Enzymatically Degradable Star Polypeptides with Tunable UCST Transitions in Solution and within Layer-by-Layer Films

Journal:	*Polymer Chemistry*
Manuscript ID	PY-COM-06-2018-000939.R2
Article Type:	Communication
Date Submitted by the Author:	13-Sep-2018
Complete List of Authors:	Zhou, Qing; Texas A&M University, Department of Materials Science and Engineering Palanisamy, Anbazhagan ; Texas A&M University, Department of Materials Science and Engineering Albright, Victoria; Texas A&M University, Department of Materials Science and Engineering Sukhishvili, Svetlana; Texas A&M University, Department of Materials Science and Engineering;

Journal Name

COMMUNICATION

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Qing Zhou, Anbazhagan Palanisamy,[#] Victoria Albright, and Svetlana A. Sukhishvili*

We report enzymatically degradable, star polypeptides with uniquely sharp and reversible upper critical solution temperature (UCST) behavior in aqueous solutions. UCST transitions of star poly(L-ornithine-*co*-L-citrulline) were tunable *via* degree of uredio modification and/or length of polypeptide arms. Moreover, star polypeptides were assembled into layer-by-layer films with tannic acid and resulting films displayed robust and tunable UCST-triggered swelling transitions.

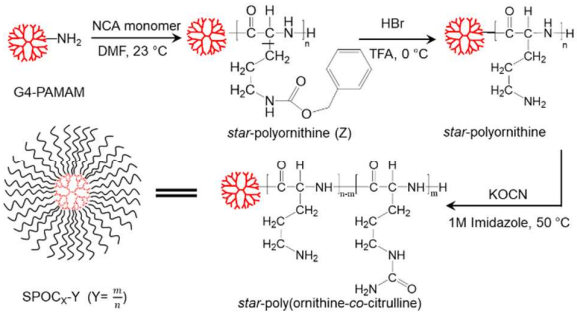
Thermo-responsive polymers have shown promise for many biomedical applications, including controlled drug delivery.[1] Polymers showing upper critical solution temperature (UCST) behavior can take advantage of a localized increase of temperature caused by tumors or inflamed tissues to release the loaded cargo in a controlled manner, and therefore, are highly desired.[1-3] Yet, UCST polymers in aqueous media are much less common and more poorly studied compared to their lower critical solution temperature (LCST) counterparts.[1-5] In fact, UCST behavior was mostly reported in organic solvent or in water/organic solvent mixtures,[1, 4, 6] with only a few reports of UCST transitions in aqueous solutions.[1, 3-5]

So far, three studies have achieved container-like morphology, which is desirable for controlled delivery, and these reports were limited to UCST polymeric micelles.[1, 2, 7] Recently, Palanisamy *et al.* developed UCST block copolymer micelles driven by hydrogen bonding between ureido-derivatized functional groups.[1] The micelles showed highly reversible UCST-type swelling/deswelling transitions when deposited within layer-by-layer (LbL) films and demonstrated on-demand release capability. However, preventing β-sheet formation of the polypeptide block of the copolymer was challenging.[1] Moreover, micelle formation itself is a dynamic equilibrium process, which depends on various factors such as solvent type, pH, and temperature, and this dynamic behavior impedes facile engineering of robust responsive material.[8] Furthermore, unlike LCST, which largely depends on polymer-water interactions, UCST involves polymer-polymer binding,[9] and therefore occurs in a wide temperature range[1, 9, 10] and is strongly dependent on polymer concentration.[4] The width and concentration dependence of UCST transitions present significant obstacles in designing functional materials with well-controlled responses for actuation and delivery applications.

In this work, we aimed to synthesize a dendritic star polypeptide, whose molecular architecture would impede the dependence of UCST transitions on concentration and environmental conditions and use it as a building block to form UCST responsive materials to overcome the above challenges. The three-dimensional molecular architecture of star polymers[11, 12] makes them ideal candidates for programmable materials, since the high and well-defined local concentration of functional groups enables precise control over their stimuli-response behavior.[13, 14] However, to date, only a few reports have used star polymers as building blocks for thermally responsive materials, with those reports focused on polymers that exhibit LCST behavior, which mostly involved non-degradable poly(N-isopropylacrylamide) (PNIPAM).[15-21]

Here, a series of *star*-like polypeptides, s*tar*-poly-L-ornithine (SPO), were synthesized *via* ring-opening polymerization (ROP) of L-ornithine N-carboxyanhydride (NCA) with carboxybenzyl protecting group (L-ornithine(Z)-NCA) initiated by the primary amino groups on the generation-four polyamidoamine dendrimer (G4-PAMAM), followed by deprotection of carboxybenzyl groups (scheme 1). The pendant primary amine groups were then functionalized into ureido groups, resulting in *star*-poly(L-ornithine-*co*-L-citrulline) (SPOC).

Department of Materials Science and Engineering, Texas A&M University, 575 Ross Street, College Station, TX 77843, USA.
[#]Current affiliation address: Physical Chemistry and Soft Matter, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands.
**Correspondence and requests for materials should be addressed to S.A.S. (email: svetlana@tamu.edu)*



Scheme 1. Synthesis route of SPOC polymers starting with a G4-PAMAM dendritic core.

Star polypeptides with various arm lengths were achieved by varying the feed ratio of NCA monomer to the primary amino groups of the G4-PAMAM. Additionally, SPOCs with different degrees of ureido modification were prepared by reacting star polypeptides with various amounts of potassium cyanate to achieve desired ratios with the primary amino groups in poly(L-ornithine) arms (Table S1). As shown in Scheme 1, polymers are represented as $SPOC_X$-Y, where X represents the initial NCA/[NH_2] ratio during ROP, while Y represents the ureido modification degree. More details of monomer and polymer synthesis and characterization are described in the Supporting Information sections, including Scheme S1, Figures S1, S2, S3. Because of the high efficiency of the ring opening of NCA,[22] the polymerization of the precursor polyornithine(Z) (SPO(Z)) proceeded until complete consumption of monomers as confirmed by the absence of the monomer NHC***H*** proton signals at 4.45 ppm in the ^{1}H NMR spectra (Figure S2a),[1] and the molecular weight of the star polymer could be calculated from NCA/[NH_2] ratio. Due to the compact architecture of star polymers, however, GPC traces calibrated using standards of linear polymers could not be used to determine the molecular weights of the star polymers, but instead corresponded to the apparent molecular weights of linear polymers with the same hydrodynamic radii (R_h).[23, 24] Note that the star architecture also suppressed segmental mobility of the dendrimer core, which is indicated by the lack of PAMAM dendrimer resonances in the ^{1}H NMR data (Figure S2). The contraction factor g of the star polymers as compared to their linear counterparts was defined as:

$$g = \frac{\langle R_g^2 \rangle_s}{\langle R_g^2 \rangle_l}$$

where $\langle R_g^2 \rangle_s$ is the mean-square radius of a star polymer and $\langle R_g^2 \rangle_l$ is the radius of a linear polymer of the same molecular weight as the star polymer.[25] For star polymers with many arms dissolved in a good solvent, $\langle R_g^2 \rangle_s / \langle R_g^2 \rangle_l$ approaches $(3f-2)/f^2$,[26] where f is the number of polymer arms. This estimate gave the contraction factor of ~0.092 for a 32-arm star polymer. This indicates that the average segmental density of the star polymer is ~35 fold higher than that in linear polymer chains. Table S1 shows that the star-to-linear density ratios calculated from GPC data using the ratio between R_h and R_g of linear and dense star polymers of 0.79 and 1.29, respectively,[27] were within less than 20% of the theoretical value for all SPO(Z) polymers, thus verifying the proposed structures.

Two groups of SPOC samples were then prepared to study the effects of NCA/[NH_2] and ureido modification degree on their transition temperature. One group had a constant NCA/[NH_2] ratio of 55 with ureido modification degrees varying from 93 to 99%. The second group had a constant ureido modification degree of 96% with variable NCA/[NH_2], ranging from 41 to 100. All six synthesized SPOCs demonstrated highly reversible UCST transitions in 150 mM NaCl aqueous solutions, which are likely supported by the formation of intramolecular hydrogen bonds as shown in Figure 1a. Figures 1b and S4 illustrate, with the example of $SPOC_{55}$-96, that SPOCs were round-shaped with average diameters of 20±3 nm and 26±2 nm, as determined from AFM and TEM images, respectively.

To study the effect of molecular architecture on UCST transitions, dynamic light scattering (DLS) was used to monitor the particle size in the temperature range from 10 to 30°C. We chose DLS as the major technique to detect UCST transitions since DLS is more sensitive to early-stage aggregation in solution as compared to the UV-Vis technique. DLS distributions of hydrodynamic diameters in 2 mg/mL $SPOC_{55}$-96 solutions at several temperatures close to the UCST transition are shown in Figure S5. The transition was defined as the temperature which showed the first signs of aggregation indicated by the occurrence of a second peak in D_h distribution upon cooling of SPOC solutions. Using this definition, the UCST transition temperature of $SPOC_{55}$-96 solution was determined as 24 ˚C. This transition was highly reversible as observed from the repeatable values of photon counts during five heating/cooling cycles of 2 mg/mL SPOC solutions between 10 and 30 ˚C (Figure S6). In comparison with DLS measurements, when the turbidity of the same $SPOC_{55}$-96 solutions was measured at 670 nm *via* UV-Vis spectroscopy as shown in Figure S7, the UCST transition temperature was

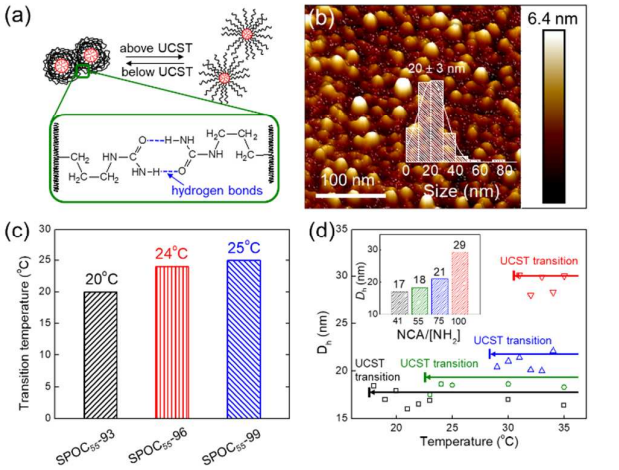


Figure 1. Hydrogen-bonding, morphology and UCST transitions of star polypeptides. (a) Illustration of hydrogen-bonding of SPOC, (b) AFM height image of $SPOC_{55}$-96 deposited on a silicon substrate *via* spin coating at 1000 RPM at room temperature (above UCST), (c) UCST transition temperatures of $SPOC_{55}$ series as a function of ureido modification degree as determined by DLS and, (d) UCST transitions and hydrodynamic diameters (the inset, measured above UCST transition) of SPOCs with NCA/[NH_2] of 41, 55, 75, and 100. All solutions contained 2 mg/mL SPOC polymers, 150 mM NaCl and were

observed at a lower temperature of ~16 ˚C. We suggest this shift in observed transition temperatures is due to the different sensitives of the techniques to early stages of aggregation in UCST solutions.

When the ureido modification degree of $SPOC_{55}$ was increased from 93 to 99%, the UCST transition changed from 20 ˚C to 25 ˚C as measured by DLS (Figure 1c) (or 14 to 18°C as measured by UV-Vis (Figure S8a)). The dependence of the UCST transition temperature of these polymers on the ureido modification degree agrees with a similar observation for linear copolymers,[4] in which an increase in ureido modification of polymers (molecular weight of 150 kDa) from 84 to 93% caused a rise in the transition temperature from ~12 ˚C to ~30 ˚C. Here, we report the first observation of a similar effect with star-shaped UCST polymers.

Interestingly, due to the high local concentration of functional groups of SPOC resulting from their covalent attachment to the core, UCST transitions of SPOC were only weakly dependent on the overall solution concentration. This is illustrated by transition temperatures of 22, 23, and 25 ˚C, observed in 0.5, 1.0, and 2.0 mg/mL $SPOC_{55}$-99 solutions, respectively (Figure S9). This behavior is in contrast with strong concentration dependence of another ureido-based non-degradable UCST linear polymer with DP of 100 reported elsewhere,[5] which showed an increase in transition temperature from 39 to 50 ˚C upon an increase in concentration from 0.5 to 2.5 mg/ml.

Moreover, UCST transitions of SPOC polymers could be controlled by the length of the polypeptide arms. Figure 1d shows that an increase in NCA/[NH_2] ratio from 41 to 100 caused the transition temperature of SPOCs to rise from 18 ˚C to 31 ˚C as measured *via* DLS (or from 16 ˚C to 24 ˚C as measured by UV-Vis, Figure S8b). The inset in Figure 1d shows that the hydrodynamic diameters of SPOCs measured above UCST transitions also increased from 17 to 29 nm. Enhanced UCST values for longer polymers are expected and are usually explained by the decrease in entropy of mixing and lowered penalty for phase separation.[28] Figure S8b also shows that $SPOC_{55}$-96, demonstrated a higher UCST transition than its two-arm linear analogue with matched length in arms and matched ureido content, ethylenediamine-*core*-poly(L-ornithine-*co*-L-citrulline) (LPOC, average arm DP of 50) (17 ˚C and 12 ˚C *via* UV-Vis for $SPOC_{55}$-96 and LPOC, respectively). This increase of UCST illustrates the effect of the increased local concentration of functional groups and enhanced polymer-polymer interactions. It is also remarkable that UCST transitions occurred within a relatively narrow temperature window (less than 5 ˚C) (Figure S8), which is much sharper than those typically observed with linear UCST polymers whose UCST phase separation usually occurs in a range wider than 15 ˚C. [1, 4, 5] Taken together, these results demonstrate that the molecular architecture of star polymer facilitates UCST transitions by locally concentrating UCST functional groups

The star architecture also showed strong effects on the formation of polypeptide secondary structures. Similarly to other previously studied star-shaped polymers with amino-acid arms,[29] FTIR of SPOCs in Figure 2a show strong amide I and amide II band absorptions, located at ~1650 and 1544 cm^{-1}, which suggests the existence of α-helical and random coil conformations.[30] However, ~ 28% of the integrated absorbance in the amide I region originated from β-sheet conformations at 1630 cm^{-1}. Importantly, unlike their linear counterparts, in which β-sheet formation progressed during multiple cooling/heating cycles and eventually resulted in irreversible precipitation,[1] the percentage of β-sheet was independent of sample history. Figure 2a shows that after at least five repeated UCST transitions shown in Figure S6, contribution of the β-sheet absorbance to the entire amide I region did not increase. We attribute this behavior to the fact that within the star architecture, the conformational freedom of polymer arms was significantly restricted and did not permit excessive formation of secondary structures. Polypeptide nature of SPOCs also enabled their enzymatically assisted degradation. Trypsin is an enzyme that is commonly found in digestion systems. Furthermore, it has been applied topically to assist the degradation of dead tissue in wounds.[31] In a clinical application, an enzymatically degradable polymeric device can be used in combination with a topical treatment that utilizes trypsin. Figure 2b shows that upon exposure of $SPOC_{55}$-96 to trypsin, a decrease in photon counts was observed by DLS. The slight decrease in photon counts of $SPOC_{55}$-96 solutions in DI water might be caused by uncatalyzed hydrolysis of the polymer at 37 ˚C.

To further study the unique properties brought by star

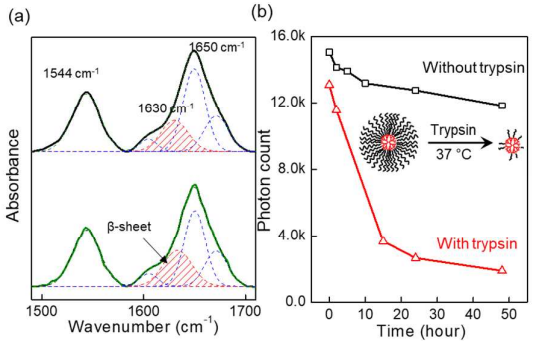


Figure 2. The β-sheet formation and enzymatic degradability of $SPOC_{55}$-96 star polypeptides. (a) FTIR spectra of dry $SPOC_{55}$-96 before and after five heating/cooling cycles between 30 and 10 ˚C, showing the presence of β-sheets and (b) time evolution of DLS photon counts during $SPOC_{55}$-96 degradation in 2 mg/ml solution at pH 7 when incubated with 0.25 % trypsin at 37 ˚C.

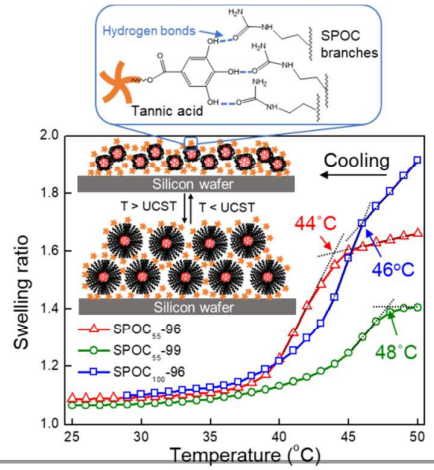


Figure 3. Temperature dependences of swelling ratios of $SPOC_{55}$-96/TA, $SPOC_{55}$-99/TA, and $SPOC_{100}$-96/TA films deposited as 6.5 bilayers on silicon substrates and exposed to 150 mM NaCl solutions at pH 7 as measured by *in situ* spectroscopic ellipsometry. The inset presents schematic illustration of UCST transitions in SPOC/TA films.

architecture and explore potential applications of the star polypeptides in functional surface coatings, we assembled SPOCs into films using the layer-by-layer (LbL) technique *via* hydrogen bonding with tannic acid (TA). LbL films were prepared by dip deposition at ambient temperature using 0.2 mg/mL SPOC and TA solutions at pH 5. Linear film growth was observed for $SPOC_{55}$-96, $SPOC_{55}$-99, and $SPOC_{100}$-96 after the third bilayer with incremental bilayer thickness of ~10 nm, as measured by ellipsometry with dry films (Figure S10a). Such films were formed by hydrogen bonding interactions, which likely occur between the ureido groups at outer periphery of arms and the phenolic hydroxyl groups on tannic acid (Fig. 3 scheme). Note that control LPOC/TA film constructed with a linear analogue of $SPOC_{55}$-96 in the same condition had bilayer thickness twice smaller than that of the star polymer LbL film (Figure S11). Interestingly, $SPOC_{55}$-93 could not form LbL films with TA, because of excessive repulsive interactions between protonated amino groups of $SPOC_{55}$-93, which are present in larger amount in this polymer.

Figure 3 shows spectroscopic ellipsometry data of various 6.5-bilayer SPOC/TA films immersed in 150 mM NaCl solution at pH 7 as a function of temperature. When exposed to heating/cooling cycles between 25 ˚C and 50 ˚C, all SPOC/TA films exhibited UCST type swelling/deswelling transitions. In contrast, control LPOC/TA film constructed with a linear analogue of $SPOC_{55}$-96 already contained ~40% of water at 25 ˚C, which was well above LPOC's transition temperature (Figure S8b), and (c) showed no obvious UCST transition upon further increase in temperature (Figure S11). Importantly, for all films of the star polymers, a UCST transition around 44-48 ˚C was observed, suggesting that hydrogen-bonding between TA and the star polypeptide did not outcompete the intramolecular hydrogen bonding within SPOC polymers, which is responsible for UCST transitions. Interestingly, UCST transitions in films occurred at much higher temperatures than in solution. Specifically, UCST transition temperature of $SPOC_{55}$-96 in solution of 24 ˚C (Figure 1c) increased to 48 ˚C in $SPOC_{55}$-96/TA films (Figure 3), where SPOC molecules were confined by binding with tannic acid. We attribute such an effect to an increased concentration of SPOC within the film, as well as to neutralization of positive charges in SPOC *via* ionization of polyphenol groups of assembled TA. Figure 3 also shows that the degree of ureido modification of SPOC polymers affected both the transition temperature and the amplitude of film swelling. As more hydrogen bonds were formed between SPOC and TA, LbL films response occurred at higher temperatures, and the films became less swellable. For example, as ureido modification degree of $SPOC_{55}$ increased from 96 to 99, the transition temperature raised from 45 to 48 ˚C, and the swelling amplitude decreased from 1.6 to 1.4. Moreover, the length of the SPOC arms also strongly affected the swelling amplitude, with an increase in the swelling ratio from 1.4 to 1.9 for $SPOC_{55}$-96 and $SPOC_{100}$-96, respectively. Swelling transitions supported by hydration/dehydration cycles of the star arms of SPOC molecules within LbL assemblies are illustrated in the inset in Figure 3. Despite non-covalent binding between SPOC and TA, LbL films demonstrated robust and reversible UCST response, showing highly repeatable swelling transitions (Figure S10b). Similarly, to the UCST behavior of SPOC in solution, the transitions occurred in a relatively narrow temperature interval of ~5 ˚C. Furthermore, the films showed low swelling ratios at temperatures below UCST. This feature might help prevent pre-mature release of payload of functional molecules within such assembled films and will be explored in our future work.

In summary, a new type of enzymatically degradable star polypeptide polymers was synthesized, which showed highly reversible UCST transitions both in solutions and at surfaces. Due to the unique star architecture, the peptide-based polymers demonstrated sharp transitions, low propensity to propagation of β-sheets/irreversible aggregation, as well as weak dependence of UCST transitions on concentration. Moreover, UCST transitions of star polypeptides were easily tunable *via* ureido modification degree as well as the length of the polymer arms. When assembled with TA within LbL films, the star polypeptides retained their reversible UCST response and enabled control over the amplitude of film swelling. We believe that LbL assemblies of star polypeptides offer a facile approach to introduce UCST-type stimuli-responsive nanocontainers to surfaces for potential applications in controlled delivery of active molecules from surfaces.

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Table of Contents Entry

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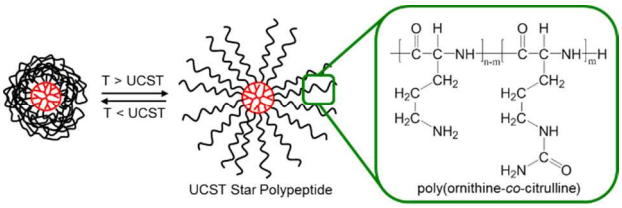
*Qing Zhou, Anbazhagan Palanisamy[#], Victoria Albright, and Svetlana A. Sukhishvili**

Department of Materials Science and Engineering, Texas A&M University, 575 Ross Street, College Station, TX 77843, USA.

*Correspondence and requests for materials should be addressed to S.A.S. (email: svetlana@tamu.edu)



Biodegradable star polypeptides with UCST-type transitions in aqueous solution and layer-by-layer assembly film.

[#] Current affiliation address: Physical Chemistry and Soft Matter, Wageningen University, Stippeneng 4, 6708 WE Wageningen, The Netherlands