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The Robust Hydrogel Hierarchically Assembled from a pH Sensitive Peptide Amphiphile Based on Silk Fibroin

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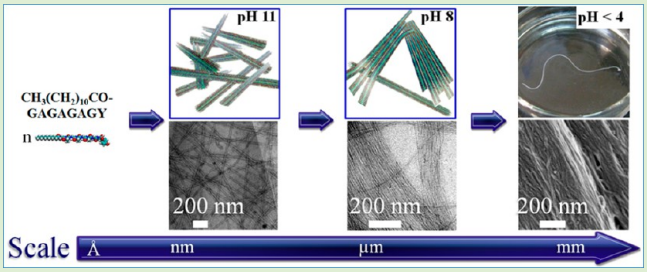
Supporting Information

ABSTRACT: Supramolecular polymers can be formed by self-assembly of designed subunits to yield highly ordered materials. In this paper, hierarchically structured materials, from molecules to nanofibers to macroscopical hydrogel, were fabricated by pH-induced assembly of C_{12}-GAGAGAGY, a peptide amphiphile (PA) based on silk fibroin. Due to the different acid dissociation constants of the carboxyl and phenolic hydroxyl groups on tyrosine residue (Y), the PAs showed unique pH sensitive assembly and aggregation behaviors. It was found that not only the molecular-scale assemblies of these PAs gradually changed from cylindrical nanofibers to nanoribbons with the decreasing of pH value from 11 to 8 but also most of nanoribbons aggregated into parallel bundles in such a case. Further decrease of pH value resulted in a hierarchically structured robust and plastic hydrogel, of which the rheological moduli reached around 10^5 Pa. Moreover, noodle-like hydrogel fibers with bundles of nanoribbons aggregated parallel along the long axis in them could be steadily prepared under shear force. Taking the pH-sensitive reversible sol−gel transition, high modulus and plasticity into account, the hydrogel is believed to have significant potential applications in tissue engineering or as the biocompatible adhesives.



INTRODUCTION

Many biomacromolecules in nature can self-assemble into functional nanostructures through noncovalent interactions such as hydrogen-bonds (H-bonds), electrostatic interaction, hydrophobic interaction and π/π stacking, etc. It is well-known that amyloid β-protein-caused neurotoxin effect assembles into nanofibers via intermolecular H-bonds,[1,2] single DNA chain couples sophisticated double helix structures via H-bonds among base pairs,[3] liposomes form bilayer structures via hydrophobic interactions,[4] and collagen chains form triple helix via electrostatic interaction and H-bonds.[5,6] Inspired by these interactions, scientists have synthesized a number of molecules that can self-assemble into various nanostructures, including micelles,[7] vesicles,[8] nanotubes[9,10] and nanofibers.[11−15] However, most of this research focused on the assembly process from molecules to nanoscaled aggregates. Although these kinds of aggregation virtually play the critical role in terms of the eventual properties,[16,17] the reports on manipulation of assembly at higher structural level, that is, further controlling the nanoaggregates to produce microstructures and macroscopical materials,[18,19] are rather sparse. In animal silk fibers, for example, silk protein (fibroin) chains fold into β-sheets, which further aggregate into crystalline region, and then both crystalline and amorphous regions are well organized to align with the long axis of silk fiber. Such hierarchical structures are considered to be the main contribution to the excellent mechanical property of the silk fibers.[20,21]

On the other hand, hydrogels have attracted great attention due to their versatile properties such as high water content and solid shape, which endow them potential applications in tissue engineering.[22,23] Until now, most hydrogels have been isotropic, which restricts their applications, such as cell scoffolds.[24,25] Recently, Zhang[26] and Huang[27] prepared gels composed of liquid-crystalline nanofiber bundles by heating and cooling peptide amphiphile solutions. In this light, fabrication of anisotropic hydrogels and one-dimensional filament gels by simple methods is required.

In our previous work,[28] a hydrolyzing method to obtain an octapeptide (GAGAGAGY) with glycine (G), alanine (A) and tyrosine (Y) from *Bombyx mori* silk fibroin and its derivative (C_{12}-GAGAGAGY) coupled with an alkyl tail was reported. It was found that such peptide derivatives behaved as the PAs and showed pH-sensitive assembling in a relatively low concentration (0.1 wt %) of aqueous solution. The C_{12}-GAGAGAGY was favored to stack into β-sheet laminates (nanoribbons) under acidic conditions (e.g., pH 4), as the terminal carboxyl groups were neutralized and the electrostatic repulsion was eliminated. Under alkaline conditions (e.g., pH 9), however, the −COOH groups of C_{12}-GAGAGAGY dissociated to $-COO^-$, and the PAs formed cylindrical nanofibers with planar β-sheets because of the electrostatic repulsion. At an even higher alkaline

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environment (around pH 11), the dissociation of phenolic hydroxyl groups had an extra effect on the assembling and aggregating of the PAs, as the pK_a of phenolic hydroxyl group on Y is about 10.[29] Following this, in consideration of the possibility to further produce the pH-sensitive organization from such nanofibers/nanoribbons, we aimed to construct the macroscopical materials with hierarchical structure via pH-induced self-assembly and organization of C_{12}-GAGAGAGY at a higher concentration (1 wt %) in this work.

EXPERIMENTAL SECTION

Materials. C_{12}-GAGAGAGY was synthesized and purified according to the literature.[28] GAGAGAGS was synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis on peptide synthesizer (PSI-200). C_{12}-GAGAGAGS was synthesized and purified in the same way as that of C_{12}-GAGAGAGY. Sodium hydroxide and hydrochloric acid were purchased from Shanghai Dahe Chemical Reagent Co. Ltd. (Shanghai, China) and were used without further purification.

Methods. *Preparation of the PAs Solutions and Gels.* C_{12}-GAGAGAGY was dissolved with 0.05 M sodium hydroxide solution to the concentration of 1 wt % and 3 wt %, respectively. Then, the pH values of the solutions were adjusted to 11 and 8 with 1 M hydrochloric acid, respectively. To prepare the hydrogel, the pH 8 solution was placed in a mold which was put into desiccators with a cup of concentrated hydrochloric acid for 24 h. C_{12}-GAGAGAGY solutions of concentrations from 0.1 to 1 wt % were also prepared to determine the lowest gelation concentration, which turned out to be around 0.2 wt %. The white noodle-like gel fiber was obtained by directly injecting pH 8 PAs aqueous solution into 0.1 M hydrochloric acid with a 1 mL syringe. Only sodium hydroxide and hydrochloric acid were used in the system to avoid the possible effect of conformation by other ions,[30] and the variations of ionic strength were quite small between solutions of different pH, compared with those of buffer systems.

Rheological Characterization. Frequency scanning experiments were performed on an Anton Paar MCR-301 rheometer. The PAs solutions of pH 11 and pH 8 were tested on a cone−plate (CP60−1/T1) at 25 °C from 100 to 0.1 rad/s, under 1% amplitude strain in linear regime. The hydrogel was tested on a plate−plate (PP-25) at the same condition. All samples were stabilized for 20 min before the measurement.

Transmission Electron Microscopy (TEM) Observation. Samples for TEM observation were prepared by pipetting one droplet of the PAs solution onto copper TEM grid and staining with uranyl acetate for 1 min. All samples were dried for 2 h in air before they were imaged on a Hitachi H-600 TEM, operated at 75 kV.

Cryo-TEM Observation. Samples were prepared in a controlled environment vitrification system (CEVS) at preset temperature. A 5 μL PAs solution with the concentration of 1 wt % was loaded onto a carbon-supported lacey TEM grid. The excess solution was blotted with a piece of filter paper, resulting in the formation of thin films suspended the mesh holes, and the grid was quickly plunged into a reservoir of liquid ethane (cooled by liquid nitrogen) at its melting temperature. The vitrified sample was then stored in liquid nitrogen until it was transferred to a cryogenic sample holder (Gatan 626) and observed by a JEM-2200 FS-TEM (200 KeV) at −174 °C. The image of which the phase contrast was enhanced by underfocus was recorded on a Gatanmultiscan CCD and processed with Digital Micrograph.

Field Emission Scanning Electron Microscopy (FE-SEM) Observation. C_{12}-GAGAGAGY hydrogel as well as fiber-like gel was frozen in liquid nitrogen, and then was freeze-dried by a FD-1B-50 freeze-dryer (Bilang, Shanghai). The sample was placed on a stub and sputtered with gold before being imaged by a Hitachi S-4800 Field Emission-SEM operated at 1.0 kV.

Small Angle X-ray Scattering (SAXS) Characterization. Samples were made by loading freeze-dried gel on sample stage. The vacuum measurements were carried on a NanoSTAR small-angle X-ray scattering system (Bruker, Germany) at 298 K using Cu Kα radiation at 45 kV and 0.65 mA. A Hi-Star detector (1024 × 1024 pixels) was positioned 108 cm behind the examined samples, resulting in a q-range of 0.14−2.0 nm^{-1}. The wave vector, q, was defined as $q = (4\pi/\lambda)\sin(2\theta/2)$, where 2θ is the scattering angle and λ is the wavelength of Cu Kα radiation (1.542 Å). The 2D SAXS patterns were azimuthally averaged and the background was subtracted using standard methods.

Polarizing Observation. A drop of C_{12}-GAGAGAGY solution was put on the glass slide gently and then sheared with a cover glass. The sample on glass slide was then observed by a BX51 Olympus microscope at room temperature. The pictures were collected by CCD at ISO 200, with exposure time 1 s.

RESULTS AND DISCUSSION

Solution/Gel Transition and Rheological Properties of the C_{12}-GAGAGAGY/H_2O System. It could be seen that the C_{12}-GAGAGAGY solution at pH 11 was stable and transparent with good fluidity (Figure 1a), while it turned to translucent

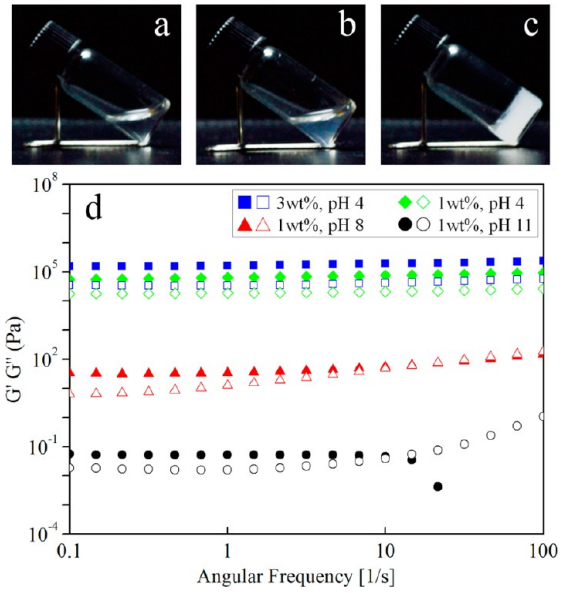

Figure 1. C_{12}-GAGAGAGY/H_2O system at (a) pH 11, (b) pH 8 and (c) around pH 4. (d) Storage modulus (G′, filled symbols) and loss modulus (G″, open symbols) versus frequency of various C_{12}-GAGAGAGY/water systems as concentration and pH noted.

fluid as the pH decreased to 8 (Figure 1b), suggesting the phase separation or the aggregation happened in the solution. In the case where the pH value of the solution was further adjusted to around 4, an opaque and self-supporting gel was formed immediately (Figure 1c). In order to make a comparison, we investigated the aggregation behavior of another peptide amphiphile, C_{12}-GAGAGAGS, which is with the similar primary structure but different C-terminal amino acid. It was noted that as to such PA, both the solution of pH 11 and pH 8 had opalescence, and it only turned into turbid liquid rather than gel at pH 4 or lower (Figure S1, Supporting Information).

The rheological measurement was carried on to further determine the situation of those PAs/water systems. It was found that both storage modulus (G′) and loss modulus (G″) of PAs solution were quite low at pH 11, and G′ was higher than G″ at most regions, which means the existence of a weak "network" self-assembled by C_{12}-GAGAGAGY molecules (Figure 1d, circle), although the solution was transparent as seen in Figure 1a. Moreover, the G′ was lower than the G″ at

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high frequency, suggesting that the sample was actually not a hydrogel at pH 11.[31]

The G′ and G″ increased by more than 2 orders of magnitudes when the pH value of the solution decreased to 8. Combined with the opalescence of the sample (Figure 1b), the aggregates get larger and tangle to networks, which leads to more pronounced properties of gel. However, these networks were still not strong enough to be real hydrogel.

When further decreasing pH to around 4, it showed that the modulus of the gel increased by 3 orders of magnitudes in comparison with those of the pH 8 sample, and the storage modulus over the entire frequency range exceeded the loss modulus by 1 order of magnitude. The complete opaqueness of the sample (Figure 1c) indicated the presence of larger aggregates and the formation of stronger networks. Furthermore, the gel was considered as physically cross-linked because it was obtained by simply changing the electric charge effect and the sol−gel process was found to be reversible.[32] Comparing with other physically cross-linked hydrogels,[33−36] interestingly, the gel showed a much higher modulus, for example, around 10^5 Pa at 1 wt %, and could be further improved to surpass 10^5 Pa at the concentration of 3 wt %.

Morphologies of C_{12}-GAGAGAGY Aggregates. The morphologies of C_{12}-GAGAGAGY aggregates at different pH values were investigated in detail by TEM. According to our previous report, cylindrical nanofibers of C_{12}-GAGAGAGY were formed at alkaline conditions. It could be observed that a number of nanofibers with diameter of 10 nm and length over several micrometers were produced at the relatively high concentration of C_{12}-GAGAGAGY, pH 11 (Figure 2a). This phenomenon was in good agreement with the rheology result which indicated the existence of C_{12}-GAGAGAGY assembly even at high pH value. Nevertheless, the nanofibers were randomly distributed in the image, suggesting the well dispersion of those nanofibers in the solution which corresponded to the fluidic and transparent properties of C_{12}-GAGAGAGY aqueous solution at pH 11. Indeed, such well dispersion of the nanofibers was independent of the concentration of C_{12}-GAGAGAGY, mainly because of the dissociation of carboxyl groups and phenolic hydroxyl groups located in the periphery of the cylindrical nanofibers. In comparison, nanofibers of C_{12}-GAGAGAGS were formed under pH 11 (Figure S2, Supporting Information).

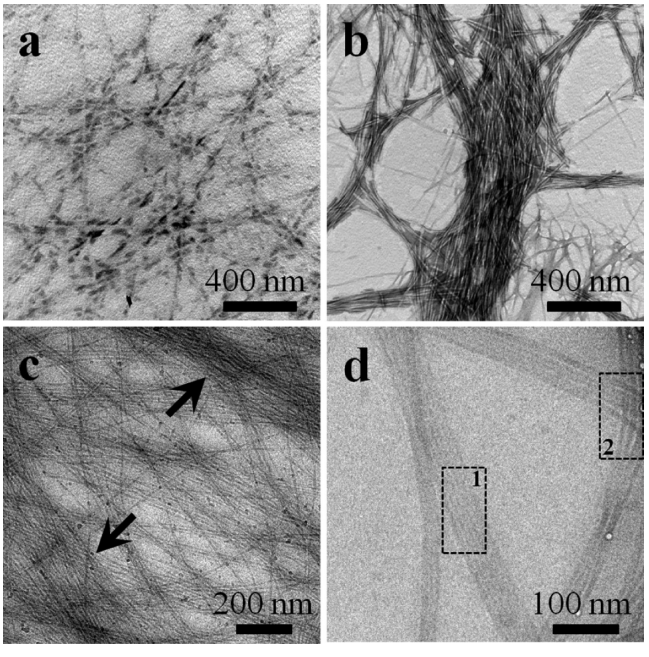

Figure 2. TEM images of C_{12}-GAGAGAGY assembly and aggregates (negatively stained by uranyl acetate) at (a) pH 11 and (b) pH 8. (c and d) Cryo-TEM images of C_{12}-GAGAGAGY assembly and aggregates at pH 8. The concentration of C_{12}-GAGAGAGY was 1 wt %.

When the pH value of the solution was decreased, the assemblies of C_{12}-GAGAGAGY tended to aggregate parallel with each other because of the neutralization of phenolic hydroxyl groups. It could be found that most assemblies parallelly aggregated to bundles with the width between 100 to 500 nm at pH 8 (Figure 2b), besides a few separated nanofibers. Compared with the cylindrical nanofibers at pH 11, these aggregates were larger in size and furcated into recognizable domains, in accordance with the opalescence of the solution at pH 8 (Figure 1b). Moreover, "network structure" formed by such bundle-like aggregates induced the notably high modulus (G′ and G″) of C_{12}-GAGAGAGY aqueous solution measured by rheometer (Figure 1d, triangle). However, the TEM samples were prepared by drying at room temperature, and the bundles of C_{12}-GAGAGAGY assemblies imaged might be high concentration dependent. Therefore, the cryo-TEM was employed to clarify this issue. It could be found that the domains parallelly aggregated by assemblies still existed in the 1 wt % concentration C_{12}-GAGAGAGY aqueous solution (Figure 2c, pointed by black arrows). According to the parallel configuration of the aggregates, it was speculated that the assemblies tend to be nanoribbons with approximate rectangular cross sections,[26] though at a weak alkaline environment. At such pH, the phenolic hydroxyl groups were neutralized while most of the carboxyl groups were dissociated, offering the possibility to form hydrogen bonds between $-COO^-$ and phenol hydroxyl. When the concentration of the solution was increased to a certain degree, the nanoribbons interacted with each other through H-bonds on both sides and assembled parallel to the larger aggregates. However, the twisted structure of the ribbons which was common in the low concentration was not found in our case, probably due to the fixed nature of H-bonds. Interestingly, the images in Figure 2d not only display the bundles of C_{12}-GAGAGAGY assemblies consisting of several parallel nanoribbons with 10 nm in width (dashed box 1 in Figure 2c) but also show the split tendency of those bundles (dashed box 2 in Figure 2c) which possibly caused the furcation of the aggregates to form a network because of the residual electrostatic repulsion between the nanoribbons.

Aggregate Structure of C_{12}-GAGAGAGY Hydrogel. In the case that the pH value was further decreased from 8, the C_{12}-GAGAGAGY aqueous solution gellated immediately. Such gelation process was concentration and temperature dependent, but it mainly relied on the pH. The intrinsic morphology of the C_{12}-GAGAGAGY hydrogel was investigated by FE-SEM after freeze-drying. It could be seen that the dried gel was composed of a large amount of microfibers with a diameter of 40−60 nm and a length over several micrometers (Figure 3), coinciding with those nanoribbon assemblies imaged by cryo-TEM (Figure 2c and d). Apparently, a few microfibers could further aggregate parallel to the domains with diameters over 200 nm in size. Different from that of the gel formed by entanglement,[37] the framework of our PAs hydrogel was

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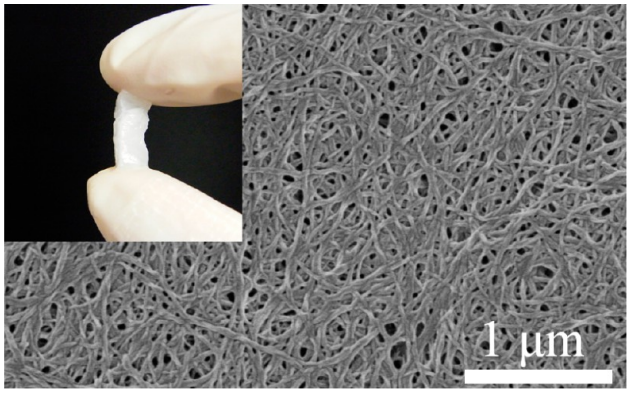

Figure 3. FE-SEM image of C_{12}-GAGAGAGY hydrogel (1 wt %) after freeze-drying. (Inset) Hydrogel.

hierarchically assembled by C_{12}-GAGAGAGY, in the terms of parallel stacking which shared large interaction areas. Therefore, such a hydrogel ultimately exhibited the significant mechanical properties. For example, both G′ and G″ of the C_{12}-GAGAGAGY gel (10^4–10^5 Pa, Figure 1d, diamond) formed at the concentration of 1 wt % were much higher than those of regenerated silk fibroin hydrogel (10^1–10^3 Pa)[38] and regenerated silk fibroin/hydroxypropylcellulose hydrogel (10^2–10^3 Pa)[39] developed from a random nanofibrillar network at the same concentration, although the molecular weight of C_{12}-GAGAGAGY was rather small. Since the generic elastic modulus of glassy polymers are around 10^9 Pa[40] and the modulus is proportional to the number of effective chains per unit volume at certain temperature,[41] the 10^5 Pa modulus of this 1 wt % concentration hydrogel suggested that the framework of the supramolecular polymer hydrogel contributed evenly to the gel strength. Moreover, this silk fibroin-based PAs system had a much better homogeneity than regenerated silk fibroin, which endowed them with higher modulus.

SAXS was employed to further investigate the framework of freeze-dried C_{12}-GAGAGAGY gel (Figure S3, Supporting Information). The peak at q = 0.120 Å^{-1} supported that the nanoribbon bundles had a periodic structure with d-spacing value of 5.2 nm, calculated by $d = 2\pi/q$.[13,42] This value was approximately equal to the height of β-sheet lamellas assembled from silk-like fibroin $(AG)_3$ reported in the literature (5.1 nm, 1.7 nm per double layer of two β-sheets).[17] As the stacking of a double layer of two β-sheets has a height of 1.6 nm, evaluated by atomic force microscope,[28] most of the nanoribbon bundles were believed to be stacked by 6 layers of β-sheet lamella, which aggregated parallel to nanoribbon bundles through hydrophobic interactions and H-bonds at the low pH value.

Regarding the birefringence of those samples we prepared at different pH values and processes, the images from a polarization microscope are provided in Figure 4. Obviously, no birefringence was observed in the aqueous solution of C_{12}-GAGAGAGY at pH 11 (Figure 4a), even when the sample was slightly sheared. However, obvious funiform birefringence texture could clearly be seen in the solution of C_{12}-GAGAGAGY at pH 8 (Figure 4b) as well as in the hydrogel at lower pH (Figure S4, Supporting Information). In combination with the observation of TEM, such anisotropy was attributed to the parallel aggregate of the nanoribbon bundles. In the case where the solution was sheared between the slide and coverslip, the birefringence texture oriented

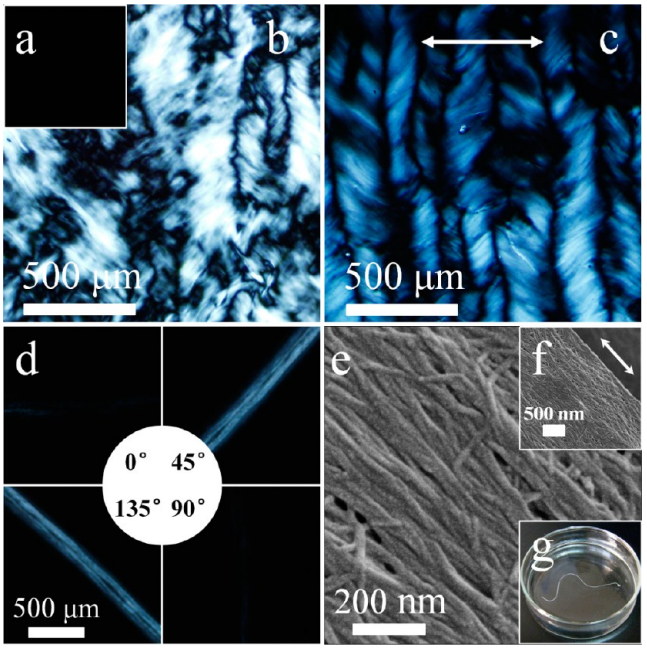

Figure 4. Polarized optical images of C_{12}-GAGAGAGY at (a) pH 11, (b) pH 8 and (c) pH 8 after shear (shear direction is showed by arrow); (d) 45 degree extinction of gel fiber under polarization microscope; (e) and (f) FE-SEM image of a gel fiber (long axis direction of the fiber is shown by arrow); (g) noodle-like gel fiber.

toward the shear direction (Figure 4c), presenting the typical characteristics of nematic liquid crystal.[43,44]

Inspired by the orientation of liquid crystal after shearing and the high modulus of the hydrogel, the macroscopical gellated fiber (Figure 4g) of C_{12}-GAGAGAGY with the significant mechanical properties was obtained by injecting the solution of pH 8 into 0.1 M HCl solution (acting as coagulating bath, Supporting Information). The evidence of 45° extinction of the birefringence in such gel fiber, that is, the maximum lightness appeared at 45° or 135° and the minimum lightness appeared at 90° or 180° was displayed in Figure 4d, indicating that the aggregates oriented well. Consistently, FE-SEM image showed that the fibrous aggregates directed along with the long axis of gel fiber (Figure 4e,f).

Unsurprisingly, the solution–gel transformation of C_{12}-GAGAGAGY was reversible as the hydrogel could be redissolved in aqueous alkali, because such hierarchical assembling mainly resulted from the dissociation and neutralization which was indeed pH sensitive. We tried to exploit the PAs solution as reversible adhesive, whose adhesive force could be adjusted by pH value. The gel was used to glue two pieces of glass slide, for example, and the adhesive force could be surpass 400 N/m^2 (Figure S5, Supporting Information). A video in the Supporting Information illustrates that the PAs solution can be used as controllable adhesive, which was durable in water and acid solution while lost efficacy in alkaline solution. Moreover, a compression test to the hydrogel showed a compression modulus of around 10^4 Pa (Figure S6, Supporting Information).

CONCLUSIONS

As described in Scheme 1, by simply adjusting the pH of the solution, hierarchical self-assemblies of C_{12}-GAGAGAGY at relatively high concentration were approached, and the

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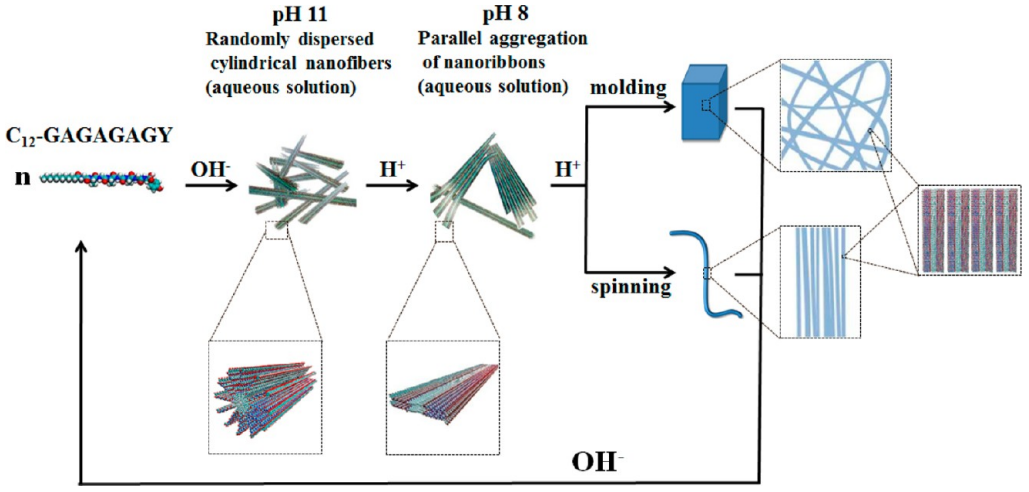
Scheme 1. pH-Induced Hierarchical Self-assembly of C_{12}-GAGAGAGY



hydrogel with significant mechanical properties was obtained. At pH 11, C_{12}-GAGAGAGY self-assembled into well-dispersed cylindrical nanofibers similar to those previously reported at low concentration. When the pH decreased to 8, however, the formed nanoribbons aggregated into parallel bundles because of the bonding of H-bonds between COO− and phenolic hydroxyl groups on both sides of the nanoribbon. Further decrease of pH value led to the formation of a hydrogel with a high modulus, which was attributed to the network structure connected by such bundles. Moreover, a kind of noodle-like gel fiber consisting of oriented fibrous nanoribbon bundles was spun when the aqueous solution of hydrogen chloride worked as the coagulating bath. These results not only shed a light on understanding the organization of various molecules at different levels but also inspired us to control the hierarchies of self-assembly in PAs-based materials by simply balancing the noncovalent interactions such as H-bond, electrostatic interactions, and hydrophobic interactions.

■ ASSOCIATED CONTENT

Supporting Information

Images of the C_{12}-GAGAGAGS/H_2O system at different pHs, TEM image of C_{12}-GAGAGAGS, SAXS data of C_{12}-GAGAGAGY hydrogel, POM image of the hydrogel, compression test result of C_{12}-GAGAGAGY hydrogel, and two videos describing PAs solution as adhesive and noodle-like gel acquiring. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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