some extent. The reduced electrostatic interaction may lead to unstable conformation.

In conclusion, the half-sequence ionic complementary peptide, P9, exhibits the properties as a smart material under the regulation of metal ions. Moreover, in the presence of metal ions, P9 can ultimately form fibers and gels, unfolding its potential applications as the traditional ionic complementary peptides with the ability to form fibers and gels as well. Consequently, half-sequence ionic complement might be an indispensable complement for peptide designing principles.

Acknowledgements. We thank Dr. Z. Zhang and Feng Qiu for helpful suggestions and stimulating discussions of the manuscript. This research is supported by the Chinese National '985 Project' of Education Ministry to Sichuan University, Chengdu, China.

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