Biotin Structures

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Ditryptophan Conjugation Triggers Conversion of Biotin Fibers into Soft Spherical Structures**

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Molecular self-assembly is a ubiquitous theme in natural systems where constituent building blocks are recruited following precise pairing patterns, to reveal an array of hierarchical architectures that are primarily supported by noncovalent interactions.[1,2] The functional role of such structures in nature can be judged from the action of self-assembled actin filaments, which are crucial for morphogenesis and cellular movement, self-organization of mitotic spindles during cell division, and the construction of complex viral capsids, to name but a few.[3–5] Inspiration derived from natural systems and natural components is increasingly being realized as a rich source of materials which imitate biological structures and function.[6,7] Despite significant advances in supramolecular chemistry and noncovalent synthesis, direct morphological control of self-assembled structures remains an enigma. Herein, we show that long biotin fibers can be morphed into soft spherical structures by simple attachment of the Trp–Trp dipeptide. The inspiration of using tryptophan as a modifier of the biotin ultrastructure emanates from the documented tryptophan interaction in the high-affinity biotin–avidin system, in which Trp indolic rings interact with biotin, presumably through hydrophobic contacts or a charge-transfer mechanism.[8–10]

Biotin, a water-soluble vitamin, acts as a cofactor in a number of important biochemical metabolic reactions and pathways related to cell signaling, gene expression, and chromatin structure.[11,12] The characterization of biotin has revealed its extended structure in the crystalline state, whereas multiple conformational ensembles ranging from extended to folded states are preferred in solution.[13,14] The latter structures are stabilized by hydrogen bonding between the ureido group and the valeryl carboxylic acid side chain. With this background, we became interested in probing the ultrastructure of the biotin supramolecular assembly in solution, by using free biotin and its methyl ester as candidate molecules (Figure 1a; Scheme SS1 in the Supporting Information). Esterification of the carboxylic acid was expected to modulate the effect of hydrogen bonding with the ureido group.

Scanning electron microscopy (SEM) and atomic force microscopy (AFM) of a freshly prepared solution of D-biotin (1 mM, 50% aqueous methanol) revealed the formation of 1–5 μm-long self-assembled fibrous structures (Figure 1a inset and Figure 1b), which were further elongated to 10–100 μm upon prolonged incubation for 10–12 days (Figure 1c). Such a morphology was also supported by AFM analysis (Figure 1d). Biotin methyl ester afforded long tubular structures, as observed by AFM (Figure 1e), which was further confirmed by SEM (Figure 1f). The formation of these self-assembled structures can be attributed to the propensity for hydrogen bonding of the ureido and carboxylic groups, and the interaction of the valeryl side chain with the ureido group of biotin, while interaction at the ureido group will predominate for the biotin methyl ester. Intramolecular hydrogen bonding of biotin in solution, with the participation of the ureido carbonyl oxygen atom as a strong hydrogen-bond acceptor, has been described.[15,16]

The high-affinity biotin–avidin interaction has been extensively investigated and mutagenesis studies implicated contributions of three Trp residues, which control the exceptionally high binding constants observed in this system.[8–10] It has been postulated that Trp residues control the equilibrium enthalpy and entropic costs of the biotin–avidin interaction. A crystal structure study of a model compound, in which biotin is connected to an indole moiety through a trimethylene linker, has suggested that a linked molecule occupies a cavity formed by two indole rings, thus suggesting the importance of hydrophobic interaction in the biotin–avidin complex.[17] In view of the crucial roles described for Trp residues, we decided to attach them to biotin to see the effect on the morphology of biotin fibers.

A remarkable change from a fibrous structure to a spherical morphology was observed in freshly prepared samples of biotin–ditryptophan methyl ester (**2**; 1 mM, 50% aqueous methanol) when analyzed by SEM on a copper surface (Figure 2a). The spherical morphology was preserved irrespective of surface variation to highly oriented pyrolytic graphite (HOPG) or under wet conditions (Figure 2b, inset) when observed by focused-ion-beam (FIB) high-resolution SEM. Transmission electron microscopy (TEM) and AFM further confirmed the transformation of biotin fibers to spherical structures by simple attachment of ditryptophan methyl ester (Figure 2c,d).

Interestingly, the appearance of spherical structures was almost instantaneous and without any indication of other common self-assembled structures, such as fibrils, filaments,

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

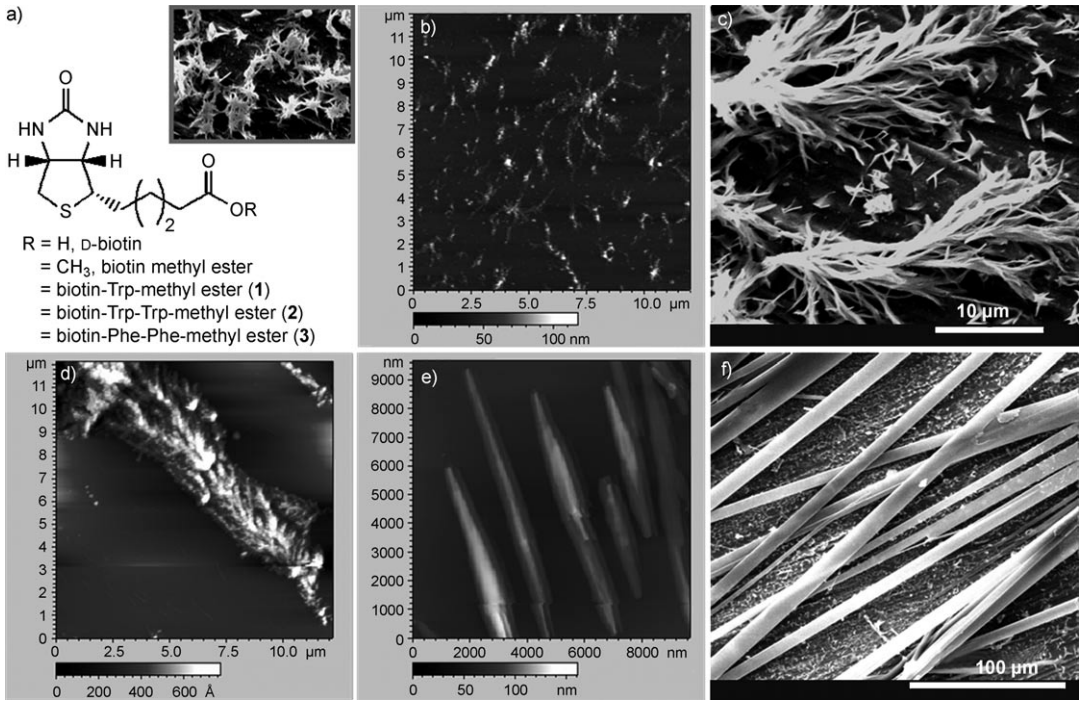

Figure 1. Self-assembly of D-biotin and D-biotin methyl ester (1 mM, 50% aqueous methanol). a) Molecular structure of biotin and its methyl ester; inset: SEM image of D-biotin (fresh sample). b) AFM image of biotin fibers (fresh sample). c) SEM and d) AFM images of biotin fibers after 12 days incubation. e) AFM and f) SEM images of biotin methyl ester (fresh sample).

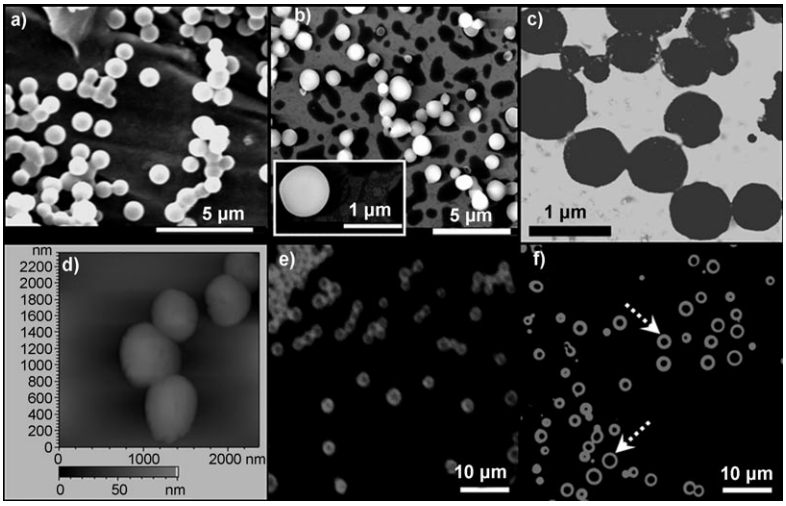

Figure 2. Microscopy images of biotinylated ditryptophan methyl ester (**2**). a) SEM image of a fresh sample (1 mM, 50% aqueous methanol) displaying spherical ball-like structures on a gold-coated copper stub. b) FIB-SEM image of structures on a HOPG surface under wet (near-native) conditions. Inset: magnified view of a single spherical structure. c) TEM image of a fresh sample. d) AFM image displaying a spherical morphology on a mica surface. Fluorescence micrographs of e) spherical balls stained with fluorescein, which reveals uniform, bright green structures, and f) release of dye upon mild acidification of the solution (pH 4.5, 24 h incubation).

and tapes. Prolonged incubation refines the structure but the gross morphology remains similar to that seen for the fresh sample, which indicates the formation of shape-persistent spherical balls under the influence of an aromatic dipeptide (Supporting Information). Biotin–Trp methyl ester (**1**) failed to provide a discernible change in the morphology (Supporting Information), thus confirming a crucial role for Trp–Trp dipeptide in controlling the morphology of biotin fibers. As dipeptide conjugation afforded interesting morphological changes, we did not pursue the conjugation of biotin with tripeptide or other higher homologues.

It was possible to stain these self-assembled structures with fluorescein dye to permit fluorescence detection (Figure 2e). Dye-stained microspheres released fluorescein and localized the dye at the periphery of circular structures when acidified to pH 4.5 (Figure 2f, arrows). We decided to investigate this property by SEM and TEM analysis. Interestingly, SEM snapshots at various pH values confirmed an abrasive effect of the medium on the spherical structures (Figure 3a–c). These observations revealed the instability of the soft spherical structures in acidic medium, possibly because of protonation of the indole nitrogen atom(s).

The effect of urea on the aggregation process of **2** was also investigated, as it may interact with the ureido portion of the biotin structure. SEM images displayed a catastrophic disintegrating effect of urea (1 mM; 1 equiv) on the spherical balls, thus suggesting a crucial role of the ureido group in the self-assembly that is hindered by the addition of urea, possibly by impairing the critical hydrogen-bonding contacts required for the stability of spherical structures (Figure 3d).

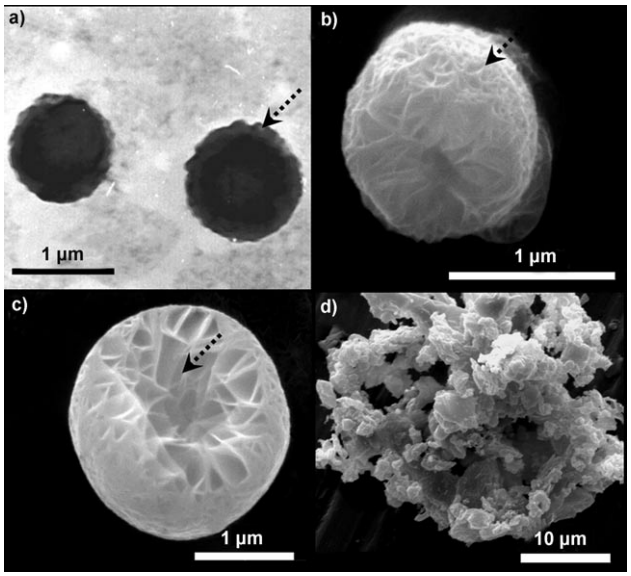

Figure 3. Effect of pH and addition of urea on self-assembled spherical structures from **2**. a) TEM and b) SEM images of spherical balls at pH 2, clearly revealing rough edges and abrasions (arrows). c) SEM image of ruptured structure at pH 1. d) SEM image showing the disintegrating effect of urea (1 M) on the spherical balls.

Acid-induced abrasion of the spherical capsules provided an impetus for us to probe the interior core of these soft structures by FIB milling experiments. Although the imaging aspects of FIB-SEM instrumentation are similar to those of SEM, the technique provides superior control in producing a finely focused ion beam of low current, which enables well-defined milling and high-resolution imaging, or higher beam currents for coarser and faster milling. We decided to mill peptide microspheres to probe the properties of their spherical core. A 500×500-nm section was milled off the surface to reach the interior of the structure (Figure 4a,b). These microspheres were also amenable to simple FIB-aided bisection, which suggests reasonable structural integrity that allows them to withstand ion currents for short time periods (Figure 4c). Interestingly, it was also possible to mill these microspheres to create different shapes, thus revealing salient features of the organization and structural integrity of the core (Figure 4d–f).

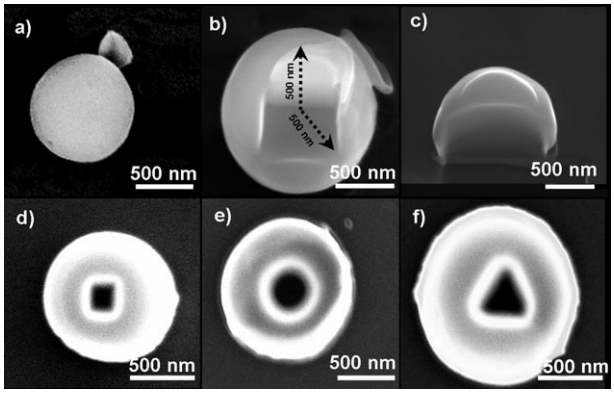

Figure 4. Observation of the exterior and interior structure of spherical balls by FIB milling. a) Intact isolated spherical structure. b) FIB milling of a 500×500-nm cross-section, which suggests a stable spherical core. c) Spherical ball bisected with a FIB of optimal intensity. d–f) Various shapes patterned on the spheres by FIB milling, which suggest stability of these self-assembled structures.

The high-affinity biotin–avidin interaction in nature is supported by the hydrogen bonding of serine and aspartic acid side chains with the NH groups of the ureido ring, while the tyrosine, asparagine, and serine side chains are within hydrogen-bonding distance of the ureido carbonyl oxygen atom.[8–10,18] Moreover, four Trp residues hydrophobically interact with the tetrahydrothiophene ring and the valeryl side chain of biotin. Individual interactions have also been confirmed by mutagenesis experiments.[9,10] In view of the various stabilizing noncovalent interactions, it can be envisioned that the self-assembly observed in this study requires an intricate interplay of hydrogen-bonding, van der Waals, and π-stacking interactions in the biotinylated ester **2**.

The participation of ureido, amido, and indolic NH protons in the self-assembly process was qualitatively estimated from the 1H NMR spectra of **2** taken in various ratios of [D_6]DMSO and H_2O (v/v). The upfield chemical shifts of the amido[19] and indolic protons was evident through the incremental addition of H_2O to [D_6]DMSO (0–40% H_2O; Figure 5 and Table 1). A marked movement of key proton

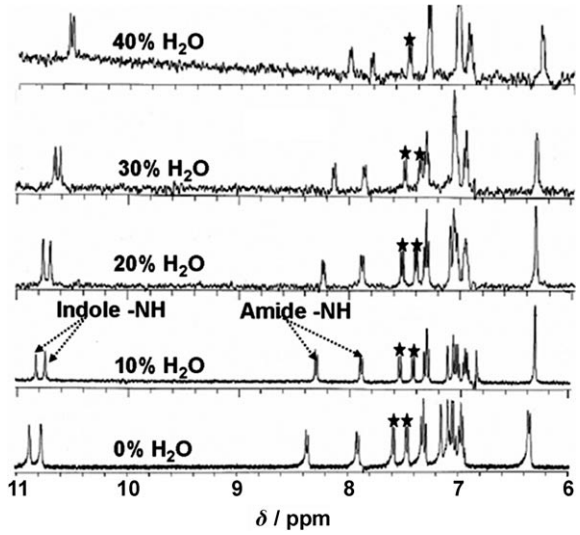

Figure 5. Solvent-dependent ^{1}H NMR studies of **2**. Changes in the chemical shifts of amido, indolic, and aromatic protons (7.5 mg mL^{-1}) in different solvent ratios (v/v) of [D_6]DMSO and H_2O (cf. Table 1).

resonances is suggestive of the crucial role of tryptophan interaction in the supramolecular ensemble. Such upfield shifts of aromatic protons may occur because of the partial face-to-face arrangement of the aromatic side chain in relation to the biotin moiety, thus causing shielding effects that result from the spatially proximal aromatic ring.[20,21]

Furthermore, a remarkable upfield shift in indolic proton resonances upon the addition of water also suggests an inextricable role of hydrogen bonding in the self-assembled

Table 1: Comparison of chemical shifts of biotin–Trp–Trp methyl ester (**2**) and biotin–Phe–Phe methyl ester (**3**).

Peptide	0% water in $[D_6]$DMSO				
	Amide-NH		Indolic-NH		Ureido-NH
	N^aH	N^bH	N^aH	N^bH	N^1H, N^3H
2	7.90	8.36	10.90	10.80	6.35
3	7.93	8.39	–	–	6.36
	40% water in $[D_6]$DMSO				
	Amide-NH		Indolic-NH		Ureido-NH
	N^aH	N^bH	N^aH	N^bH	N^1H, N^3H
2	7.72	8.00	10.52	10.54	6.30
3	7.99	8.36	–	–	6.34

spherical structures. In contrast, biotinylated Phe–Phe dipeptide (**3**) and biotinylated Tyr–Tyr dipeptide exhibit either no discernible change in morphology or irregular oblong morphologies (Figures 9–11 in the Supporting Information), thus indicating the crucial role of Trp–Trp dipeptide conjugation as a morphological trigger that results in uniformly sized spherical structures of biotin.

Thus, the conjugation of tryptophan dipeptide dramatically influences and alters the morphology of biotin fibers into spherical ball-like structures. Curiously, such morphogenesis is not achieved in a controlled fashion by other aromatic amino acids. This finding suggests that tryptophan elicits a specific morphological response not only to aromatic hydrophobic interactions, but also perhaps because of its inherent affinity for biotin, as demonstrated by key molecular contacts operating in the biotin–avidin system. Interestingly, a capping interaction of tryptophan with biotin has been proposed as the molecular basis of slow streptavidin–biotin dissociation kinetics.[22]

In conclusion, we have revealed ditryptophan as a bioinspired morphological control trigger, which transforms biotin fibers into compact, spherical, ball-like structures upon conjugation. It is believed that natural strategies of binding and recognition may be exploited to control the morphologies of soft materials derived from biological components.

Experimental Section

Fresh and aged peptide, biotin, biotin methyl ester, and N-biotinylated peptide samples were imaged with an atomic force microscope (Molecular Imaging, USA) operating in the acoustic ac mode, with the aid of a cantilever (NSC 12(c), MikroMasch). Sample-coated mica was dried for 30 min at room temperature before AFM imaging. SEM images were acquired on an FEI Quanta 200 microscope equipped with a tungsten-filament gun and operated at a working distance of 10.6 mm and 20 kV. For TEM, a fresh sample or a specimen incubated for 12 days was placed on a 400-mesh copper grid. After 1 min, excess fluid was removed and the grid was negatively stained with uranyl acetate solution (2%). Excess stain was removed from the grid and the samples were viewed with a JEOL 1200EX electron microscope operating at 80 kV.

Dye-stained structures were examined under a fluorescence microscope (Leica DM2500M) with a fluorescence illuminator and a fluorescein filter (494/521 nm). This filter optimized visualization of fluorescein-treated (positive resolution) compared with untreated (negative resolution) spherical structures.

FIB-SEM: A HOPG surface was incubated in a solution of **2** (1 mM) for 12 h at 4°C and the sample was directly mounted on the stage under wet (native) conditions. An image was obtained with a FIB scanning electron microscope dual-beam system (Nova 600 NanoLab, D97, FEI).

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