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SHORT COMMUNICATION

Template-directed self-assembly of a designed amphiphilic hexapeptide on mica surface

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Abstract Self-assembly of small molecules into highly ordered nanostructures offers many important potential applications in science research and industry. Precise self-assembling with the assistance of inorganic substrate is considered as an ideal strategy. In this experiment, the highly ordered mica surface was used to template the assembling of a novel designed amphiphilic hexapeptide to form orderly parallel fibers. The nanostructure and the self-assembly mechanism were investigated by atomic force microscopy (AFM), transmission electron microscopy, Fourier transform infrared spectroscopy, and circular dichroism techniques. By the experimental results, a dramatic conformation transition from random coil and/or α-helix into β-sheet was found after the peptide assembled on the mica surface under certain conditions, which was considered as a key factor for the ordered nanostructure. Finally, according to the AFM images and the simulated length of peptide molecules, a trilaminar β-sheet structure model was proposed to explain the hierarchical self-assembly mechanism.

Keywords Self-assembly · Template-directed · Parallel fibers · Conformational transition

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Introduction

The self-assembled nanostructure has recently attracted much attention because of its scientific importance and widespread applications in the field of modern nanotechnology [1, 2]. Generally, the formation of highly ordered nanostructures by a process of self-assembly or self-association represents the essence of this field. Ordered self-assembled nanostructures can be formed by a variety of building blocks, such as a simple peptide molecule [3–6]. In such "bottom-up" approach, simple peptide molecules interact with each other in a coordinated way to form large and more complex functional supramolecular assemblies, by a combination of many different noncovalent interactions, such as nanowires [7]. In particular, the template-directed method has good performance in arranging objects with the aim of influencing the outcome of the self-assembly process. Therefore, inorganic substrates had been widely utilized to react with peptide as templates to induce large-scale order in nanostructures, such as dot, wire, rod, tube, fiber, helix, and sphere [8–11]. And this strategy had recently gained more and more interests of scientists. However, the fundamental mechanism of reaction between the template-directed process of building blocks and inorganic substrate is still not very clear so far. Accordingly, a deep understanding of these assembling processes is required to gain an insight into the fundamental nature of the interactions between building blocks and inorganic templates [12–14].

Herein, we have carried out a series of experiments to study the template-directed self-assembly behavior of a designed peptide GAGAGS (where G represents glycine, A is alanine, and S is serine), which controls crystallinity in silk fibers to adopt a β-sheet structure in native silk proteins and plays a critical role in the spinning process of silkworm silk [15–17]. This hexapeptide, [CH_3CO]-Gly-Ala-Gly-Ala-Gly-Ser-[$CONH_2$] (GA6), was designed to be acetylated at N termini and amidated at the C termini to obtain an amphiphilic

nature. In this experiment, it was reported that a highly ordered nanostructure of parallel fibers was formed by the peptide molecules on the mica surface under certain conditions. Different influencing factors such as metallic ion, pH, and substrate were utilized to investigate how the template-directed self-assembly behavior of peptide molecules was performed. In the present work, atomic force microscopy (AFM), transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FTIR), and circular dichroism (CD) were used to study the self-assembly morphology and the conformational transition of peptide under different conditions. To the best of our knowledge, this was the first example of utilizing natural protein motif from silk fibroin to study the template-directed assembling behavior.

Experimental section

Materials

The peptide GA6 was commercially synthesized and purified (>95 %) (Bootech BioScience & Technology Co., Ltd., Shanghai, China). The peptide was acetylated at the N termini and amidated at the C termini and lyophilized into powder. The solution of GA6 was prepared by mixing the peptide powder with deionized water (Elix Water Purification System, Millipore) and vortexing for about 1 min. The solution was transparent, and all sample solutions were stored at 4 °C overnight before use. Potassium chloride, sodium chloride, calcium chloride, magnesium chloride, sodium hydroxide, hydrochloric acid, ethyl alcohol, and acetone (Changzheng Chemical Reagents Co., Chengdu, China; A.R. grade) were used as received.

Methods

After preparation, GA6 solution at 1 mg/ml was used. Five microliters of the sample was deposited onto freshly cleaved mica, each aliquot was left on the mica for about 1 min, and dried with a flow of nitrogen gas. The images of the nanostructure were obtained by AFM (spa400, SII) operating in tapping mode. Soft silicon cantilevers were chosen with cantilever length of 200 μm, spring constant of 12 N/m, and tip radius of curvature of 10 nm. Typical scanning parameters were as follows: vibrating frequency, ~124 kHz; integral and proportional gains, 0.1–0.4 and 0.01–0.03, respectively; amplitude reference, −0.1 to −0.25; and scanning speed, 0.8–1.2 Hz. Every sample was scanned more than three times to ensure similar results. FTIR data were gathered in solid state, utilizing FTIR at 4 cm^{-1} resolution (Spectrum One, PE). Spectra were taken by dried peptide dropped from an aqueous solution onto a $ZnBr_2$ crystal. Possibly, a higher sample concentration was used to ensure sufficient thickness and signal intensity for FTIR detection. The CD spectra were obtained by using the sample solution diluted into 0.125 mg/ml and immediately transferred into a quartz cell of 0.1 cm optical path length, which was placed in a chamber flushed with N_2. The far-ultraviolet CD spectrum between 190 and 260 nm was collected with the wavelength scan mode on a spectropolarimeter (Model 400, Aviv) at 20 °C. As to the solid-state CD experiments, 300 μl of sample solution was dried in a quartz cell at room temperature overnight before measurement. Every sample was scanned three times, and the spectra signal was averaged and smoothed. The nanostructure of the samples was investigated by a transmission electron microscope (H-600, Hitachi) as well. About 5 μl of the sample with a concentration of 1 mg/ml was negatively stained with 1 wt% aqueous solution of phosphotungstic acid and naturally dried on a copper grid coated with a holey formvar membrane before the experiment.

Results and discussion

Behaving like a surfactant, peptides are a class of amphiphiles that are known to assemble into a variety of nanostructures. Generally, peptide amphiphiles are self-assembled into one-dimensional nanostructures in solution. In this experiment, AFM and TEM were utilized to investigate the self-assembly structure of the peptide. Figure 1a showed the simulated space-filling molecular model of the GA6 molecule which had a length of 2.5 nm, width of 0.3 nm, and height of 0.4 nm in extended β-sheet conformation. After being dissolved in deionized water overnight, at the concentration of 1 mg/ml, the feather-like aggregations were observed by AFM observation performed on the mica surface, shown in Fig. 1b. AFM surface analysis showed that the aggregations of GA6 had diameters about 73 nm, lengths up to 360 nm, and heights of about 2.2 nm.

To our knowledge, metallic ion was involved in the silk formation of silkworm and spider, such as K^+ [18–21]. According to early reports, there is a plausible theory to account for the effect of K^+ on the conformation change of protein or peptide, that the K^+ ions coordinate to specific domains such as the carbonyl oxygen atoms of amino acid residues with an O–K^+ coordination channel structure [22–24]. These channels allow only the passage of dehydrated K^+ ions, and at a high concentration of K^+, the channel is quite inflexible. The dehydrated K^+ ion fits snugly with proper coordination to the backbone carbonyl oxygen atoms in a stiff conformation as sheet-like structures [25]. It was known that metallic ions also influenced the conformation transition in proteins such as amyloid β-peptide (Aβ) [26].

In the current experiment, the GA6 motif is a core protein unit of silkworm fibroin; thus, it would be interesting to know whether metallic ions have a special effect on the

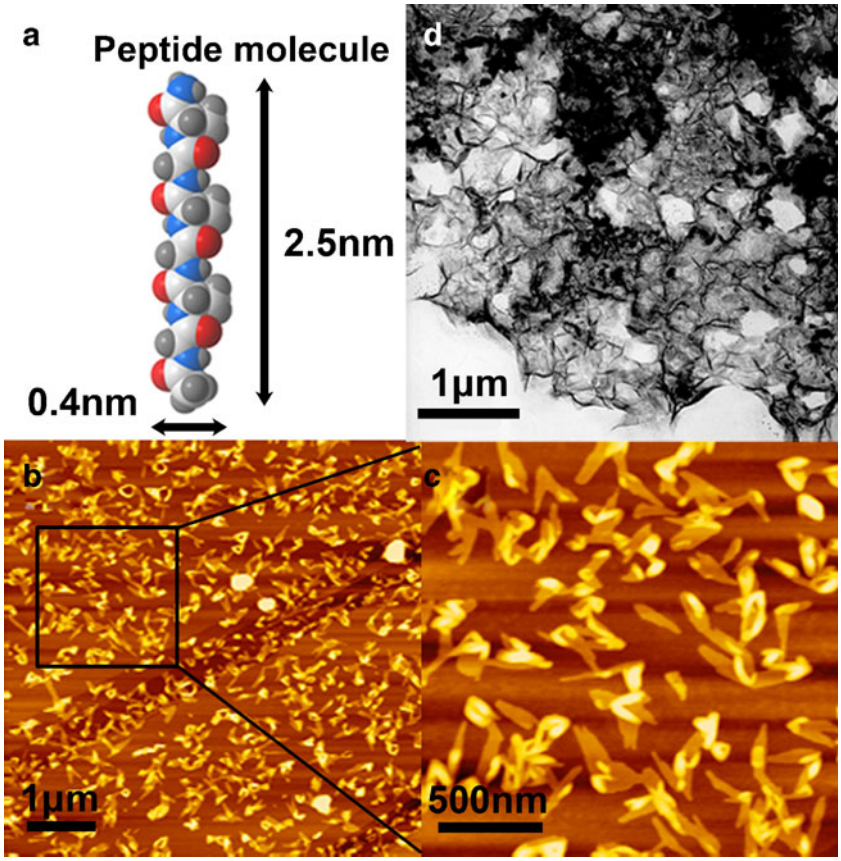

Fig. 1 The simulated space-filling molecular model of the peptide (**a**). The typical AFM morphological image of the peptide (**b**) and the magnified image inside the *black frame* (**c**). **d** TEM microphoto of the sample stained by phosphotungstic acid

self-assembly behavior of the peptide amphiphiles. Accordingly, K^+ ion was selected and added into the sample solutions. The typical concentration of GA6 solutions was 1 mg/ml with ion concentration of 20 mM. After the addition of KCl, the sample solution was stored at 4 °C overnight. After preparation of the sample on mica, AFM observation was carried out. It was observed that a kind of parallel, uniform fiber was formed by AFM images, shown in Fig. 2. According to AFM surface analysis, the aggregations of GA6 have diameters up to 300 nm and heights about 7.5 nm (calculated between two colored lines in Fig. 2d). The aggregations were quite uniform, in which the height difference of each filament is <0.4 nm on average, indicating a rather same inner nanostructure. Moreover, these fibers could extend to lengths ranging from several to tens of microns, but they never overlapped with one another, although some disconnections happened in the growth direction. Other ions were also added to GA6 solutions to investigate the specific ion effect of K^+. Samples with Na^+, Ca^{2+}, and Mg^{2+} ion at concentrations of 10, 20, 50, 100, and 200 mM in GA6 solution were studied. However, no directed aggregations were found in these samples. A sample with Na^+ is shown in Fig. 2e. Similar results had been reported before in the literature [27, 28].

pH was considered as another important factor in the natural silk spinning process, and it was reported to have effects on the conformational transition from silk I to silk II in *Bombyx mori* fibroin [29–31]. Therefore, we investigated the effect of pH on the directed self-assembly behavior of GA6 in the presence of K^+ ion by AFM observations. In order to avoid the influence of extraneous ions, all sample solutions were adjusted using HCl and NaOH in order to obtain pH values from 2.82 to 8.76. The AFM observations showed that the parallel fibers were formed at pH from 2.82 to 5.63, and no parallel fibers were detected at pH above 8.81, as shown in Fig. 3. AFM observation of the sample without K^+ at pH 8.61 was also undertaken, and no ordered structures were observed. It was indicated that an acidic pH environment possibly promotes the formation of the ordered structure; however, the shapeless deposition only appeared in the basic pH environment. When GA6 directly assembled across the mica surface, it sometimes showed three preferred orientations at 120° to each other, as shown in Fig. 3a, and the same pattern was sometimes observed in the other samples as well. According to previous reports [32, 33], it was indicated that the orientations of the peptide was assembled consistently with the underlying hexagonal atomic lattice of the mica surface [34].

It still remained unknown whether the conformation transition happened in solid or solution. CD spectroscopy is a useful method to study the conformation of proteins both in solution and solid. Therefore, CD measurement was carried

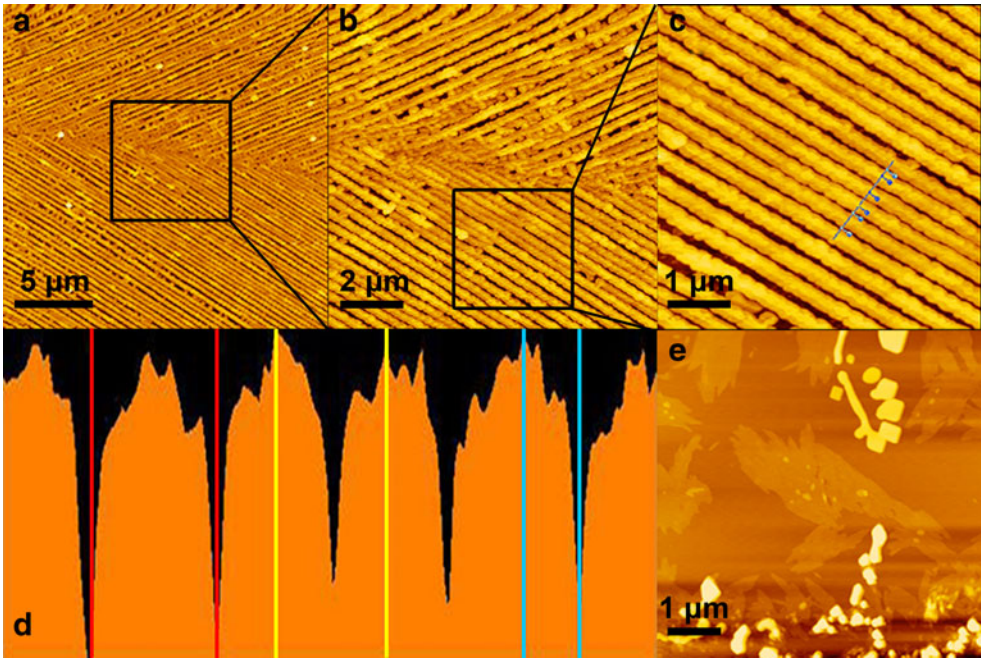

Fig. 2 The AFM images of parallel fibers (**a**). **b**, **c** Higher magnification of the images inside the *black frame*. **d** Longitudinal section profile for the corresponding selected line in **c** (adopting the scale scope of 1.57 μm×9.3 nm). **e** The sample prepared in Na^+ solution

out to study the secondary structure of the peptide in solution. The CD spectra of samples in solution shown in Fig. 4 revealed a strong negative peak near 194 nm and a weak negative peak near 224 nm. This spectrum indicated the absence of either α-helix or β-sheet structure in the sample solution. It had been concluded in some literatures that this type of CD spectrum was indicative of random coil structure [14, 35]. The solid-state CD measurement was carried out as well. The typical β-sheet spectrum was only observed in the presence of K^+ at acidic pH. Therefore, it was proved that, similar to those previous reports [36, 37], the β-sheet structure was only formed at a dehydrated state.

In order to investigate the interaction between peptide and substrate surface, AFM experiments were performed on a Mn^{2+}-modified mica. Before sample preparation, the freshly cleaved mica was immerged in solution of Mn^{2+} at a concentration of 0.01 mol/L for about 10 min and then washed several times with deionized water to obtain a positively charged surface [38]. However, no ordered structure was found on the modified mica surface. It was

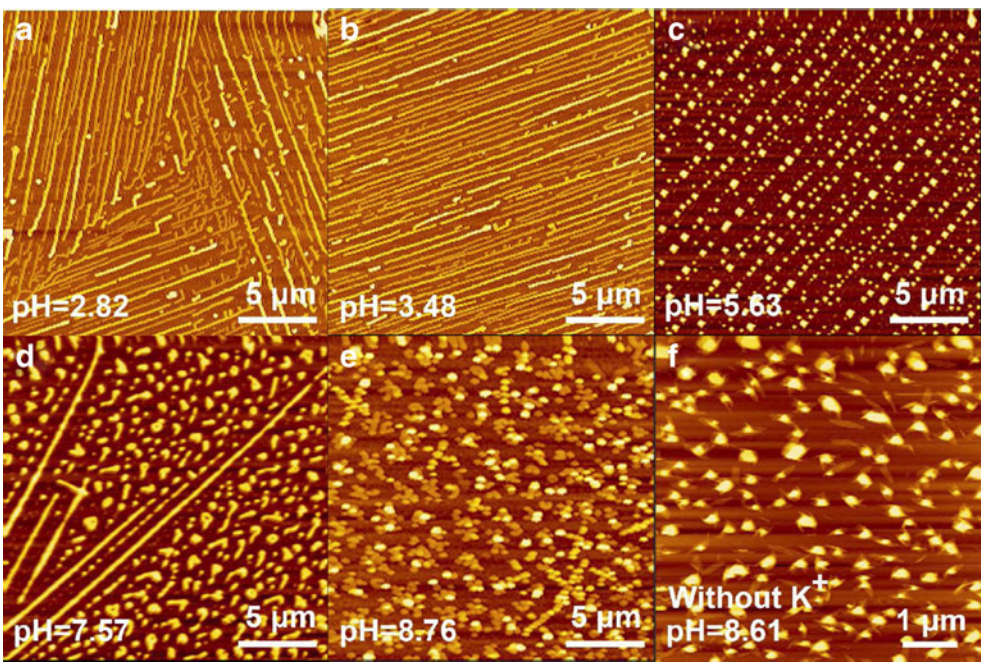

Fig. 3 Self-assembly morphology of peptide amphiphiles at different pH values on mica

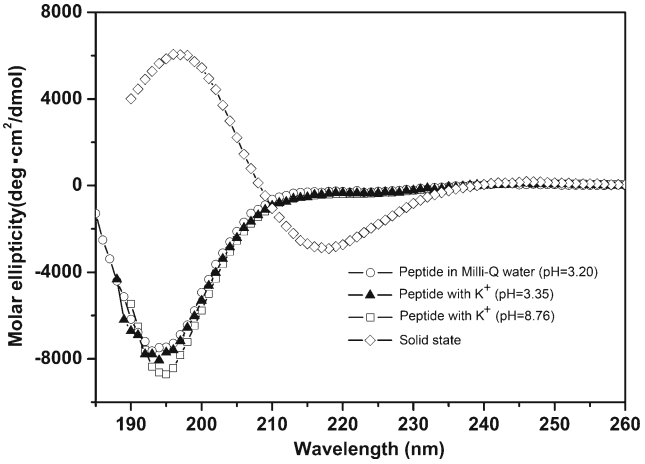

Fig. 4 CD examinations of the peptide in various conditions

indicated that the electrostatic interaction between positively charged peptide and negative mica surface might be the driving force for the directed self-assembly behavior.

It was reported that potassium ion and pH are both evolved in the conformation transition in silk protein [39, 40]. This may provide us a clue to further investigate the mechanism of the directed self-assembly. In the present work, FTIR spectroscopy was used to investigate the possible conformation transition of the peptide molecules due to its wide application in molecular conformation identification. Figure 5 shows the FTIR spectra of GA6 sample solutions with or without K^+ ion at different pH values. Generally, the region of 1,600–1,700 cm^{-1} for amide I band (stretching C=O) was investigated because of its strong characteristic absorbance and low interference from other chemical group vibrations. This band had been widely used to distinguish β-sheet from random coil or α-helix structure in natural biomaterial. The following attributions were commonly employed: 1,625–1,630 cm^{-1} to β-sheet; 1,650–1,655 cm^{-1} to α-helix; 1,660–1,665 cm^{-1} to random coil conformation [41, 42].

Figure 5a, b shows the spectra of GA6 in water at pH 8.61 and 3.20, while Fig. 5c, d shows the spectra of GA6 solution in the presence of K^+ ion at pH 8.76 and 3.35. In Fig. 5, according to the peak attribution in amide I band, the peak round 1,626 cm^{-1} was assigned to β-sheet, 1,650 cm^{-1} to α-helix, and 1,661 cm^{-1} to random coil. In Fig. 5a, two absorbances of C=O vibration assigned to α-helix and random coil were shown, which were stronger than that of the β-sheet, indicating the coexistence of α-helix, random coil, and β-sheet structure, while GA6 assembled in a basic solution. In Fig. 5b, c, absorbance around 1,626 cm^{-1} was increased, which indicated the increase of the content of the β-sheet structure. In Fig. 5d, the absorbance of β-sheet became predominantly strong. Therefore, it was evidenced that acidic environment and K^+ ion would help to facilitate the conformation transition from α-helix and random coil into β-sheet. In addition, the appearance of the peak around 1,668 cm^{-1} also revealed the predominance of β-sheet structure. According to the spectra previously discussed, it was indicated that the GA6 molecules underwent a poorly ordered assembly process in water because of the coexistence of α-helix, random coil, and β-sheet. However, acidic pH and K^+ ion facilitated the formation of only the β-sheet structure, causing a well-ordered packing of peptide molecules.

In order to interpret the fundamental mechanism of the template-directed process between peptide and mica surface, a hierarchical self-assembly model was proposed. Generally, the experimental results indicated that GA6 formed a template-directed ordered structure only in acidic environment and in the presence of K^+ ions on mica, and the conformation transition from the α-helix and/or random coil

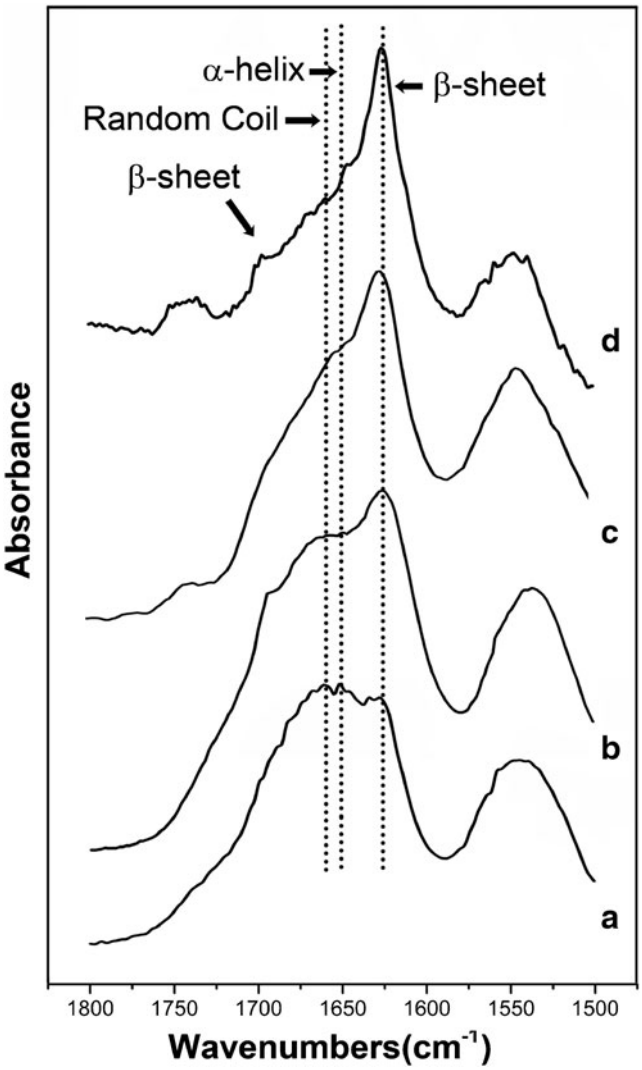

Fig. 5 FTIR spectra of GA6 in deionized water at pH 8.61 (*a*) and pH 3.20 (*b*) and with K^+ at pH 8.76 (*c*) and pH 3.35 (*d*)

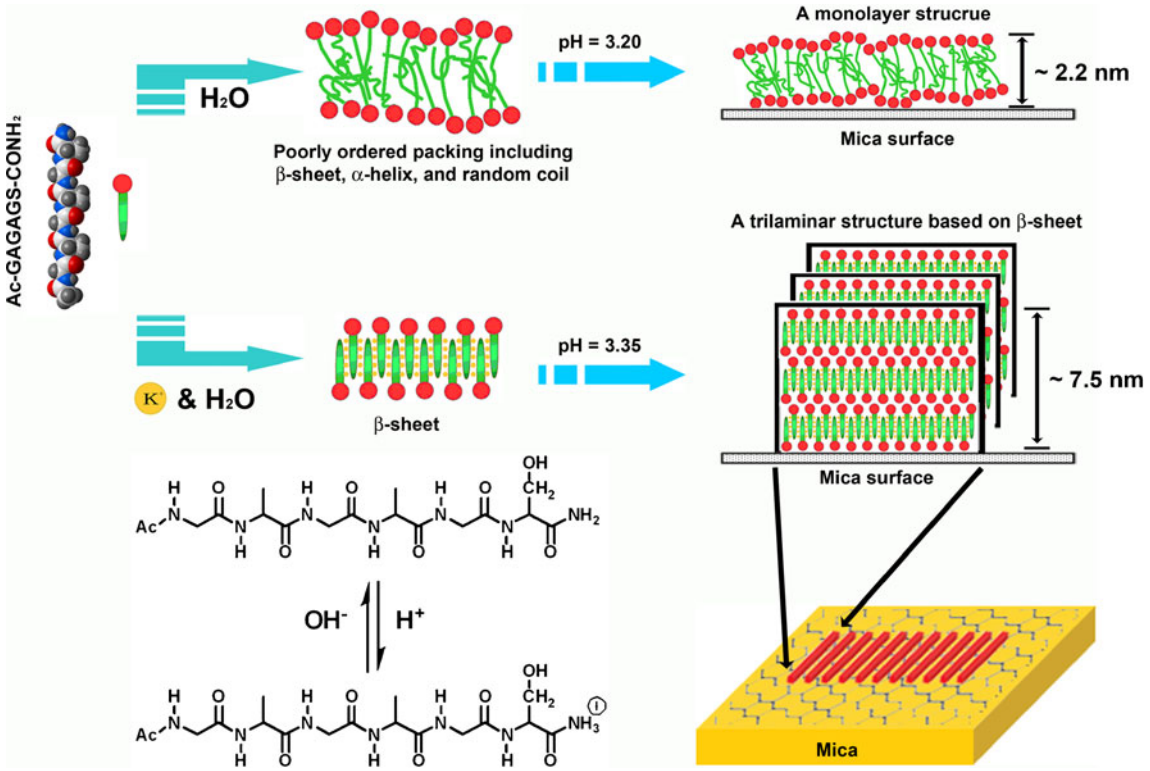

Fig. 6 Schematic representation of the proposed model for the template-directed self-assembly behavior

into β-sheet was the key point for this directed self-assembly behavior. It was because, in basic environment, hydrophobic interaction between peptide monomers resulted in the formation of spherical aggregations due to the absence of a charged group in the peptide monomer. In other words, no electrostatic interaction happened between the peptide and mica surface. On the contrary, in acidic environment, the peptide terminal ($-HH_2$) is positively charged and attracted by the negative mica surface by electrostatic interaction. However, the coexistence of α-helix, random coil, and β-sheet conformation retarded the ordered arrangement of the peptide molecules. Consequently, the height of feather-like structures is about 2.2 nm that is less than the length of GA6 (2.5 nm in length), indicating a poorly ordered packing structure. According to the FTIR spectra and the calculated length of AFM, it appeared reasonable that the peptide molecules may abut against an adjacent peptide chain adopting a monolayer structure and form a lamellar micelle aggregation. The micelle lied with their hydrophilic side in contact with the hydrophilic surface of mica. Finally, the addition of K^+ ion induced a conformation transition from random coil and α-helix into β-sheet. The interlocking structure of micelle put their hydrophilic side against the mica surface and oriented upright to the substrate, which is because the formation of β-sheet causes a stiff conformation of the molecule backbone. Then, the positively charged peptide molecules assemble into a uniform structure aligned along the atomic lattice of mica. Interestingly, the height of the parallel nanostructure is about 7.5 nm, which is just three times the length of the peptide. Therefore, the stiff monolayer structure overlapped into a trilaminar micelle structure (shown in Fig. 6).

As to the interaction between amphiphiles and the inorganic surface, it was reasonable that, because the hexapeptide was designed to be acetylated at the N termini and amidated at the C termini, the primary amine group at the end of the peptide has a pH-sensitive structural feature. Therefore, the molecule can bear positive charges or zero charge with changing of pH value. In this experiment, the peptide solution was adjusted to an acidic pH in the presence of K^+ so that the primary amine group was positively charged. Accordingly, the peptide became a charged amphiphile. On the other hand, the freshly cleaved mica surface was negatively charged due to the absence of K^+ on the surface and the excess of hydroxyl group 5 nm below its surface [43], and the atom lattice was considered as a hexagonal symmetry packing structure by previous reports [33]. While the peptide sample was in acidic environment, the positively charged amine group was attracted by the mica surface; thus, the peptide molecules were tightly absorbed on the mica surface. Furthermore, because of the hexagonal atomic lattice of the mica surface, it was probable that the peptide molecules were absorbed regularly along the atomic lattice and formed a parallel fiber structure. Therefore, the electrostatic interaction might be the driving force for the process of ordered assembly.

Conclusion

In this experiment, an ordered parallel nanostructure formed by template-directed self-assembly of a hexapeptide on mica surface was reported. Based on the experimental results, three influencing factors were involved in this assembly behavior: (1) K^+ ion was considered to promote the conformation transition of the peptide molecules from random coil and/or α-helix rich structure into a β-sheet predominant structure. (2) An acidic pH environment made the peptide molecule positively charged, which facilitated the well-ordered deposition on the negatively charged mica surface by electrostatic interaction. (3) The ordered hexagonal atomic lattice of the mica surface helped to enable the orientated deposition of peptide amphiphiles. Finally, a reasonable hierarchical self-assembly mechanism was proposed to interpret this phenomenon. Although further works are required to investigate the potential application of this kind of nanomaterial, this experiment provides a simple method to research the relationship between biomaterial and inorganic substrate and also brings us a potential physicochemical method to fabricate biological nanodevice just using simple molecular building blocks.

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