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Photo-crosslinking of a self-assembled coumarin-dipeptide hydrogel†

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The self-assembly and photo-crosslinking of a lysine dipeptide functionalized with 7-(diethylamino)-3-coumarin carboxylic acid (7-DAC) is described. Self-assembly of the dipeptide in water and PBS produces a hydrogel comprised of uniform, micrometer length nanofibers. Irradiation of the nanofibers at 365 nm induces dimerization of the coumarin groups, which crosslinks and stabilizes the noncovalent nanofibers and enhances the mechanical properties.

The self-assembly of peptides is an efficient method to synthesize functional materials of interest for applications in *in vivo* imaging,[1] drug delivery,[2] and tissue engineering.[3] This approach offers a versatile strategy to create nanostructures of high complexity, with the potential for self-healing and structural responsivity.[4] Noncovalent structures are generally less robust than covalently formed analogs, and thus self-asssembled nanostructures often degrade or reorganize with changes in temperature and solvent. The utility of these materials in many applications requires greater physical robustness and mechanical stability. One strategy to improve the stability of soft materials is to chemically crosslink the nanostructure following the self-assembly process. This strategy is currently being used to stabilize hydrogels,[5] micelles,[6] liposomes[7] supramolecular gels,[8] dendrimers,[9] polymeric nanoparticles[10] and amphiphilic nanotubes.[11] Herein, we report the self-assembly and photocrosslinking of a coumarin–dipeptide conjugate in aqueous media as a method to increase the robustness of the assembled nanostructure.

The β-sheet self-assembly of short peptides[12] provides a potential strategy to create nanostructured materials from simple, accessible building blocks. The challenge in creating robust assemblies from short dipeptides stems from the difficulty in designing structures with limited functionality to be jointly capable of undergoing controlled self-assembly and subsequent chemical crosslinking.[13] Previously, we described the β-sheet assembly of dilysines, functionalized with naphthalenedimide (NDI) appended to the α-amino position, assembled into 1-D nanostructures in aqueous medium.[14] The β-sheet assembly of these dipeptides in water was driven by hydrophobic effects and π–π interactions of the NDI group. Intermolecular electrostatic interactions among adjacent lysine ammonium groups attenuate the assembly process and reduce formation of insoluble aggregates. Furthermore, we found that *N*-Fmoc derivatives of these dipeptides formed self-supporting hydrogels comprised of well-defined nanobelts.[15]

Coumarin is ideally suited to serve as the crosslinking functionality for dipeptide structures. The planar structure of the chromophore strongly promotes intermolecular π–π stacking to drive self-assembly in water, similar to the NDI chromophore. 7-Diethylamino coumarin (7-DAC) also undergoes a reversible photodimerization upon irradiation at 365 nm, and dimer cleavage at 265 nm.[16] Photodimerization requires the coumarin groups to be positioned within 4.2–4.5 Å of each other.[17] Thus, the self-assembly process must proximally position the coumarin groups to permit dimerization, thereby circumventing the formation of nonspecific crosslinks between randomly positioned chromophores.[11a] Coumarins also find broad utility as fluorescent probes[18] in biology and medicine due to their photostability, low cytotoxicity, and high fluorescence.[19]

Accordingly, 7-DAC chromophores were appended to both the N-terminal and *N*-ε side chain positions of a dilysine peptide, as shown in Fig. 1. Two 7-DAC chromophores were conjugated to the dipeptide, resulting in compound **A**, in an analogous synthetic scheme as that used in constructing Fmoc-KK(NDI)-NH_2 dipeptides. In this case, the Fmoc groups were replaced with 7-DAC units, which should be similarly capable of intermolecular π–π interactions. Indeed, self-assembly was observed for these compounds in water, saline, and PBS. The critical aggregation concentration (CAC) of freshly dissolved solutions of **A** in PBS (w/o calcium and magnesium), prepared

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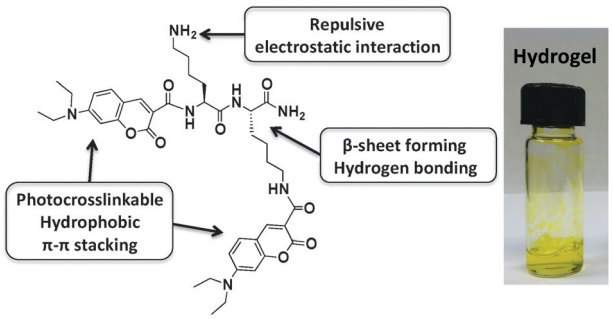

Fig. 1 Structural design of coumarin dipeptide hydrogel (10 mM in water, wt% = 0.76%), DAC-KK(DAC)-NH_2 (**A**).

without preincubation, was 380 μM, measured using the solvochromatic dye Nile Red (Fig. S4 in the ESI†).

The self-assembly of **A** was investigated in both HPLC-grade water (pH 7.0) and PBS (pH 7.4) by transmission electron microscopy (TEM). Samples were prepared by diluting 10 mM solutions after 24 h to 250 μM. TEM images of **A** in H_2O and PBS showed the formation of micron-length nanofibers with uniform diameters of 18 ± 1 nm (Fig. 2a and b). In contrast, no observable aggregates were identified by TEM in 2,2,2-trifluoroethanol (TFE) (0.25 mM), even after 1 week aging at 10 mM. Although fibers formed in both PBS and water exhibited similar dimensions, there was more apparent intertwining and bundling of the fibers in PBS, compared with the mostly parallel arrays of fibers formed in water. This increased bundling likely arises from salt-induced electrostatic screening experienced by the ammonium side-chain of **A** in PBS, compared with water. These repulsive electrostatic interactions attenuate aggregation and would be greater in pure water, leading to less interfiber associations and entanglements.[20]

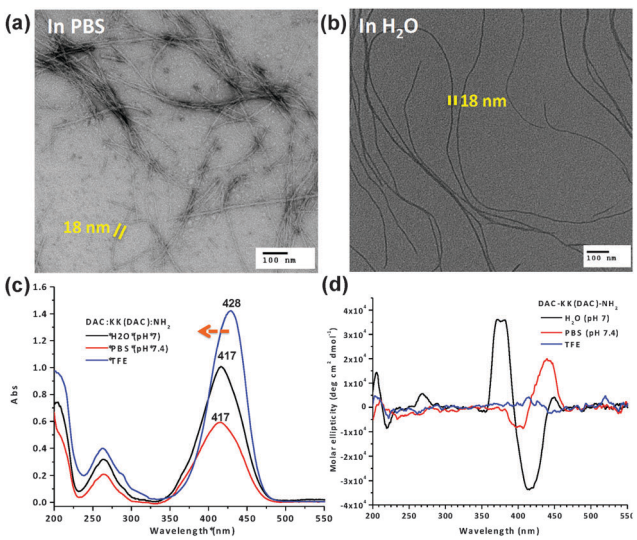

Fig. 2 TEM images of self-assembled dipeptide **A** in (a) PBS (pH 7.4) and (b) water (pH 7.0). (Carbon-coated copper grid, 2 wt% uranyl acetate as negative stain) (c) UV-vis spectra and (d) CD spectra of **A** in H_2O, PBS and TFE. All solutions were prepared at 10 mM and freshly diluted to 0.25 mM after 1 day aging.

The UV-vis absorption spectrum of **A** in TFE (0.25 mM) displayed absorption maxima at 264 and 428 nm (Fig. 2c). Changing solvent from TFE to either water or PBS resulted in reduced intensity and blue-shifted absorptions at 417 nm, indicative of the presence of H-type face-to-face π-stacking within the fibers. Similarly, the circular dichroism (CD) spectra exhibited excitonic couplets with zero-crossings in the range of 400–425 nm in PBS and water (Fig. 2d). In contrast, a flat line was recorded in TFE, consistent with the monomolecular state of **A** in this solvent. Given the absence of an induced CD effect in TFE, the couplets observed in PBS and water must arise from the relative intermolecular orientation of the coumarin chromophores within the aggregate. The electric transition moment of the excitation band at 417 nm for 7-DAC runs approximately along the axis connecting the 3- and 7-positions of the coumarin structure.[21] Accordingly, the opposite sign of the couplets in water and PBS indicated the presence of an M- and P-type helical packing orientation of the coumarin chromophores in water and PBS, respectively (Fig. 3). Deconvolution of the Fourier-transform infrared (FTIR) spectroscopy of **A** in PBS (10 mM, D_2O) displayed an amide I band at 1630 cm^{-1}, consistent with the presence of β-sheet structure as a stabilizing feature of the nanofibers (Fig. S1, ESI†).[22]

The photoinduced dimerization of the coumarin chromophores was explored as a method to crosslink and stabilize the self-assembled nanofibers. Accordingly, solutions of **A** in TFE (250 μM), a solvent in which no aggregation takes place, did not show significant changes in UV spectra after irradiation at 365 nm over 8 hours (Fig. 4c). In contrast, the absorption bands at 260 and 417 nm progressively decreased by 50% and 30% in PBS and water, respectively, upon irradiation for 8 h (Fig. 4a and b). Without further UV exposure, the bright yellow hydrogel remained intact for >3 weeks; however, after irradiating for 48 h, the hydrogel slowly darkened in shade (Fig. 4a). Prolonged irradiation at 365 nm (>7 d) resulted in an insoluble yellow precipitate as dimerization further progressed (Fig. 4; inset, Fig. S2, ESI†), resulting in flat-lines in both CD and UV-vis spectra. Fully crosslinked water-insoluble nanostructures were collected by centrifugation and washed with water to remove any dipeptide monomers or oligomers. The crosslinked solid was highly soluble in TFE, a solvent that could be expected to disrupt the self-assembled nanofibers, based on the monomolecular nature of **A** in this solvent. However, TEM imaging of the crosslinked materials that were dissolved with TFE revealed the presence of nanofibers with uniform diameters of 15 ± 1 nm (Fig. 4d), comparable to the nanofiber dimensions produced in water or PBS.

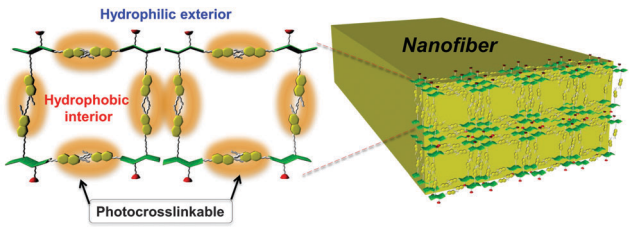

Fig. 3 Notional depiction of cross-linked, β-sheet nanostructure of **A**.

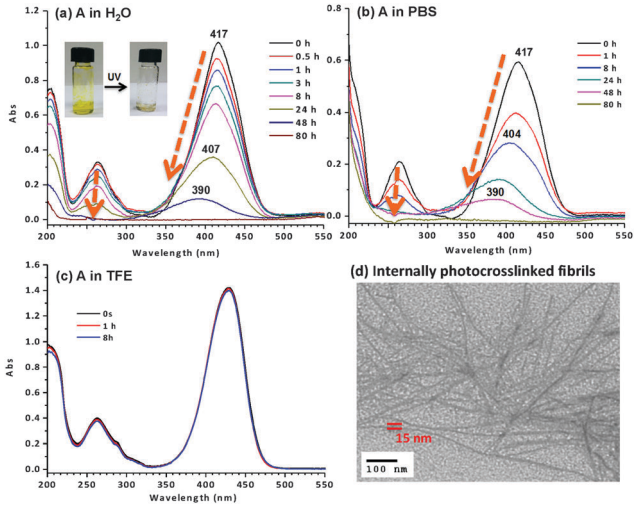

Fig. 4 Time dependent UV-vis spectra of **A** (a) in H_2O (pH = 7.0), (b) in PBS (pH = 7.4), and (c) in TFE upon UV irradiation. Inset pictures display the hydrogel (10 mM) of **A** in water and the formation of insoluble precipitation after UV irradiation at 365 nm. (d) TEM image of internally photocrosslinked fibrils. All samples (250 μM) were irradiated at 365 nm with UV lamp (6 W/0.16 Amps) for 80 hours. TEM samples were prepared from the insoluble polymeric system (after UV irradiation) that was redissolved in TFE.

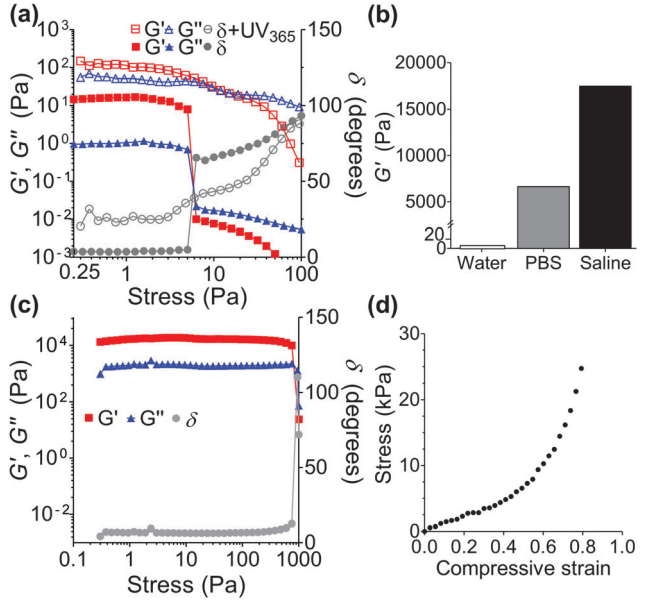

Fig. 5 Oscillatory shear testing of hydrogels prepared from **A** at 10 mM showing stress sweeps of gels in pure water for both non-irradiated and irradiated hydrogels (a); summarized elastic storage moduli (G') for non-irradiated hydrogels prepared in various aqueous solutions (*i.e.*, water, PBS, and saline) (b); non-irradiated hydrogels in saline (0.9% NaCl) (c); and compressive properties of non-irradiated hydrogels prepared in saline (d).

Thus, although extensive photocrosslinking disrupts the hydrogel structure, the self-assembled nanofibers remain intact in otherwise denaturing solvents such as TFE.

The mechanical properties of hydrogels prepared from **A** were investigated by oscillatory shear testing (Fig. 5). In pure water (10 mM, 23 °C), the hydrogels were weak and reversible with an elastic storage modulus, G', of ~20 Pa (Fig. 5a). UV-irradiation provided a modest increase in stiffness ($G' \approx 150$ Pa), with a loss of their reversibility characteristics. Reversibility was determined by subjecting the same sample to a subsequent stress sweep and comparing these two values (Fig. S6, ESI†). While more mechanical and spectroscopic studies will help determine the supramolecular effect of photodimerization within the hydrogel (*i.e.*, intrafiber *versus* interfiber linkages), the oscillatory shear data suggest a majority of intrafiber crosslinks are due to the close association and positioning required for individual molecules to dimerize. As such, the majority of individual nanofibers stiffen, but these large fibers are incapable of reassembling after being disrupted by excessive shear strains. Gels (10 mM) prepared in ionic environments (*e.g.*, saline/0.9% NaCl) exhibited 100-fold greater elastic moduli ($G' \approx 20$ kPa) (Fig. 5b) and possessed a compressive modulus, E, of 11.4 kPa (Fig. 5c), whereas hydrogels prepared in water were too weak for compression tests. The effect of increasing peptide hydrogel stiffness by increasing ionic strength and/or changing pH has been described by other researchers in detail,[23] and is due to the increasing hydrophobicity of the di-lysine peptides, which lowers their solubility and increases their solid-like (precipitate) characteristics. Unlike those prepared in pure water, these hydrogels did not experience any appreciable changes in stiffness after UV-irradiation. This was due to the reduced solubility, optical transparency, and molecular mobility within these gels, which was not an issue with the lower concentrations (250 μM) used for spectroscopic analysis. Nonetheless, important variables such as ionic strength and UV irradiation can tune the mechanical properties of these gels, and further studies are warranted to understand these triggers on self assembly and/or reorganization.

In summary, we describe a facile method to cross-link self-assembled, β-sheet forming dipeptides. The structural design follows that of Fmoc-KK(NDI)-NH_2 dipeptide, whereby the Fmoc and NDI chromophores are replaced with 7-DAC. The resultant dipepetide **A** self-assembles into β-sheet nanofibers that efficiently gel in aqueous media. The mechanical properties of hydrogels prepared from **A** are strongly dependent on their aqueous environment, suggesting an additional means to tune their physical properties. Irradiation at 365 nm crosslinks the coumarin moieties and retains the nanofiber structure. Although partial crosslinking enhances the mechanical stability of the hydrogel, extensive irradiation disrupts the hydrogel structure, resulting in a water-insoluble material. However, the nanostructure remains intact in TFE, a potentially denaturing solvent, which dissolves the non-crosslinked material. This versatile strategy to stabilize nanostructures formed by the β-sheet assembly of small dipeptides will facilitate the design of additional soft materials for applications that require more robust or responsive physical properties.

Experimental

For the synthesis of 7-(diethylamino)-3-coumarin carboxylic acid (7-DAC), a solution of 4-diethylaminosalicylaldehyde (193.25 mg, 1 mmol) and dimethyl malonate (158.5 mg, 1.2 mmol) in MeOH (1 mL) was prepared. Piperidine (0.01 mM, 0.1 mmol) with

catalytic amount of glacial acetic acid (1 drop) was added to the reaction mixture. After 3 h at reflux, 2 mL H_2O was added and cooled to 0 °C. 1 N NaOH (aq) solution was added and refluxed for 20 min with vigorous stirring. After cooling to r.t., 2 N HCl (aq) was added and the orange crude solid was collected by filtration, washed with 50% aqueous cold ethanol and recrystallized with ethanol to obtain the bright orange crystalline product (206 mg, yield = 79%). Compound **A** was manually prepared using Fmoc/*t*-Bu solid-phase peptide synthesis on rink amide resin. Compound **A** was cleaved from the resin by the treatment with TFA/water/triethylsilane (95/1/4) at room temperature for 2 h and purified by reversed-phased HPLC on preparative Varian Dynamax C18 column eluting with a linear gradient of CH_3CN/water containing 0.1% TFA and stored as lyophilized powers at 0 °C. Rotational shear rheometry of hydrogels was performed on a TA Instruments AR1000 stress-controlled rheometer. Compound **A** was prepared at 10 mM in pure HPLC-grade water, PBS (without Ca^{2+} and Mg^{2+}), or saline, and incubated at room temperature for 24 hours. The complex shear storage (G') and loss (G'') moduli of these self-assembled hydrogels were then measured as a function of oscillatory stress. For photo-crosslinking gels in pure water, the gels were first prepared in humidified, sealed glass dishes for 24 h and subsequently exposed to UV light (λ = 365 nm) for 70 h before performing shear testing.

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