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Hierarchical Self-Assembly from Nanometric Micelles to Colloidal Spherical Superstructures

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Abstract

We report sequential self-assembly of low molecular weight asymmetric star-like polymers from nanometric spherical micelles (core diameter 8-12 nm) to colloidal spherical superstructures ($d > 500\text{--}1000$ nm) in aqueous media, as directed by quadruple hydrogen bonding moieties activated by solvent exchanges. The molecules consist of a rigid asymmetric steroidal cholic acid (CA) core, whose hydroxyl groups are first grafted with four oligomeric allyl glycidyl ether (AGE) chains involving six repeat units. The allylic double bonds are thiol-clicked with cysteamine (HS-(CH₂)₂-NH₂), and in the final step their amino end groups are conjugated with 2-ureido-4[1*H*]-pyrimidinone (UPy) using UPy-(CH₂)₆-NCO via isocyanate chemistry to obtain asymmetric star-like facially amphiphilic molecules denoted as CA[(AGE-NH₂)₆]₄-(UPy)_n. They self-assemble into spherical micelles in dimethyl sulfoxide (DMSO), which suppresses the dimerization of UPys. Solvent exchange from DMSO to water activates the inter-micellar hydrogen bonds via the peripheral UPy units, which triggers the formation of spherical submicron to micron scale structures. Based on three-dimensional (3D) reconstruction using transmission electron tomography (ET), we show that such structures are composed of densely packed micellar network. Our work shows that surfactant-like molecules, characteristically leading to micelles, can undergo complex and hierarchical self-assembly to microscale by equipping the solubilising chains with hydrogen bonding units that can be switched ON and OFF by sequential solvent exchanges.

Keywords

Self-assembly, Hydrogen bonding, Micelles, Supramicelles, Electron tomography

1. Introduction

In order to tailor materials properties, concepts to control the structures at different length scales have extensively been pursued, where self-assemblies due to competing interactions provide a feasible platform [1-3]. Well-defined block copolymers [4-6], block copolypeptides [7], and dendronic molecules [8], allow rational routes for solvent-based molecular/supramolecular self-assemblies, where micelles, co-micelles, polymersomes, dendrosomes, and their superstructures have been observed [9-17]. Using colloids, i.e. larger self-assembling structural units, significant progress has recently been made for colloidal self-assembly to e.g. one-dimensional (1D) micellar superstructures, two-dimensional (2D) supermicelles, co-micelles, and colloidosomes in solvent media [18-31]. Therein, challenges include controlling the size dispersities, directionalities, and aggregation behavior. The presence of various colloidal level forces makes the colloidal self-assembly significantly different from that of molecular or supramolecular self-assembly [22]. For example, it has been demonstrated that upon controlling the repulsive interactions by restricting the free volume of submicron or micrometer sized colloidal particles below certain threshold, colloidal crystals with long-range order can be obtained [18]. Colloidal particles having oppositely charged surface charges allow co-crystals or binary superlattices [20, 27]. By controlling the inter-nanoparticle hydrogen-bonding (H-bonding) of atomically precise gold nanoparticles or cobalt nanoparticles of narrow size distribution, either hexagonally arranged 2D colloidal crystals or spherical capsid-like structures have been reported [30,31]. The key to obtain well defined self-assemblies is to precisely control the balance between attractive and repulsive interactions. In general, controlling self-assembly across the length scales is subtle but could offer a potential route

towards engineering of functions, where biomolecules and biological concepts could provide inspiration.

Among small biomolecular building blocks, bile acids and their salts have been extensively studied in supramolecular and materials chemistry [32]. Because of their facial amphiphilicity and the hydroxyl groups, which are asymmetrically functionalizable by side chains, they show tunable aggregation properties. Unlike conventional surfactants, bile salts self-assemble to bilayer-like micelles with low aggregation number. Structurally diverse morphologies such as vesicles, tapes, fibers, and helical ribbons have been observed using bile acid based supramolecular building blocks [33-37]. Polymeric constructs based on bile acids are promising for dental composites [38]. Oligoethylene glycol appended bile acid derivatives lead to thermoresponsive micelles [39]. Further, cysteamine derivatives of oligo allyl glycidyl ether (AGE) asymmetrically grafted cholic acid (CA) star-like polymers show thermosensitive aggregation behavior [40]. The self-assembly occurs due to the aggregation of lipophilic backbones of the steroid followed by H-bonding of hydroxyl and side chain carboxylic acid groups. Thus, the modification of side chain and hydroxyl groups with functionalized allyl glycidyl ethers also provides facially amphiphilic macromolecules. Therefore, the resulting structures have a similar aggregation tendency, however, due to structural modification and long oligomeric units the functionalized molecules tend to form spherical micelles unlike natural bile salts [40].

We hypothesized that, cholic acid-based micellar aggregate can lead to higher order assembly by introducing structure directing units. In supramolecular chemistry, 2-ureido-4[1*H*]-pyrimidinone (UPy) is useful due its quadruple hydrogen bonding motif [41-48]. The arrangement of H-bonding donors (D) and acceptors (A) in a DDAA array minimizes the secondary repulsive interactions and maximizes the attractive interactions. This quadruple H-bonding provides high association constant (10^6 M^{-1}) in chloroform [41], and the UPy derivatives exist as hydrogen bonded dimers in solid state. However, using relatively polar and H-bonding competing solvents, such as dimethyl sulfoxide

(DMSO), the equilibrium can be shifted to monomers. Recently, we have shown that functionalizing the hydroxyl groups of CA(AGE₆)₄ with UPy units leads to transition from micelles to supramolecular fibers which, in turn, wrap into spheres, as triggered by controlled solvent exchanges [48].

Here, we first report synthesis of amine-terminated asymmetric star-like macromolecules with rigid cholic acid cores (see Fig.1 for the chemical structures) by using thiol-ene click reaction to functionalize CA(AGE₆)₄ with cysteamines. In the next step, the amino groups are reacted with different molar equivalents (*n*, where *n* = 3, 6, 9) of UPy hexamethylene isocyanate (UPy(CH₂)₆CNO) to yield final asymmetric star-like polymer with peripheral UPy-groups, denoted as CA[(AGE-NH₂)₆]₄-(UPy)_{*n*} having molecular weights ~5 - 6 kDa. We show that that the self-assembly of such star-like polymers can be controlled in aqueous media, where the micelles undergo sequential and hierarchical assembly into micrometer size spheres. We further demonstrate using transmission electron tomographic reconstruction that the internal structure of the spherical superstructures is made up of highly densified network-like structure, probably due to H-bonded micelles.

2. Experimental

2.1 Synthesis of CA[(AGE-NH₂)₆]₄ (**3**)

The synthesis of compound **3** was performed according to the reported literature procedure [40]. In detail, a mixture containing 1/20 w/v CA(AGE₆)₄ (**2**), 5.0 equiv of cysteamine hydrochloride and 0.5 equiv of azobisisobutyronitrile (AIBN) per double bond was dissolved in methanol in oven-dried round-bottom flask (50 mL) equipped with a condenser and CaCl₂ guard tube. After refluxing the reaction mixture at 80 °C for 46 h it was allowed to attain room temperature and the solvent was evaporated under reduced pressure using a rotary evaporator. The residual syrup was dissolved in chloroform and sodium hydroxide pellets were added to neutralize the ammonium chloride salt resulting in a cloudy precipitate. The liquid layer was decanted to a separatory funnel and extracted once with water. The semi-solid phase was dissolved in 40 mL water and more NaOH pellets were

added until the mixture was basic. The aqueous phase was extracted with 3x20 mL chloroform and all organic phases were combined. The organic phase was washed with water and chloroform was evaporated under reduced pressure. The yellow residual solid was purified by dialyzing against chloroform. After 24 h the solvent was changed to methanol. Finally, the solvent was evaporated resulting solid material. ^1H NMR (400 MHz, CDCl_3): δ (ppm) 1.77 (2H, $\text{CH}_2\text{CH}_2\text{SCH}_2$), 2.55 (4H, CH_2SCH_2), 2.80 (2H, CH_2NH_2), 3.46 (7H, $\text{HOCHCH}_2\text{OCH}_2\text{O}$). ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ (ppm) 1.74 (2H, $\text{CH}_2\text{CH}_2\text{SCH}_2$), 2.55 (4H, CH_2SCH_2), 2.51 (4H, CH_2SCH_2), 2.70 (2H, CH_2NH_2), 3.35-3.53 (7H, $\text{HOCHCH}_2\text{OCH}_2\text{O}$).

2.2 Synthesis of 2-ureido-4[1H]-pyrimidinone hexamethylene isocyanate (UPy- $(\text{CH}_2)_6\text{-NCO}$) (**4**)

The synthesis was performed according the literature procedure (Supporting Information Scheme S1) [48]. In a typical experiment a mixture of 2-amino-4-hydroxy-6-methylpyrimidine (1.6 g, 5.54 mmol) and excess 1,6-diisocyanatohexane (18 mL, 111.3 mmol) was taken in a dry 250 mL 2-neck round-bottom flask equipped with a magnetic stir bar and a condenser under nitrogen atmosphere. After refluxing the reaction mixture for 19 h at 100 °C, it was allowed to attain room temperature and transferred to 50 mL hexane. The precipitate was filtered using Büchner funnel and washed with 100 mL hexane. The white solid was dried under vacuum oven at 50 °C overnight (yield: 3.33 g, 84%). ^1H NMR (400 MHz, CDCl_3): δ (ppm) 11.86 (s, 1H), 10.19 (s, 1H), 5.81 (s, 1H), 3.28 (m, 4H), 2.23 (s, 3H), 1.63 (m, 4H), 1.40 (m, 4H). ^{13}C NMR (100 MHz, CDCl_3): δ (ppm) 173.22, 156.73, 154.83, 148.43, 120.55, 106.83, 77.16, 43.02, 39.92, 31.32, 29.45, 26.31, 19.10.

2.3 Synthesis of $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ (**5a-5c**)

To a clear solution of $\text{CA}[(\text{AGE-NH}_2)_6]_4$ (**3**) (50.0 mg, 0.01 mmol) in chloroform (1.0 mL) in a glass vial, UPy- $(\text{CH}_2)_6\text{-NCO}$ (**4**) dissolved in chloroform was added with varying molar equivalent w.r.t the number of amino ($-\text{NH}_2$) groups. In detail, 8.7 mg (0.03 mmol in 1.0 mL CHCl_3), 17.3 mg (0.06 mmol in 2.0 mL CHCl_3) and 26.0 mg (0.09 mmol in 3.0 mL CHCl_3) of UPy- $(\text{CH}_2)_6\text{-NCO}$ (**4**) was added dropwise for the synthesis of **5a**, **5b** and **5c** respectively. Upon adding **4** to the polymer

solution, the reaction mixture turned into a white turbid dispersion instantaneously. After refluxing the reaction mixture for 24 h, 10 mL of chloroform was added and the reaction mixture was centrifuged at 9000 rpm for 15 min. Supernatant was decanted and re-dispersed the solid residue in 1,4-dioxane (2.0 mL). The sample was freeze-dried under the vacuum over the night to obtain solid powders. ^1H NMR (400 MHz, $\text{DMSO-}d_6$): δ (ppm) 1.25-1.45 (8H, $\text{NH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2$), 1.73 (2H, $\text{CH}_2\text{CH}_2\text{SCH}_2$), 2.10 (3H, CH_3), 2.79 (2H, NH_2), 2.96 (2H, $\text{SCH}_2\text{CH}_2\text{NHCO}$) 3.15 (4H, $\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO}$).

The final polymers (**5a-c**), obtained upon reacting with varying amounts of UPy units is denoted as $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ (*Note: n represents the molar equivalent of UPy-(CH_2) $_6$ -NCO (**4**) used to react with $\text{CA}[(\text{AGE-NH}_2)_6]_4$ (**3**), not the number of UPy units in the final polymers. Theoretical Mw of **5a**, **5b** and **5c** 5.9 kDa, 6.8 kDa and 7.7 kDa). Schematic of synthesis with the chemical structures and illustrative image of **5** is presented in Figure1 (see Supporting Information, Figure S1 for the macroscopic appearance of solid products).*

Fig. 1. Synthesis with representative chemical structures of cholic acid (CA) (**1**); CA(AGE₆)₄ (**2**); CA[(AGE-NH₂)₆]₄ (**3**); UPy-(CH₂)₆-NCO (**4**) and CA[(AGE-NH₂)₆]₄-(UPy)_n (**5**). In addition, an illustrative graphic is presented for the final product.

2.4 Nuclear magnetic resonance (¹H NMR) spectroscopy

The solution state ¹H NMR spectra were recorded with a Bruker Avance 400 MHz spectroscopy with 100 scans. CA[(AGE-NH₂)₆]₄ (**3**) spectrum was recorded in dimethyl sulfoxide-*d*₆ and in CDCl₃. All other samples were recorded in dimethyl sulfoxide-*d*₆. NMR spectra were processed with MestReNova software. The chemical shifts were referenced to trace of DMSO-*d*₆ (2.50 ppm) or CDCl₃ (7.26 ppm). The calibration for integration calculations was done using the signal at 2.55 ppm (4H) in CDCl₃ and the signal at 1.73-1.74 ppm (2H) in DMSO-*d*₆ (Supporting Information Figures S2-S7). The polymers exists as spherical micelles in DMSO with a core diameter of 8-12 nm. Therefore, approximate molecular weight of a micelle exceeds 40 kDa. This is beyond the limitation of solution NMR spectroscopy experiments as its sensitivity is lost due to slow molecular reorientation. Slow molecular tumbling decreases the efficiency of coherence transfer processes and broaden the resonance lines of observed signals. These effects become quite severe even for well explored biological samples larger than about 30 kDa. Therefore, our attempts to extensive characterization based on NMR spectroscopy and to obtain ¹³C NMR spectra was not successful as it requires large amount of samples and acquisition time. Even, quantitative determination of the number of UPy units is challenging due to micelles (d~10 nm) and even their aggregation in DMSO.

2.5 Fourier transform infrared spectroscopy (FT-IR) spectroscopy

Transmission spectra were recorded with a Thermo Nicolet Avatar 380 FT-IR spectrometer by averaging 64 scans. Background spectra were acquired before measuring each sample. Solid samples were used for the measurements. (Supporting Information Fig. S8)

2.6 Self-assembly via dialysis

Dialysis was performed to allow polymers (**5**) slowly self-assemble during the solvent exchange of polymer dispersion from DMSO to water. The initial dialysis against DMSO also purified low molecular weight impurities and unreacted UPy-(CH₂)₆-NCO from the final product. The polymer dispersions of 0.01 % w/v and 1 % w/v in DMSO were prepared by dissolving (**5a**), (**5b**) and (**5c**) with heating. In a typical experiment, 100 μ L of solutions were dialysed using Slide-A-Lyzer MINI Dialysis device Floats (3.5 K MWCO). Dialysis was performed using solvent exchange from DMSO to water as shown in Table 1.

DMSO:H ₂ O ratio (v/v)	DMSO (mL)	H ₂ O (mL)
1:0	150	-
3:2	150	100
1:1	150	150
2:3	150	250
0:1	-	300

Table 1. The ratio of DMSO:H₂O (v/v) and total volumes of the solvents used in each step. The samples were stirred for 1 hour in each step before the next solvent change.

2.7 Transmission electron microscopy (TEM)

The morphologies of self-assembled superstructures were imaged with a FEI Tecnai 12 transmission electron microscope (120 kV) to study the internal structure of the self-assembled polymers. In a typical experiment, 5 μ L of 0.01 % w/v and 1 % w/v solutions were drop casted on to a 300 mesh hexagonal copper grid with ultrathin carbon support film and after two minutes the excess liquid was blotted using filter paper before drying under ambient conditions. The images with different sample concentrations were studied to compared the effect of concentration for the structure of the self-assembled polymers.

2.8 SerialEM and Transmission electronm tomography

Tilt series for Electron Tomography (ET) reconstruction was acquired using the JEM 3200FSC transmission electron microscope operated at 300 kV with zero loss energy filter. The

sample (3.0 μL) was placed on a 300 square mesh copper grid with ultrathin carbon support film. Before adding the polymer samples, the TEM grid was plasma cleaned and treated with *11-mercapto-1-undecanol* ligand capped 5 nm fiducial gold markers. The grid containing sample was subjected to sequential washing by dipping the grid in 50% MeOH/H₂O and 3 x MeOH for 15 seconds each. Further, the washing was continued with 50% MeOH/*tert*-BuOH and 3 x *tert*-BuOH and placed the grid in an Eppendorf containing 100 μL of *tert*-BuOH. The excess *tert*-BuOH was removed using micropipette just before placing the samples under vacuum in a lyophilizer for 30 minutes. The dried sample was used for collecting tilt series between between $\pm 69^\circ$ with 2° increment steps were acquired with the SerialEM-software package [49]. Prealignment, fine alignment and the cropping of the tilt series was executed with IMOD [50]. The images were binned four times to reduce noise and computation time. Maximum entropy method (MEM) reconstruction scheme was carried out with custom made program on Mac or Linux cluster with regularization parameter value of $\lambda=1.0\text{e}^{-3}$ [51].

2.9 Scanning electron microscopy (SEM)

Self-assembled structures were imaged with a Zeiss Sigma VP scanning electron microscope (acceleration voltage of 3 kV) to investigate the surface structures of self-assembled polymers. Prior to the imaging, a thin gold coating was sputtered onto sample with Emitech K950X/K350 (30 mA 1.45 min and for freeze-dried sample 2.15 min). SEM samples were prepared by three different methods. First, 5 μL of dialyzed 1.0 % w/v samples were placed on the carbon tape and after two minutes, excess sample was blotted using filter paper. Second, dialyzed 1.0 % w/v samples were centrifuged with VWR Ministar Silverline centrifuge and after removing the supernatant, the solid pellet was transferred on to the carbon tape placed on an aluminium stub. Third, 1.0 % w/v dialyzed samples were freeze-dried in the liquid propane to confirm the polymer structures in water. Freeze-dried samples were compared to the other methods to study the drying effects of the polymers and identify the possible self-assembly during the drying.

2.10 In-situ self-assembly on hydrophilic substrates

In situ self-assembly of the polymers was studied on hydrophilic substrates during the solvent exchange. Study was done with compound (**5b**), as it tends to form distinct spherical morphology of the self-assemblies. Muscovite mica sheets and silicon wafer were used as substrates while solvent was changed from DMSO to water via dialysis. Mica and silicon wafer substrates were plasma cleaned for 2.0 minutes prior to use. In a typical experiment, 100 μ L of 1.0 % w/v solutions in DMSO were heated and dialyzed in Slide-A-Lyzer MINI Dialysis device Floats (3.5 K MWCO) against water with mica or silicon wafer immersed in the dialysis tube. The substrates were washed from the excess of the samples by dipping the samples in MilliQ water after 2.0 h dialysis, freeze-dried in liquid propane under vacuum dried and imaged using Zeiss Sigma VP scanning electron microscope (acceleration voltage of 3 kV) with 5 nm platinum coating.

2.11 Polarizing optical microscopy studies

The polymer self-assembly was visualized using polarizing optical microscopy experiments. In a typical experiment, self-assembled structures of 1.0 % w/v in water were imaged with a Leica DM4500 optical microscope after the dialysis. The dispersion (20 μ L) was pipetted into a Grace Bio-Labs SecureSeal imaging spacer on the glass slide and covered with a cover slip. Samples were imaged using optical microscope at magnification 40x equipped with Canon EOS 70D camera.

2.12 Reverse dialysis of self-assembled superstructures

Reverse dialysis of self-assembled superstructures from water to DMSO was performed to investigate the solvent induced reversibility. This study was performed by first dialyzing the polymer from DMSO to water to obtain self-assembled spherical superstructures. After the primary dialysis, 100 μ L of aqueous dispersion was dialysed again using Slide-A-Lyzer MINI Dialysis device Floats (3.5 K MWCO) but this time from water to DMSO.

2.13 Thermal response of self-assembled superstructures

The effect of heating on self-assembled superstructures in aqueous dispersion, 100 μ L of aqueous dispersion containing self-assembled spherical particles was subjected for heating in a screw capped

glass vial. The sample was heated until the turbid dispersion turned to a translucent dispersion. The sample was then analyzed using TEM and optical microscopy imaging techniques.

3. Results and discussion

3.1 Solubility studies

First, we discuss the polymer solubilities. In our previous work [48], it has been shown that upon equipping compound **2** with UPy units through hydroxyl (-OH) groups, the resulting polymer undergo sequential assembly and transformation from micelle to fibers to spherical superstructures. There in, micellar structures in DMSO was expected due to packing parameter of the star-like molecule which adopts a cone like structure. The formation of fibers is attributed to the decreased solubility upon adding water creates a lamellar array of micelles or even the packing attains cylindrical structures. Therefore, instead of hierarchical structure formation, a sequential assembly with structural transformation was achieved. On the other hand, compound **3** CA[(AGE-NH₂)₆]₄, having peripheral polar amino groups in addition to hydroxyl functionalities allow better solubility in water compared to that of compound **2**. Therefore, a better dispersion of the the polymer CA[(AGE-NH₂)₆]₄-(UPy)₃ (**5a**) is expected. Accordingly, CA[(AGE-NH₂)₆]₄-(UPy)₃ (**5a**) dissolved in DMSO upon heating. However, the solubility decreased upon increasing the molar amount of UPy units (n), and the compounds, **5b** and **5c** required sonication prior to heating to obtain a clear solution. Obviously heating in DMSO disrupts the hydrogen bonds between UPy groups and thus improves the solubility in DMSO. As the solubility at higher concentration (>1.0 % w/v) was found to be low, all subsequent studies are conducted at low concentrations of 0.01 % and 1.0 % w/v. The two different concentrations were choosen to study the effect of concentration on the superstructure formation as well as to facilitate the microscopic characterization. The compounds (**5a-c**) as dissolved in DMSO tend to phase-separate after storing at room temperature for several hours. On the other hand, the amine terminated polymer CA[(AGE-NH₂)₆]₄ (**3**) has been shown to display reverse solubility

behavior in water [40]. It dissolves readily at lower temperatures, and at higher temperatures undergoes phase separation and shows a cloud point (CP). This is attributed to the disruption of hydrogen bonds between the polymer and the water molecules [40].

3.2 Solvent exchange by dialysis

In order to activate the supramolecular self-assemblies, polymers **3** and **5a-c** were dissolved in DMSO and were next dialyzed against water. After complete solvent exchange, CA[(AGE-NH₂)₆]₄ (**3**) remained as a clear solution due to the presence of polar amino groups. However, the solubilities of the polymers **5a-c** in water were found to be concentration dependent. When the dialyses of **5a-c** were performed at 0.01 % w/v, no macroscopic changes were visible (Fig. 2). However, when the concentration was increased to 1.0 % w/v, all the UPy-decorated polymer (**5a-c**) solutions turned turbid during the dialysis (Fig. 2) upon starting water addition to DMSO. The turbidity increased when the ratio of water was increased, finally producing a milky suspension. The turbidity was also increased with increasing equivalents of UPy leading to either as translucent (**5a**) or opaque (**5b**, **5c**) dispersions. The turbid dispersions at 1.0 % w/v concentration tend to sediment at the bottom of the vials after keeping them undisturbed for several days (Fig. 2). Sedimentation indicates that the particles are relatively large in size ($\geq 1\ \mu\text{m}$) or aggregations of several smaller particles due to the of gravity has an impact on the particles.

Fig. 2. Solubility behavior of CA[(AGE-NH₂)₆]₄ (**3**) and CA[(AGE-NH₂)₆]₄-(UPy)_n (**5a-c**) in water after the self-assembly via solvent exchange during the dialysis. In 0.01 % w/v solutions, no macroscopic changes were visible (top). However, all of (**5a-c**) in 1.0 % w/v were turbid suspensions (middle) which sediment at the bottom of the vials after keeping them undisturbed for several days (bottom).

3.3 Transmission electron microscopy

Micellar self-assembly of CA[(AGE-NH₂)₆]₄ based on its amphiphilicity could be expected, as the polymer side chains remain hydrophilic even by addition of the NH₂-groups and the lipophilic steroidal backbone form the cores of the micelles [40]. Indeed, CA[(AGE-NH₂)₆]₄ undergoes micellar self-assembly in DMSO based on TEM micrographs (Fig. S9a). Also the UPy-conjugated polymers (**5a-5c**), showed spherical micellar structures in DMSO with 8-12 nm core diameter (Fig. 3a-3d). They also showed the tendency to aggregate into larger (~100 nm) disordered structures (see Fig. 3d for a characteristic example) as well as sedimentation upon standing at room temperature for longer times (Fig. 2). Therefore, a conclusive size determination by dynamic light scattering (Supporting Information Fig. S10), as well as extensive structural characterization using NMR spectroscopy remained a challenge (See section 2.4). Interestingly, solvent exchange from DMSO to water using dialysis showed only spherical superstructures irrespective of the amount of UPy (Fig. 3e-3g) suggesting that, even a few UPys units can direct the higher order assemblies (> 1 μm). However, larger particles were observed at 1.0 % w/v, compared to those at 0.01 % w/v concentration (see Supporting Information Fig. S11) indicating the effect of concentration. A systematic analysis of the TEM micrographs revealed that, at 0.01% similar superstructures were formed as in the case of 1.0%. However, the size (100-500 nm) distribution of the particles at lower concentrations have significantly reduced, which suggests a more controlled assembly process. Further, decrease in the size of the spherical superstructures at 0.01% upon increasing number of UPy groups was observed.

Fig. 3. TEM micrographs of micelles and spherical superstructures for CA[(AGE-NH₂)₆]₄-(UPy)_n in DMSO and water. a) Micelles of CA[(AGE-NH₂)₆]₄-(UPy)₃ (**5a**) in DMSO (0.01 % w/v); b) Micelles of CA[(AGE-NH₂)₆]₄-(UPy)₆ (**5b**) in DMSO (0.01 % w/v); c,d) Micelles of CA[(AGE-NH₂)₆]₄-(UPy)₉ (**5c**) in DMSO (0.01 % w/v). Higher order superstructures in water upon dialysis from DMSO (1.0 % w/v): e) for (**5a**); f) for (**5b**); and g) for (**5c**).

3.4 3D reconstruction by transmission electron tomography

As conventional TEM micrographs reveals only the 2D projections of the samples, we performed three dimensional (3D) reconstruction of the aqueous spherical superparticles in order to gain more insight on their internal structures. Importantly, due to relatively large size (up to ~1.0 μm), cryo-TEM imaging was not suitable, and an alternative sample preparation method had to be identified which would still maintain the structural features of spherical particles. In our method, a rapid and sequential solvent exchange was performed (see Experimental Section), and the sample was imaged after lyophilization. Interestingly, the spherical shape of the particles was retained (Fig. 4). A tilt series was collected between +69° to -69° with an increment angle of 2° between the

projections, which was then subjected for alignment with fiducial gold markers followed by reconstruction. The tilt series shows that the spherical particle retained its shape as shown by representative snapshots in Figs. 4a-c (Video S1). The 3D structure of the sphere obtained after electron tomographic reconstruction is shown in Fig. 4d (see Video S2). It suggests that the solid sphere is made up of highly interconnected network (Fig. 4e) and the spherical superstructure is a supermicellar structure, where the UPy's drive the intermicellar H-bonding (Fig. 4f).

Fig. 4. Electron tomographic reconstruction of a spherical particle: a-c) snapshots from the tilt series at $+67^\circ$, 0° and -67° respectively (arrows indicate ~ 5 nm fiducial gold markers, *Note*: a dark circle observed inside 4b is the area attached to the grid and should not be mistaken for vesicle); d) 3D reconstructed structure of a sphere showing spherical nature of the particle (isosurface); e) a cross-section of the tomogram of a spherical particle (*Note*: the thick surface layers seemingly not containing network structures results from imaging an artifact due to missing tilting wedge in tomography) and f) 3D schematic illustration of inter-micellar network.

3.5 Scanning electron microscopy

Further investigation on the morphology was carried out using scanning electron microscopy (SEM) for the starting material (**3**) and the UPy conjugates (**5a-c**). Three different methods were used: (i) drying the aqueous dispersions under ambient conditions; (ii) drying pellets from centrifuged samples under ambient conditions; and (iii) freeze drying using liquid propane. Related to the first drying method (i), the SEM images of samples prepared under ambient drying (see Experimental Section for details) are shown in Fig. 5. The polymer CA[(AGE-NH₂)₆]₄ (**3**) formed relatively continuous film on the carbon tape, and only faint signatures suggested existence of colloidal structures in SEM (Fig. 5a,b) due to their small size. By contrast, polymers with UPy (**5a-c**) formed distinct spheres (Fig. 5c-k), in agreement with TEM. However, the superstructures were collapsed to some extent during the drying, but the spherical morphology was clearly evident. The superstructures from compound (**5a**) (Fig. 5c) formed arrays with local hexagonal close packings. Also the compounds (**5b**) (Fig. 5d) and (**5c**) (Fig. 5e) formed spherical superstructures. This behavior might be explained with the solubility or amount of water in the sample during the drying: The compound **5a** was better dispersible in water due to its high number of polar –NH₂ end groups. For the second drying method (ii), the samples were first centrifuged and then the pellets were drop-casted on a carbon tape and dried under ambient conditions. As shown in Fig. 5f-5h, the SEM images revealed close packed arrays of spherical superstructures. Interestingly, some of the superstructures show holes presumably due to drying artefact. Importantly, the internal network structures are visible in SEM in a collapsed spherical particles (Supporting Information, Fig. S12), further supporting our observation in electron tomography. Centrifugation was not done with CA[(AGE-NH₂)₆]₄ (**3**) as it was a clear solution. In the last drying method (iii), uncollapsed superstructures are observed for samples (**5a-c**) upon freeze drying (Figs 5i-k)

Fig. 5. SEM micrographs of $\text{CA}[(\text{AGE-NH}_2)_6]_4$ (**3**) micelles and $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ (**5a-c**) spherical superstructures prepared from water after the self-assembly via solvent exchange by the dialysis from DMSO (1 % w/v). a) $\text{CA}[(\text{AGE-NH}_2)_6]_4$ (**3**) dried in ambient conditions; b) Freeze dried $\text{CA}[(\text{AGE-NH}_2)_6]_4$ (**3**); c-e) $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ (**5a-c**) dried in ambient conditions; f-h) Pellet of $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ (**5a-c**) dried in ambient conditions after centrifugation; i-k) Freeze dried $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ (**5a-c**).

3.6 *In situ self-assembly at surfaces*

We envisaged that, the facile self-assembly of $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ from micelles to spherical superstructures triggered by sequential solvent exchange can also be used to fabricate self-assembled colloidal layers on various surfaces. This would also allow a potentially simpler characterization of the spherical superstructures as the assembly can occur directly at the surfaces, which can be then imaged under electron microscopy. We studied the in-situ self-assembly at surfaces using mica sheets and silicon wafers. Studies were performed for the compound (**5b**) since the self-assembly of (**5b**) and (**5c**) were the most promising due to distinct spherical morphology but water dispersibility was better with (**5b**) than with (**5c**).

The self-assembly was carried out by placing silicon wafer or mica sheet inside the dialysis tube containing a clear DMSO-solution of polymer. The solution was then subjected for dialysis against water. During this process the self-assembled spheres deposit on silicon or mica substrate. The substrate was then removed after the solvent was completely exchanged to water and freeze-dried using liquid propane prior to imaging. Fig. 6 shows the in-situ self-assembled spherical superstructures on mica and silicon wafer surface. Single layers of colloids were observed in both cases (Fig. 6), albeit not complete coverage, and no significant difference was observed between these two cases. Self-assembly at surfaces allows a better control over sample preparation method to provide regular and uniform colloidal single layer surface coatings. This approach might offer a facile route to achieve nano- or micro surface patterning of substrates. This approach is also a useful method for preparing colloidal particles for imaging, which generally have the tendency to form non-uniform deposition at the substrates.

Fig. 6. SEM micrographs of 1 % w/v CA[(AGE-NH₂)₆]₄-(UPy)₆ (**5b**) self-assembled a) on mica sheets and b) on silicon wafer during the solvent exchange in dialysis.

3.7 Polarizing optical microscopy studies

Because of their large size, the spherical superstructures offer the possibilities to image using polarizing optical microscopy under ambient condition. Spherical super structures of UPy conjugates (**5a-c**) were soberved in optical microscope whereas micelles of CA[(AGE-NH₂)₆]₄ (**3**) was not visible due to the small size (see Supporting Information Fig. S13). Similarly, when the polymers 5a-c dispersion in DMSO was images, no structures features were observed as the size of the micelles are beyond the resolution limit of the optical microscopy. Interestingly, when a water was added dispersion of 5a in DMSO instantaneous aggregation leading to superstructures was observed as supported by real time video (see Video S3).

3.8 Reversible assembly of superstructures: effect of solvent and temperature

The self-assembly of polymers **5a-c** was due to the activation of UPy dimerization upon solvent exchange from DMSO to water. Therefore, it is relevant to investigate whether, the superstructures are reversible. When an aqueous dispersion of superstructures obtained from polymers after dialysis was subjected for reverse dialysis (i.e. from water to DMSO), a clear solution was obtained (see

Supporting Information Fig. S14). TEM micrographs suggests that the superstructures are reversible and only micelles were observed. Similarly, when an aqueous dispersion of superstructures obtained from polymer was heated, the turbid dispersion turned translucent. TEM analysis suggests that the spherical particles did not disintegrate, instead a slightly reduced size was observed (Supporting Information Fig. S15). This suggest that the superstructures can be switched ON and OFF using solvent. However, they are thermally stable once dialyzed to water.

3.9 Suggested sequential hierarchical self-assemblies

Fig. 7 illustrates the proposed mechanistic pathway for the spherical superstructures. Natural bile salts are known to undergo self-assembly into non-spherical micelles in water due to their facially amphiphilic nature [32]. However, the modified star-like molecules have been shown to form spherical micelles in water [40]. Similarly, the star-like $\text{CA}[(\text{AGE-NH}_2)_6]_4\text{-(UPy)}_n$ molecules undergo self-assembly to spherical micelles in DMSO with the polar arms on the surface and lipophilic steroidal units at the core of the micelles (Fig. 7a-b), as evidenced by TEM (Fig. 3a-3d). Upon exchanging to water, higher order supermicellar structures (Fig. 7c,d) are formed due to the activation of the hydrogen bonds between the UPys as promoted by the hydrophobic hexyl pockets.

Fig. 7. Schematic illustration of the proposed self-assembly. a) Cartoon representation of the asymmetric star-like amphiphilic CA[(AGE-NH₂)₆]₄-(UPy)_n; b) Its self-assembly to spherical micelles in DMSO; c-f) Upon dialyzing to water the H-bondings between UPy units are activated leading to spherical microspheres; e) *In-situ* self-assembly to colloidal single layers on mica surface in water.

4. Conclusion

In this work, we explore minimal modifications to achieve complexity to classic micellar structures. Micelles have been typically obtained by amphiphilic molecules or polymers involving hydrophilic and hydrophobic ends. Here we equip the hydrophilic chain ends of asymmetric star-like amphiphilic polymers with hydrogen bonding ureidopyrimidinone moieties whose mutual supramolecular interactions can be switched ON by solvent exchange from DMSO to water. This leads to formation of micrometer-sized supermicelles having internal network structures consisting of hydrogen bonded micelles. The 3D structural details were explored using electron tomographic

reconstruction, where the conventional techniques such as cryo-TEM are not suitable due to the limitations of ice thickness (≤ 130 nm). The results show that combining competing supramolecular interactions to amphiphilic polymers can allow complexity and self-assembly through the length scales.

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