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Optical Property Modulation of Fmoc Group by pH-Dependent Self-Assembly

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The modification of short peptides with the 9-fluorenylmethyloxycarbonyl group (Fmoc) results in a very efficient self-assembly propensity of these building blocks. Nevertheless, the influence of self-organization on the optical properties of the Fmoc group *per se* is still not fully understood. We envision that Fmoc-modified 5-aminopentanoic acid (Fmoc-5), which has similar molecular dimensions to the highly studied Fmoc-diphenylalanine peptide, could serve as a simple non-peptide model that possesses inherently profound self-organization properties without amide-backbone contributions. Herein, we demonstrate that Fmoc-5 molecules self-assemble to form plate-like crystals at pH 2.0, where Fmoc groups are mainly organized in anti-parallel arrangements and form 2-D quantum-well confined structures (2-D QW), exhibiting a dominant fluorescent emission peak at 324 nm and a step-like absorbance from 260 to 300 nm. At pH 10.0 Fmoc initially exhibits its inherent optical properties, since Fmoc-5 hydrolyses and cleaved Fmoc groups self-assemble to form nanovesicles which further coalesce with each other, a new emission peak at 467 nm, and a quasi-continuous absorbance emerges and dominates in fluorescence and UV-vis absorption. Our findings suggest that the optical properties of Fmoc could be modulated through pH-dependent self-assembly.

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Introduction

9-Fluorenylmethyloxycarbonyl (Fmoc) is well-known as the main amine protecting group in peptide synthesis.[1-4] Owing to its intrinsic hydrophobicity and aromaticity that promote hydrophobic and π-π stacking interactions, Fmoc-modified dipeptides, which are much more advantageous than longer peptides or proteins due to their simple and cost-effective synthesis, possess excellent self-assembly properties and can be employed as ideal low-molecular-weight hydrogelators.[5-9] Since Fmoc-modified diphenylalanine (Fmoc-FF), a dipeptide derived from the core recognition motif of Alzheimer's disease β-amyloid polypeptide,[10] was found to self-assemble to form a rigid hydrogel and utilized as a notable cell culture scaffold,[5,11-14] the self-assembly of Fmoc-modified dipeptides have attracted increasing interest. By now, a variety of Fmoc-modified dipeptide sequences have been designed and their self-assemblies, especially hydrogelation, have been studied.[5-8,11-26] In addition, the introduction of enzymatic response,[27-31] referring to enzyme-triggered hydrolysis and coupling, endowed the self-assembly of Fmoc-modified dipeptides in a controllable manner, such as spatiotemporal dependent organization and disassociation.[21,22,29,32,33]

However, despite the extensive work performed regarding the hydrogelation and mechanical properties of Fmoc-modified dipeptides, investigations on their optical properties,[34] especially the influence of self-assembly on the optical properties of Fmoc, specifically referring to the highly UV-active and fluorescent fluorenyl ring,[35,36] are not fully understood. It should be noted that a previous study by Kaminski *et al.* reported that peptide-based self-assemblies possess an intrinsic fluorescence, which is probably overlap with the optical phenomenon of Fmoc.[37] Therefore, in this work, to avoid the potential interference by the intrinsic optical properties of peptides, Fmoc-5 was chosen as a simple non-peptide model to investigate the influence of self-assembly on the optical properties of Fmoc. By using EM and spectroscopic characterizations, the different fluorescence and UV-vis absorbance of Fmoc mediated by pH-dependent self-assembly were elucidated.

Materials and Experimental Methods

Materials

Fmoc-5-aminopentanoic acid (Fmoc-5) was purchased from GL Biochem (Shanghai) Ltd. and was used as received without further purification. Hexafluoroisopropanol (HFIP) was purchased from Sigma-Aldrich. The water used was processed by a Milli-Q purification system (EMD Millipore, Billerica, Massachusetts, USA) with a minimum resistivity of 18.2 MΩ•cm.

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Sample preparation

50 µL of a fresh HFIP stock solution of Fmoc-5 at 100.0 mg mL^{-1} was mixed with 950 µL of water to form a 1 mL sample solution with Fmoc-5 at a concentration of 5.0 mg mL^{-1}. The solution pH was adjusted to 2.0 ± 0.2 with a 1.0 M HCl or 10.0 ± 0.2 with a 5.0 M NaOH aqueous solution.

Scanning electron microscopy

20 µL solution samples were placed onto a clean glass slide. After being allowed to adsorb for 5 min and removing excessive liquid with filter paper, the slide was coated with Cr and observed under a JSM-6700 field emission scanning electron microscope (JEOL, Tokyo, Japan) operated at 10 kV.

Transmission electron microscopy

10 µL solution samples were dropped onto a 400-mesh copper grid covered with carbon-stabilized Formvar film (SPI, West Chester, PA, USA) and allowed to adsorb for 2 min before blotting of excess fluid. Afterwards, 10 µL 2% (wt.%) uranyl acetate solution was dropped on the grid and allowed to stain for another 2 min before blotting of excess fluid. TEM micrographs were recorded under a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

Fluorescence

600 µL solution samples were pipetted into a 1.0 cm path-length quartz cuvette and a fluorescence emission spectrum was collected on a FL3-11 Spectrofluorometer (Horiba Jobin Yvon, Kyoto, Japan) at ambient temperature. The excitation and emission wavelengths were set at 280 nm and 300-550 nm, respectively, both with a slit of 2 nm. A HFIP solution of Fmoc-5 at 5.0 mg mL^{-1} was used as a control.

UV-vis absorbance

50 µL solution samples at pH 2.0 or 2 µL solution samples at pH 10.0 were diluted with a 150 µL HFIP/water mixture (1/20) at pH 2.0 or a 198 µL HFIP/water mixture (1/20) at pH 10.0 in a 96-well UV-Star UV transparent plate (Greiner BioOne, Frickenhausen, Germany). UV-vis absorbance was then recorded under a Biotek Synergy HT plate reader (Biotek, Winooski, VT, USA), with a normal reading speed and calibration before reading. Blank HFIP/water mixtures (1/20) at pH 2.0 and pH 10.0 were used as backgrounds and subtracted. A HFIP solution of Fmoc-5 at 5.0 mg mL^{-1} was used as a control.

Figure 1. (A) Visual observation of the white precipitate at pH 2.0 after 20 days of incubation and (B) the solution evolution vs. time at pH 10.0 after dissolving Fmoc-5 in a HFIP/water mixture

Results and Discussion

Characterization of the self-assembly behaviour of Fmoc 5-aminopentanoic acid

The self-assembly of Fmoc-5 was induced by briefly dissolving the building block in HFIP to form a stock solution, then dilute it with water at an acidic pH (pH 2.0), a white precipitate immediately emerged, as shown in Figure 1A. As the pH increased, however, the precipitate gradually dissolved until it completely disappeared when a stronger basic pH was used (pH 10.0), inducing a transformation to a transparent solution, as shown in the first picture (0 d) in Figure 1B. However, the solution was not thermodynamically stable, and it gradually became yellow and turbid with time, which made it impossible to obtain a pH-dependent phase diagram. After 20 days at ambient temperature, the solution became thoroughly opaque and filled with yellow suspensions, followed by massive precipitation after 30 days, as shown in Figure 1B.

It is well-known that the amide bond between Fmoc and amino groups is resistant to acid treatment but is easily cleaved by bases.[1,2] Correspondingly, we speculated that the phase evolution at pH 10.0 results from Fmoc cleavage and aggregation. To test our hypothesis, mass spectroscopy was utilized for the analysis of the centrifuged precipitates after 20 days of incubation. For the specimen at pH 2.0, the molecular ion peaks of Fmoc-5 and its dimers were easily observed in the MS spectrum, despite the fact that free 5-aminopentanoic acid was also detected (we assumed that it was produced by the fragment ion peak of Fmoc-5 during the MS experiment). However, for the specimen at pH 10.0, no signal of Fmoc-5 could be detected. Instead, numerous peaks corresponding to fluorenyl-based oligomers were detected (as shown in Figure S1 and Table S1 in SI). The results indicate that, as opposed to its stability at pH 2.0, Fmoc was indeed cleaved from Fmoc-5 and aggregated to precipitate at pH 10.0.

The precipitate morphologies were further studied using

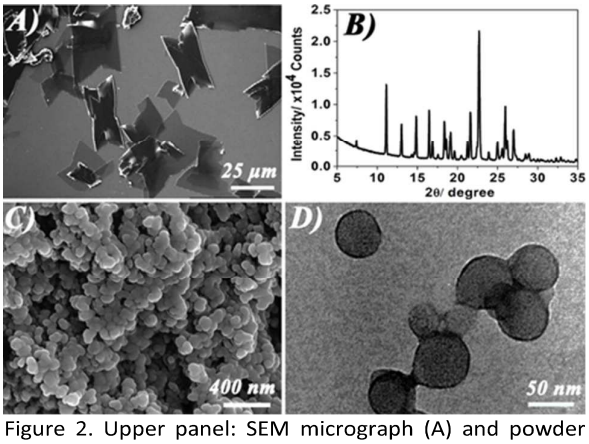
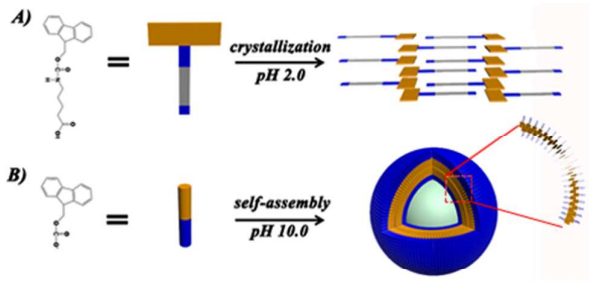


Figure 2. Upper panel: SEM micrograph (A) and powder XRD spectrum (B) of the white precipitate after 20 days of incubation at pH 2.0. Lower panel: SEM (C) and TEM (D) micrographs of the yellow precipitate after 20 days of incubation at pH 10.0.



Scheme 1. Schematic representations of Fmoc organization to anti-parallel arrangements in Fmoc-5 crystals at pH 2.0 (A) and hollow nanovesicles at pH 10.0 (B). The yellow moiety represents the aromatic fluorenyl ring, the blue moiety represents the hydrophilic amine and carboxyl groups, and the grey moiety represents the aliphatic chain.

electron microscopy (EM). Figure 2A shows that the white precipitate at pH 2.0 is composed of plate-like structures, which were proved to be crystals by the powder XRD spectrum presented in Figure 2B. Moreover, the yellow precipitate formed at pH 10.0 was indeed composed of aggregates of nanospheres, as shown in Figure 2C, which was also confirmed by observing individual nanospheres and discrete, smaller nanoclusters from SEM imaging of the sample after 7 days of incubation (as shown in Figure S2 in SI). In addition, the TEM result illustrates that the nanospheres have a distinctly darker contour and a relatively paler inside (Figure 2D), with a statistical outer diameter of 55.0 ± 14.1 nm and a wall thickness of 1.9 ± 0.5 nm (as shown in Figure S3 in SI), suggesting that these nanospheres are actually hollow nanovesicles. Considering the dimensional length of the Fmoc group (~8.6 Å),[20] we assume that at pH 10.0 cleaved Fmoc groups self-assemble to a tail-to-tail bilayer (~8.6 Å × 2= ~1.7 nm, similar to that measured at 1.9 nm), with fluorenyl rings encapsulated inside and arranged through hydrophobic and π-π stacking interactions, while the hydrophilic carboxyl groups are distributed outside. The bilayers then self-closed to form nanovesicles that decrease the exposure of the hydrophobic region, as shown in Scheme 1B. The coalescing properties indicate that the nanovesicles can further aggregate to form massive clusters. As a control, no nanoscale self-assemblies were detected under AFM or DLS of a 5-amino-pentanoic acid solution at pH 10.0 (data not shown), suggesting that the nanovesicles were indeed self-assembled by cleaved Fmoc.

Fluorescence analysis of Fmoc moiety

The different precipitate colours suggest that the optical properties of the self-assemblies must differ at pH 2.0 and pH 10.0. Fluorescence spectroscopy was performed to investigate the effect of pH-dependent self-assembly on the optical properties of Fmoc, due to the fact that the different arrangements of aromatic rings (H- and J-aggregates) can induce distinct shifts of the emission peak positions (blue- and red-shifts). The sample at pH 2.0 showed an emission peak at 324 nm, characteristic of an anti-parallel arrangement of fluorenyl rings,[23,38] accompanied by a weak, wider peak at 387 nm, characteristic of a parallel arrangement of fluorenyl rings,[23] as shown in Figure 3A. As a control, Fmoc-5 in HFIP exhibited only one emission peak at 315 nm, characteristic of free Fmoc group without self-assembly (Figure 3A).[23] Combined with the EM result, we believe that during Fmoc-5 crystallization, Fmoc groups aggregate with each other mainly in anti-parallel arrangements through hydrophobic and π-π overlapping interactions, among which some parallel arrangements are also formed at intervals, as shown in Scheme 1A. The spectral shape did not change with time, indicating that the self-assemblies were considerably stable, consistent with the results above. However, for the sample at pH 10.0, at the beginning (0 d) the two peaks were located at 317 nm and 370 nm, respectively, as shown in Figure 3B. The blue-shift relative to those at pH 2.0 indicate that at this time point a large part of the Fmoc groups remained free and did not participate in the aggregation. With time, however, the former peak was red shifted towards 330 nm and the later peak gradually vanished. Instead, a new dominant peak at 467 nm emerged, characteristic of an extensive J-aggregation of the aromatic fluorenyl rings.[10,23,38] By plotting the intensity ratio at 467 nm and at 317 nm vs. time, we found that the value exponentially reached ~32-fold within 16 days; thereafter, it slowly decreased with sedimentation (as shown

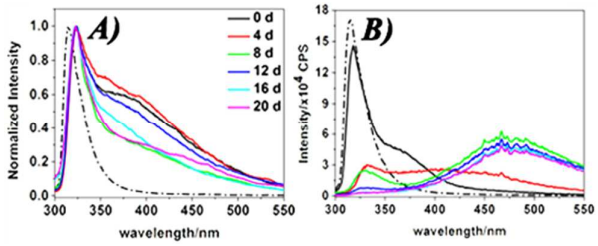


Figure 3. Fluorescent emission spectra vs. time for the samples at pH 2.0 (A) and pH 10.0 (B). Note that the excitation wavelength was set at 280 nm, and the spectrum of Fmoc-5 in HFIP was added and denoted as a dash-dot line as a control.

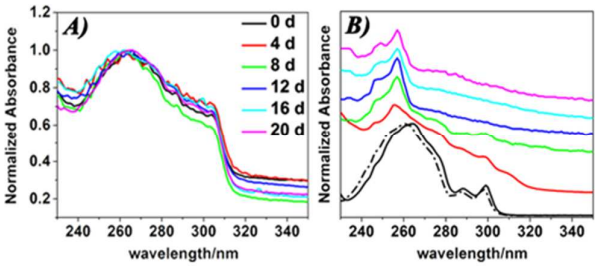


Figure 4. UV-vis absorption vs. time for the samples at pH 2.0 (A) and pH 10.0 (B). Note that the spectrum of Fmoc-5 in HFIP was added and denoted as a dash-dot line as a control in (B).

in Figure S4 in SI). We assume that this phenomenon occurs due to the fact that as cleaved Fmoc self-assembles, it allows nanovesicles to form and coalesce, and increasingly more fluorenyl rings participate in π-π stacking and form extensive J-aggregates, resulting in the fluorescence red-shifts and intensity evolution. As a control, the ratio for the sample at pH 2.0 was constantly near 0 regardless of time.

UV-vis absorption analysis of Fmoc Moiety

For the UV-vis absorption, Fmoc-5 in HFIP exhibited the intrinsic UV-vis absorbance of Fmoc groups, displaying three main peaks at 264, 288, and 299 nm, as shown in Figure 4B.[34] After crystallization at pH 2.0, it exhibited a step-like absorption spectrum in the range of 260-300 nm, accompanied by a weak peak at 304 nm, as shown in Figure 4A. The absorption feature remained stable independent of time. However, the sample at pH 10.0, initially (0 d) exhibited the intrinsic UV-vis absorbance of Fmoc with a spectrum similar to that of the Fmoc-5 monomers in HFIP, indicating that Fmoc was free and did not aggregate, consistent with the fluorescence result and the transparency of the solution. However, with time, the main peak at 264 nm blue shifted to 257 nm and the other peaks gradually disappeared, and instead, the absorption curve became smooth, as shown in Figure 4B. It is known that the optical absorption is determined by the density of the electronic states (DOS),[39] which is characterized by a quasi-continuous variation as a function of energy for 3-D crystalline bulky materials while by a step-like behaviour for 2-D QWs.[14,39] Consequently, we can conclude that Fmoc groups form 2-D QW structures in Fmoc-5 crystals at pH 2.0, leading to the observed step-like absorption. It is likely that the confined dimensions are located along the thickness direction, which is in the nanometric range (as shown in Figure S5 in SI). The additional peak at 304 nm, which originated from the excitons resulted by strengthened Coulomb interactions between electrons and holes, further confirmed the QW character.[14,39] We assume the arrangements of the fluorenyl entities are the key parameters in the determination of the electronic QC structures, and now working on the theoretical calculations to estimate these parameters.[14,40] While at pH 10.0, the solution initially exhibits the absorption characteristics of free Fmoc; however, as nanovesicles are self-assembled and coalesce extensively, cleaved Fmoc groups aggregate into 3-D bulky materials, resulting in quasi-continuous absorption properties. And the blue-shift may be contributed to the formation of H-aggregates by Fmoc groups during self-assembly.

Conclusions

In conclusion, a new non-peptidic model of Fmoc-modified building blocks was developed, and the effect of its self-assembly on the optical properties of Fmoc was studied. We demonstrarted that the fluoresscence and UV-vis absorbance of Fmoc could be easily controlled through pH-dependent crystallization or hydrolysis-assembly of Fmoc-5-aminopentanoic acid. Our findings offer an optical approach for investigating the dynamics and mechanism underlying self-assembly, and also offer an inexpensive, organic alternative for substituting traditional QC materials, such as GaAs, in photonic devices.

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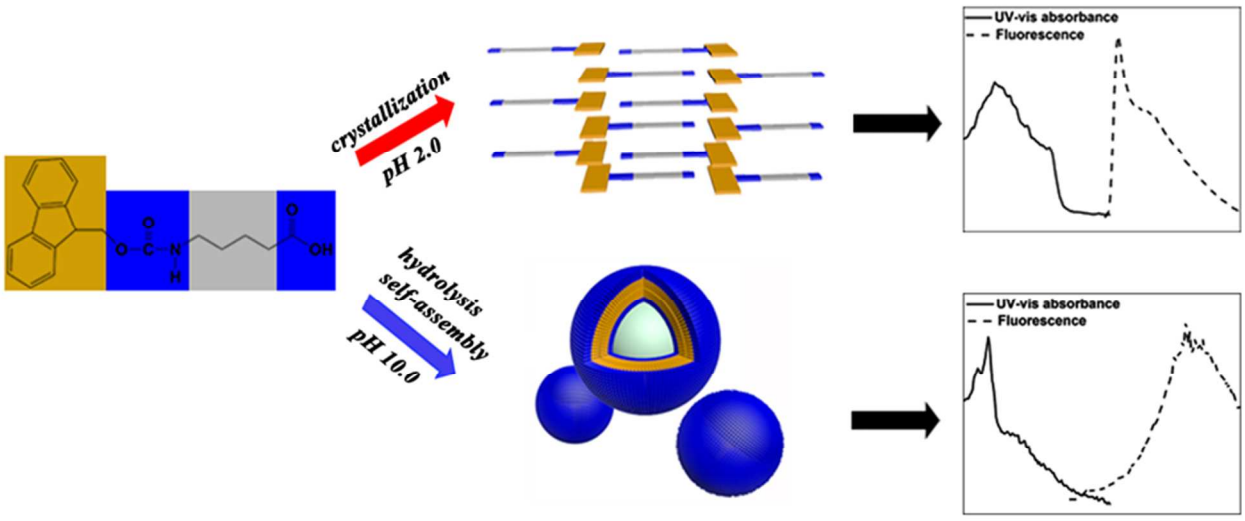

The photophysical features of the Fmoc group can be modulated by pH-mediated self-assembly.