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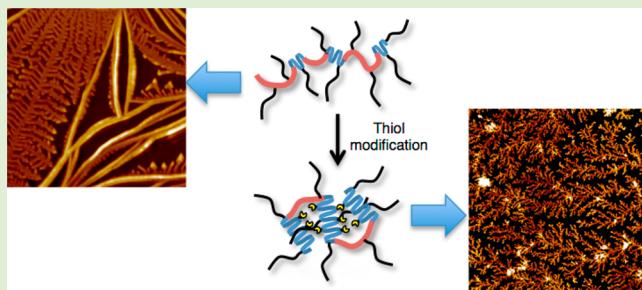
Self-Assembly of High Molecular Weight Polypeptide Copolymers Studied via Diffusion Limited Aggregation

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 Supporting Information

ABSTRACT: The assembly of high molecular weight polypeptides into complex architectures exhibiting structural complexity ranging from the nano- to the mesoscale is of fundamental importance for various protein-related diseases but also hold great promise for various nano- and biotechnological applications. Here, the aggregation of partially unfolded high molecular weight polypeptides into multiscale fractal structures is investigated by means of diffusion limited aggregation and atomic force microscopy. The zeta potential, the hydrodynamic radius, and the obtained fractal morphologies were correlated with the conformation of the polypeptide backbones as obtained from circular dichroism measurements. The polypeptides are modified with polyethylene oxide side chains to stabilize the polypeptides and to normalize intermolecular interactions. The modification with the hydrophobic thioctic acid alters the folding of the polypeptide backbone, resulting in a change in solution aggregation and fractal morphology. We found that a more compact folding results in dense and highly branched structures, whereas a less compact folded polypeptide chain yields a more directional assembly. Our results provide first evidence for the role of compactness of polypeptide folding on aggregation. Furthermore, the mesoscale-structured biofilms were used to achieve a hierarchical protein assembly, which is demonstrated by deposition of Rhodamine-labeled HSA with the preassembled fractal structures. These results contribute important insights to the fundamental understanding of the aggregation of high molecular weight polypeptides in general and provide opportunities to study nanostructure-related effects on biological systems such as adhesion, proliferation, and the development of, for example, neuronal cells.



INTRODUCTION

Aggregation of polypeptides into insoluble aggregates is a process of tremendous fundamental and practical importance. Aggregates of high molecular weight polypeptides of partially unfolded or misfolded proteins are related to several neurodegenerative and other diseases but also play an important role in vital processes such as biosynthetic pathways and hormone storage.^{1–5} Current aggregation studies mainly focus on the more readily accessible low molecular weight polypeptide derivatives with up to 40 amino acid residues. In addition, the low solubility of the partially unfolded polypeptides and the concurrent intermediates hamper the investigation of aggregation.⁶ Gaining fundamental insights in the underlying aggregation mechanisms of high molecular weight polypeptides is of high interest not only for the development of therapeutic strategies against proteinopathies, but also for the preparation of advanced polypeptide based nanomaterials.

Polypeptides recently gained interest as building blocks to synthesize highly ordered nanostructures and complex multiscale materials.^{7–12} These biomacromolecules exhibit an unraveled structural precision in terms of amino acid sequence, molecular weight, and secondary structure elements. Inter- and intramolecular interactions result in multiscale assemblies with structural features ranging from the nano- to the macroscale,

which share structural similarities with pathogenic aggregates.¹³ Aggregation of low molecular weight polypeptides has been studied using fractal analysis of the superstructures formed under diffusion limited aggregation (DLA), which allowed for important insights into the aggregation process.^{14–16} DLA is a process typically found at interphases and depends on concentration, temperature and on inherent molecular properties such as particle shape and the anisotropy of intermolecular interactions.^{17–20} Depending on these parameters, different superstructure morphologies with high orientational order, so-called compact and dendritic fractals (high anisotropic interphase interactions) or without apparent orientational order, so-called compact and dendritic seaweed structures (for vanishing anisotropy) are formed.^{21,22} Thus, detailed analysis of the formed structures reveals important information on aggregation-determining features such as the isotropy of intermolecular interactions and structural characteristics. Furthermore, DLA is a promising approach for the “bottom-up” assembly of nanostructured surface coatings with great potential for biotemplating, modulating cell adhesion, or to

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engineer neuronal networks.^{14,15,23–27} So far, the investigation of self-assembly of high molecular weight polypeptides by means of fractal analysis is hampered by their poor availability and low solubility. However, newly developed modification strategies provide polypeptides with functional residues to overcome known limitations, for example, their low solubility.

Recently, we introduced a new approach to stabilize high molecular weight polypeptides in aqueous buffers utilizing side chain modifications.²⁸ This approach allows one to prepare partially unfolded yet water-soluble high molecular weight polypeptides derived from naturally occurring proteins such as human serum albumin (HSA), bovine serum albumin (BSA), and hen egg white lysozyme (LY). The polypeptide copolymers containing polyethylene oxide (PEO) side chains exhibit a monodisperse backbone length, defined molecular compositions, and secondary structure elements. These PEO-grafted polypeptides are versatile macromolecular carriers that, for example, efficiently stabilizes lipophilic guest molecules.^{29–31} The synthetic strategy allows introducing orthofunctional groups such as PEO, primary amines and thioctic acid (TA) to the polypeptide backbone, which impart improved stability, water solubility and enhanced cellular uptake.^{28,32–34}

Here, we study the aggregation of partially unfolded high molecular weight polypeptides derived from LY, cBSA, and HSA into multiscale ordered surface structures by means of circular dichroism (CD), dynamic light scattering (DLS), atomic force microscopy (AFM), and fractal analysis. The investigated polypeptides exhibit essential differences in their macromolecular features such as backbone length, net charge as well as secondary structure. They form surface supported aggregates, which allow gaining insight into the aggregation process of high molecular weight polypeptides. We found that a less compact polypeptide conformation characterized by high random coil content favors anisotropic interactions, which is manifested in a highly directional self-assembly, whereas a more compact polypeptide conformation results in more isotropic intermolecular interactions and the formation of branched structures. Our findings suggest that the aggregation in solution and on surfaces can be controlled and fine-tuned via modifications at the macromolecular level, which is highly attractive for the development of bioactive polypeptide materials. We demonstrate that the fractal structures are even stable toward postassembly modification in aqueous solutions, which opens up unique opportunities to explore these polypeptide structures as, for example, bioactive and biocompatible surface coatings.

MATERIALS AND METHODS

All chemical reagents were obtained from commercial suppliers and used without further purification unless otherwise noted. Albumins from human (HSA, lyophilized powder, ≥96% (agarose gel electrophoresis) Sigma) or bovine serum (BSA, ≥98% (agarose gel electrophoresis), lyophilized powder) and lysozyme from hen egg white (LY, lysozyme from chicken egg white powder (crystalline), ~70 000 U/mg, Fluka) were obtained from Sigma-Aldrich. cBSA (cBSA-147) was synthesized according to our previous report.³⁰ Bio-Gel P30 from Bio-Rad was used for desalting. Complete removal of salt was verified via monitoring the absorbance at 280 nm and conductance during chromatography and with XPS analysis of the purified compounds (Supporting Information Figures S3 and S4). Vivaspin centrifugal concentrators were purchased from GE Healthcare. The synthesis of dHSA-PEO(5000)₂₇, dcBSA-PEO(5000)₂₇, and dLY-PEO(5000)₈ has been described recently.²⁸ The N-hydroxysuccinimide ester of thioctic acid (NHS-TA) was prepared according to the

literature.³⁵ The synthesis of dHSA-PEO(5000)₂₇-TA₂₂ can be found in our previous report.³³ The other TA-derivatives were synthesized according to the protocol as described below, purified with column chromatography and characterized with MALDI-ToF and SDS-PAGE. ÄKTA Purifier FPLC and Sephadyl S-100 HR gel filtration column were used for copolymer purification. Precast NuPAGE TA 3%–8% gel and NuPAGE Bis-Tris 4%–12% gel were purchased from Invitrogen, and gel electrophoresis was performed in an Invitrogen Novek Mini-Cell apparatus. The matrix-assisted laser desorption/ionization time-of-flight mass spectra (MALDI-ToF MS) were recorded on a Bruker Reflex III MALDI-ToF spectrometer (Bruker Daltonics).

Synthesis of dcBSA-PEO(5000)₂₇-TA₂₆. dcBSA-PEO(5000)₂₇ (5 mg, 6 μmol amino groups) was dissolved in water (1 mL). NHS-TA (2.4 mg, 8 μmol) was dissolved in DMF (0.2 mL) and added to the solution. After addition of NaHCO₃ (0.2 mL, 5 mg/mL), the reaction mixture was vigorously stirred overnight. The insoluble thioctic acid-NHS residue was filtered off, and the clear solution was concentrated by ultrafiltration (Vivaspin 20, 30 kDa MWCO), washed five times with water, and desalted (Bio Gel P-30 desalting column). After lyophilization, dcBSA-PEO(5000)₂₇-TA₂₆ (4.1 mg) was obtained as a white solid in 80% yield. SDS-PAGE indicates a molecular weight of about 200 kDa (Supporting Information).

Synthesis of dLY-PEO(5000)₈-TA₈. Denatured dLY-PEO(5000)₈ (5.4 mg, 0.7 μmol amino groups) was dissolved in water (3 mL). NHS-TA (2.4 mg, 8 μmol) was dissolved in DMF (0.2 mL) and added to the reaction mixture. After addition of NaHCO₃ (0.6 mg), the reaction mixture was vigorously stirred overnight. The insoluble residue was filtered off, and the solution was concentrated by ultrafiltration (Vivaspin 20, 10 kDa MWCO), washed five times with water, and desalted (Bio Gel P-30 desalting column). After lyophilization, dLY-PEO(5000)₈-TA₈ (4.2 mg) was obtained as a white solid in 75% yield. MALDI-ToF MS: *m/z* 56,367(M⁺)

zeta Potential. Zeta potentials were measured on a Malvern Zetasizer ZEN3600 instrument (Malvern Ltd., Malvern, U.K.). The respective polypeptide copolymers were dissolved in water (1 mg mL⁻¹), and aliquots (50 μL) were diluted with a solution of potassium chloride (1 mL, 1 mM) to a final concentration of 0.05 mg mL⁻¹ and filtered through a syringe filter (0.2 μm, cellulose acetate membrane, VWR international GmbH, Germany). Prior to its use, the disposable capillary cell (DTS1060, Malvern) was filled with deionized water, equilibrated for 30 min, and then rinsed with the appropriate sample solution twice. A sufficient sample volume was used to completely cover the electrodes of the cell. The cell was equilibrated at 25 °C in the instrument for 1 min prior to measurements. Data were collected 10 times for each sample, and the results are plotted as mean values with error bars of standard deviation.

DPS Test. The number of thioctic acid groups of TA-derivatives was quantified with the DPS assay using 4,4'-dithiodipyridine according to the literature.²⁸ The detailed results are listed in the Supporting Information.

Circular Dichroism (CD). The copolymers were dissolved in pure water (2 mg/mL). An aliquot of 0.1 mL of this solution was diluted with 0.75 mL of Tris buffer (pH 7, 50 mM). The sample was measured in a 1 mm cuvette with a volume of 700 μL. The CD signal was measured from 240 to 190 nm. The bandwidth was set to 1 nm with a response of 1 s. Standard sensitivity was used with a data pitch of 0.1 nm and a scanning speed of 100 nm/min. During the measurements, the temperature was kept constant at 20 °C. The data was accumulated using five subsequent measurements. The secondary structure composition was calculated using the CDSSTR program, available in the CDPro software package (<http://lamar.colostate.edu/~sreeram/CDPro/main.html>).

One should note that proteins could undergo structural transitions upon adsorption on solid surfaces. For instance, adsorption of BSA and Lys on hydrophilic silica particles reduces the α-helix content.³⁶ It was also shown that the decrease is more significant for BSA than for LY and becomes less pronounced with increasing surface coverage. Unfortunately, characterizing the secondary structure of the

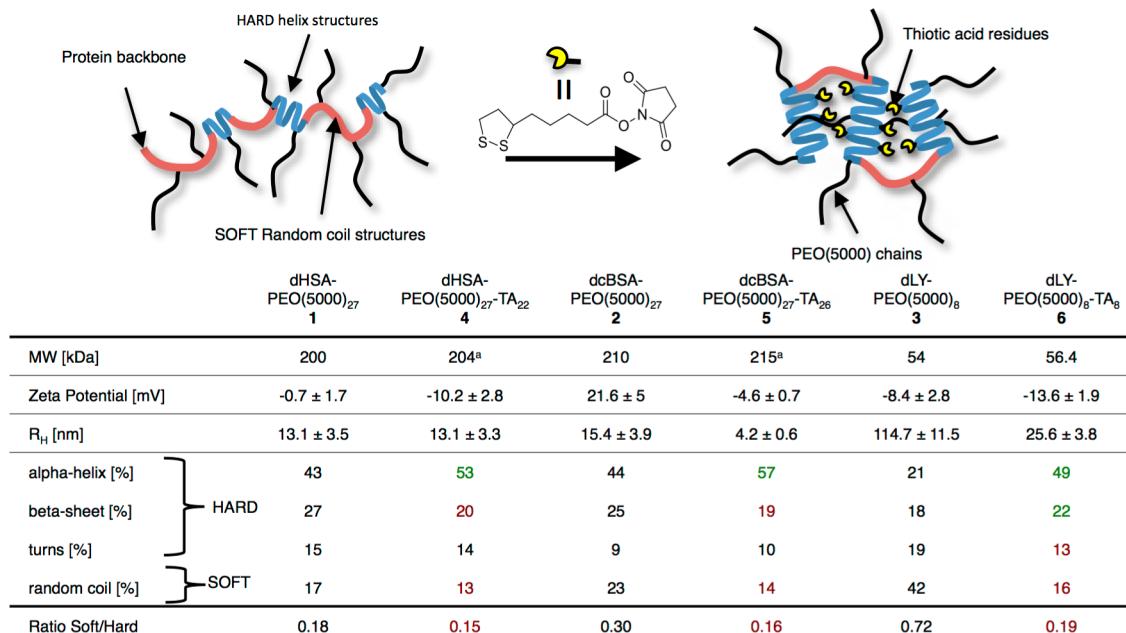


Figure 1. Schematic representation and table of physicochemical properties of the investigated polypeptide copolymers comprising a denatured peptide backbone and PEO chains grafted to cysteine residues. Primary amines are modified with an amine-reactive thioc acid NHS-ester to increase backbone hydrophobicity. Molecular weights (MW) except for those of dHSA-PEO(5000)₂₇-TA₂₂ and dcBSA-PEO(5000)₂₇-TA₂₆ were assessed by both SDS-PAGE and MALDI-ToF; the MWs and the number of sulphydryl groups of the thioc acid modified albumin derivatives were estimated from quantitative DPS thiol assay and SDS-PAGE (Supporting Information). The zeta potential was measured at pH 7.4. The number average of the hydrodynamic radius R_H was determined by DLS and the relative amount of secondary structure elements was deduced from CD experiments. The increase and decrease in values are highlighted green and red, respectively.

polypeptide copolymers on mica is not feasible with available methods due to limitations in sensitivity.

Atomic Force Microscopy (AFM). The AFM samples were prepared by drop-casting aliquots of 10 μL of 0.3 mg/mL solutions in pure water on freshly cleaved mica and drying in air. AFM measurements were performed on a Veco Multimode IIIa atomic force microscope using silicon nitride cantilevers (Olympus) with a spring constant of 1.7 N/m. The raw data was processed (plane correction only) and analyzed with the SPIP 5.1.5 software (Image Metrology, DK). The fractal analysis was performed with the ImageJ 1.46 software from <http://rsb.info.nih.gov/ij/> (National Institutes of Health) with the FracLac plug-in, version 2.5. The AFM images were converted into binary images, and the fractal dimension D_F and lacunarity λ were calculated using the standard box counting (SBC) algorithm provided by the FracLac plug-in. In the SBC algorithm, a series of grids of different sizes were overlaid over a binary image, and the foreground pixels were counted to estimate scaling and to calculate D_F and λ.

Preparation of HSA-TMRh. Rhodamine labeled HSA (HSA-TMRh) was prepared by reacting tetramethylrhodamine-5-maleimide (TMRh-MI) with the Cys34 residue of HSA according to a reported procedure.³⁰ To a solution of HSA (68 mg, 1 μmol) in 60 mL phosphate buffer (pH 6.5, 50 mM), 0.22 mL of a freshly prepared aqueous solution of tris(2-chloroethyl)phosphate (TCEP, 1 mM, 0.22 μmol) was added under argon, and the reaction mixture was stirred for 10 min at RT. A solution of TMRh-MI (1 mg) in DMSO (1 mL, 2 μmol, 200% excess) was added, and the reaction mixture was stirred at RT for 1 h under argon atmosphere. The nonreacted TMRh-MI was removed by ultrafiltration (Vivaspin 20, 30 kDa MWCO) and washed five times with water, and then the crude product was purified by size-exclusion chromatography (Bio-Gel P 30). The first colored layer was lyophilized to afford HSA-Rho. The labeling ratio of Rhodamine was determined by comparing the absorbance at 280 nm (protein) and 543 nm (Rhodamine), and about 30% of HSA was labeled with Rhodamine. It is known that some of the Cys34 residues in native HSA are blocked by mixed disulfide formation.³⁷

Fluorescence Microscopy. The coassembly of the protein copolymer layer with HSA-TMRh was carried out as follows. To a mica surface decorated with dLY-PEO(5000)₈-TA₈, a saturated solution of HSA-TMRh in EtOH/H₂O (3:1 v/v) was added, and the solvent was evaporated in air. The modified surface was imaged using a fluorescence microscope (Zeiss AxioScope 2 mot plus).

RESULTS AND DISCUSSION

The polypeptide copolymers were obtained from the high molecular weight proteins cBSA (70 kDa), HSA (65 kDa), and the smaller protein LY (14 kDa) via denaturation under reducing conditions and stabilization via cysteine modification with maleimide-functionalized PEO(5000). The proteins have been selected due to their differences in sequence length, size, and charge. The PEO-residues serve the purpose to minimize intermolecular interactions between the polypeptide backbones and to stabilize the denatured polypeptides; thus, intermolecular interactions emerging from the chemical properties of the polypeptide sequence are reduced. To stimulate a change in the secondary structure and thus aggregation of the polypeptide copolymers, we increased the hydrophobic character of the peptide backbone by conjugating the amine reactive hydrophobic thioc acid ester with the hydrophilic primary amines of the PEO-modified polypeptide backbone (Figure 1).³⁰ We have chosen thioc acid over other hydrophobic molecules as it additionally introduces thiol moieties to the backbone, which open up the possibility for further surface modification with e.g. metal surfaces or maleimide-functionalized compounds.^{28,30} The successful modification was verified with the DPS test indicating the reaction of sulphydryl groups, SDS-PAGE and MALDI-ToF (Supporting Information). MALDI-ToF analysis has been accomplished for the albumin-derived copolymers as well as the lysozyme and TA-modified lysozyme copolymers.^{28,33} No MALDI-ToF analysis has been achieved for both

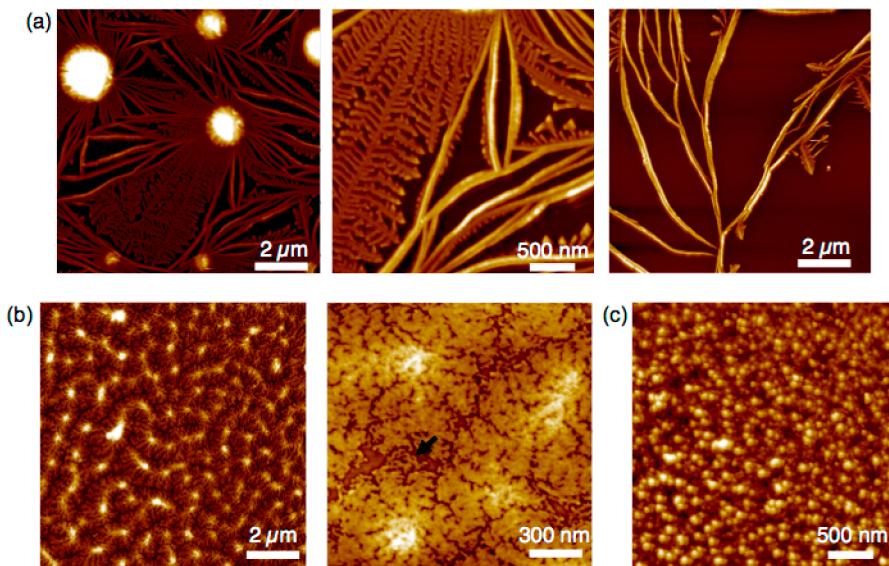


Figure 2. AFM images of surface coatings obtained via drop casting on mica. (a) Negatively charged dLY-PEO(5000)₈. The third panel shows an area with mainly primary branches (“trunks”). (b) Cationic charged dcBSA-PEO(5000)₂₇. In the vicinity of the branch tips, linear features are visible (black arrow) which indicate that the polypeptides presumably adopt an elongated chain conformation (see also Supporting Information). (c) Neutrally charged dHSA-PEO(5000)₂₇ deposits as spherical objects, which coincides with a compact polypeptide conformation.

TA-modified albumin copolymers as reported previously.^{28,38} All the protein derived polypeptide copolymers are prepared with highest degree of PEO and TA modification. This helps to ensure a reproducible distribution of the modifications, which was predicted by analyzing the sequence of the proteins (Supporting Information). For instance, the PEO modifications are equally distributed along the protein backbone, which ensures a sufficient stabilization in aqueous solutions. The copolymers and their modified derivatives are denominated according to the number of PEO and TA residues, namely, dHSA-PEO(5000)₂₇ and dHSA-PEO(5000)₂₇-TA₂₂, dcBSA-PEO(5000)₂₇ and dcBSA-PEO(5000)₂₇-TA₂₆, and dLY-PEO(5000)₈ and dLY-PEO(5000)₈-TA₈ (Figure 1).

The investigated copolymers differ significantly in their physicochemical properties and structural features. They exhibit differences in charge, molecular weight and the nature and distribution of secondary structure elements (Figure 1). The PEO side chains render the polypeptides water-soluble and determine the intermolecular interactions, whereas the charges and functional groups in the polypeptide backbone are responsible for the intramolecular interactions.

The hydrodynamic radii R_H for the albumin derived copolymers dHSA-PEO(5000)₂₇ and dcBSA-PEO(5000)₂₇ are similar, which is in accordance with their comparable molecular composition. Interestingly, the significantly smaller dLY-PEO(5000)₈ copolymer shows a rather large R_H , despite a similar relative PEO modification ratio (number of PEO molecules per amino acid) and charge compared to the albumin derivatives. This aggregate formation in solution indicates the presence of weak intermolecular interactions despite the PEO modification. The TA modification affects R_H and zeta potential of all polypeptide copolymers (Figure 1). The zeta potential of the HSA-derived copolymers decreases, whereas their R_H remains constant. For the BSA-derived copolymers, the zeta potential decreases from about 22 mV to -5 mV, whereas the R_H decreases from 15 to 4 nm. For the LY derived copolymers, the zeta potential decreases from -8 to -14 mV, which is accompanied with a significant decrease in R_H .

Circular dichroism (CD) spectra of the polypeptides revealed that all polypeptide copolymers exhibit different secondary structure elements, which we classified into soft and hard secondary structure elements (Figure 1 and Supporting Information). Random coil elements can be considered as soft, whereas α -helix, β -sheet, and β -turn structures result in a higher structural rigidity and a more compact folded polypeptide chain.^{14,39–43} The ratio of soft to hard secondary structures is further denoted as random coil ratio $R_{S/H}$. The secondary structure elements in dLY-PEO(5000)₈ differ significantly from the albumin derived polymers. In the LY derivative, the content of hard α -helices, β -sheets, and β -turns is lower compared to the case of the albumin derived polymers, whereas the amount of random coil elements is higher. As a result, the LY polypeptide chain is more flexible, which most likely allows for intermolecular interactions and thus aggregate formation in solution, which is in accordance with the observed R_H . After modification with the hydrophobic thioctic acid groups, the relative content of α -helical structure elements in the HSA and cBSA derivatives increases by 25%, whereas the β -sheet content decreases by 15%. The random coil content decreases by 14% for the HSA derivatives and 40% for the BSA derivatives. Thus, the amount of hard and compact structural elements increases for both albumin derivatives after modification with the hydrophilic thioctic acid. Similarly, the modification of the LY-derived copolymer with TA residues leads to a higher content of hard structural elements. The amount of α -helical subunits increases by 2.5-fold, whereas the β -sheet content remains unchanged. The β -turn and random coil contents are reduced by 30% and 60%, respectively.

Although this pronounced secondary structure transformation of the polypeptide copolymers is barely understood, it is reasonable to assume that the hydrophobic TA motifs contribute to the formation of lipophilic patches along the polypeptide backbone. As a result, the more compact α -helical conformation is favored over the random coil conformation to minimize exposure of hydrophobic patches to the aqueous medium.⁴⁴ Another driving force is the decrease in backbone

charge upon reaction of TA with charged amino acid residues. The number of primary amines and thus cationic charges decreases, allowing the polypeptide chain to adopt a more compact conformation, which should favor more compact α -helix and β -sheet conformation over the less dense packed random coil conformation.

However, the less pronounced aggregation of the TA-modified polypeptide copolymers cannot be explained with the common model for electrostatic stabilization of colloidal solutions, which predicts aggregation and thus an increase in R_H with decreasing zeta potential. For the polypeptide copolymers, the decrease in R_H is accompanied with an increase in compact secondary structure elements. In particular, the partially unfolded derivatives with high random coil content (high soft to hard ratio) show a higher tendency to aggregate (larger R_H) than the more folded derivatives with lower random coil content.

Polypeptide Copolymers Form Fractal-like Structures on Mica. To further investigate the observed aggregation behavior in solution, we deposited the copolymer peptides onto mica from aqueous solution under DLA conditions and performed fractal analysis on the formed structures. Several concentrations between 0.03 and 3 mg/mL were investigated. At a concentration of 0.3 mg/mL all polypeptides reproducibly form fractal structures on mica. Higher concentrations lead to multilayer formation, whereas at lower concentrations deposition of individual particles was observed (Supporting Information). Concentration fluctuations also have a significant effect on fractal morphology.^{15,21} During preparation, the evaporation of the solvent entails a concentration gradient, resulting in different conditions at the beginning and the end of the deposition. Thus, the formed structures at the outer area of the wetted surface are formed under different conditions than the structures formed in the inner part.¹⁵ To rule out any artifacts due to this phenomenon and to ensure comparable preparation conditions, we analyzed and compared the structures found in the center of the mica substrate.

Interestingly, we have found that dcBSA-PEO(5000)₂₇ and dLY-PEO(5000)₈ form dendritic structures, whereas dHSA-PEO(5000)₂₇ deposits as spherical aggregates (Figure 2). The negatively charged derivative dLY-PEO(5000)₈ forms fractal structures with a fractal dendrite morphology exhibiting two different types of structural features, main branches with a height of 12 nm and side branches with a height of 6 nm (Supporting Information). This indicates a hierarchical growth mechanism, which is supported by the AFM image recorded at the outer parts of the substrate, showing mainly the branches and only a few secondary nucleation points for side branches (Figure 2).

The fractal structures of dcBSA-PEO(5000)₂₇ can be described as compact dendrites, characterized by the dense branches. The nucleation density is much higher as for the LY polypeptide, which is due to electrostatic interactions between the cationic polypeptides and the negative mica surface. The structures have a height ranging from 4 to 7 nm (Supporting Information). The deviance from the R_H of 16 nm in solution is due to collapse of the polypeptide as a result of dehydration. High magnification images of the dendritic branches indicate the presence of small rod-like objects in the vicinity of the branch tips (Supporting Information). From lower concentrations, the BSA polypeptide deposits as worm-like objects, indicating an elongated chain conformation due to electrostatic

repulsion in the backbone and the high random coil content (Supporting Information).

The dHSA-PEO(5000)₂₇ does not form fractal structures when deposited on mica. Instead, spherical objects with heights ranging from 5 to 15 nm were found (Supporting Information), corresponding well to the R_H of 14 nm. This finding is in agreement with the low random coil content, which should favor a compact polypeptide conformation.

After TA-modification, the HSA derivative dHSA-PEO(5000)₂₇-TA₂₂ does not show significant changes in R_H , ratio of hard-to-soft secondary structure elements and surfaces structures (Supporting Information). The modified as well as the unmodified copolymers deposit as spherical particles. For the BSA and LY derivatives, however, a significant difference in fractal morphology can be observed (Figure 3).

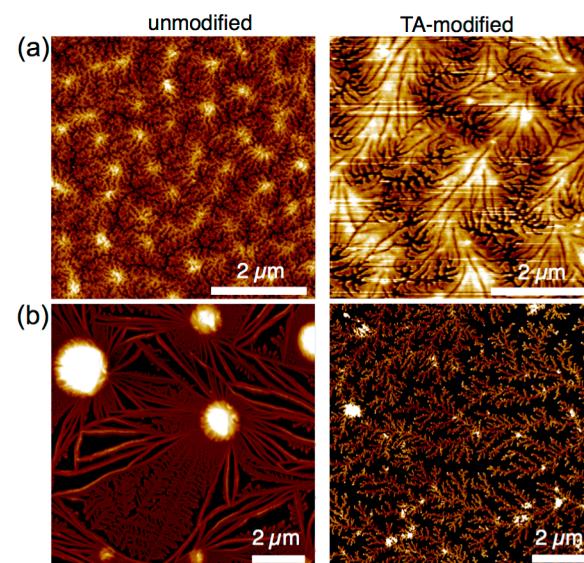


Figure 3. AFM images of the surface layers formed by the TA-modified PEO-Copolymers. (a) dcBSA-PEO(5000)₂₇-TA₂₆ and (b) dLY-PEO(5000)₈-TA₈.

The copolymer dLY-PEO(5000)₈-TA₈ self-assembles into patterns with a fractal seaweed morphology, characterized by the high degree of branching. The highly branched structures show a uniform height of ~6 nm, which corresponds to the height of the side branches of the fractal structures obtained with the unmodified copolymer. The polypeptide copolymer dcBSA-PEO(5000)₂₇-TA₂₆ forms aggregates with “double finger” features, so-called doublons, which are characteristic for a compact seaweed morphology.²¹ The surface structures exhibit heights ranging from 6 to 10 nm (Supporting Information).

To gain insight into the growth mechanisms and the intermolecular interactions, we performed a fractal analysis of the surface nanostructures (Figure 4). Fractal structures can be described with the fractal dimension D_F , which gives insight into the formation mechanism and the intermolecular interactions.⁴⁵ For instance, the rapid particle collisions (strong interactions) in a binary polymer/DNA system result in a low D_F (~1.5), whereas structures obtained from slow particle collisions (weak interactions) show a higher D_F (~1.7).²² Noteworthy, the D_F is not a unique value to describe the complexity of different fractal structures. It is very common that structures, which appear visually different, show a similar D_F .⁴⁶

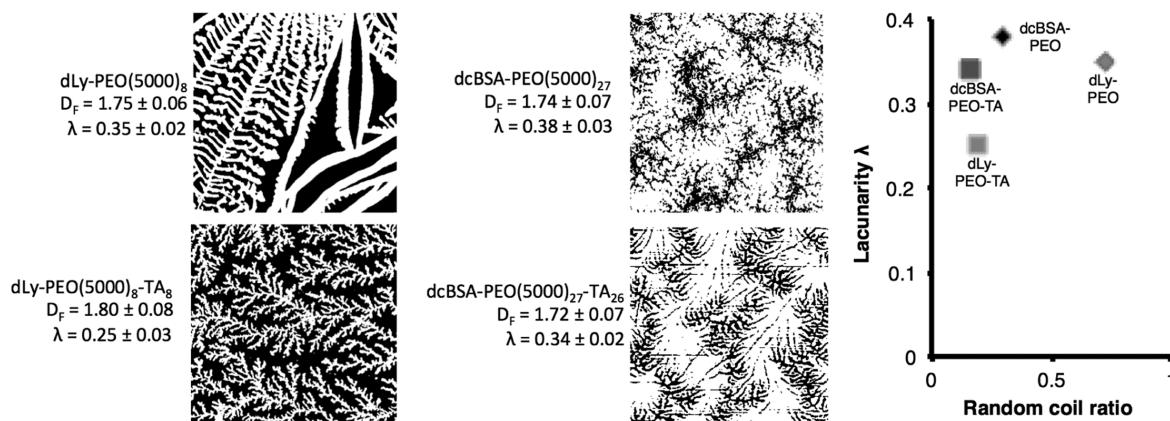


Figure 4. Binary images of the surface topographies prepared for the analysis of the fractal structures and the corresponding results of the box counting analysis for fractal dimension D_F and lacunarity λ . Plotting λ over the ratio of soft to hard secondary structure elements (random coil ratio) reveals that the polypeptides with less random coil content form less heterogenic fractal structures than their analogues with higher random coil content.

Table 1. Summary and Comparison of the Macromolecular Characteristics and Aggregation in Solution and on the Mica Surface of the BSA and LY Derived Polypeptide Copolymers^a

	MW [kDa]	zeta potential [mV]	R_H [nm]	ratio soft/hard	D_F	lacunarity	morphology
dcBSA-PEO(5000) ₂₇	210	21.6 ± 5	15.4 ± 3.9	0.30	1.74 ± 0.07	0.38 ± 0.03	compact dendrites
dcBSA-PEO(5000) ₂₇ -TA ₂₆	215	-4.6 ± 0.7	4.2 ± 0.6	0.16	1.72 ± 0.07	0.34 ± 0.02	compact seaweed
dLY-PEO(5000) ₈	54	-8.4 ± 2.8	114.7 ± 11.5	0.72	1.75 ± 0.06	0.35 ± 0.02	fractal dendrites
dLY-PEO(5000) ₈ -TA ₈	56.4	-13.6 ± 1.9	25.6 ± 3.8	0.19	1.80 ± 0.08	0.25 ± 0.03	fractal seaweed

^aHSA has been omitted, as it does not form structured aggregates on mica.

The lacunarity λ , however, is a measure that allows distinguishing objects that are similar in D_F but reveal a difference in texture. Lacunarity analysis is an established method to analyze fractal structures abundant in biological systems, for example, the morphology of neurons.⁴⁷ In brief, lacunarity λ describes the nonuniformity (heterogeneity) of a structure or the degree of structural variance within an object. Descriptively, a structure or fractal of high λ exhibits wide gaps, holes, or voids, whereas objects of low or vanishing lacunarity are more homogeneous and depleted in such features.⁴⁶

The obtained results from the image analysis, fractal dimension D_F and lacunarity λ , allow for a qualitative interpretation of the self-assembly (Figure 4). The unmodified copolymer dLY-PEO(5000)₈ and the TA-modified dLY-PEO(5000)₈-TA₈ show relatively high fractal dimensions D_F of 1.75 ± 0.06 and 1.80 ± 0.08 , indicating that, in both cases, weak intermolecular interactions govern the self-assembly. The lacunarity λ of the fractal structures decreases from 0.35 ± 0.02 to 0.25 ± 0.03 for the TA-modified copolymer, which is in accordance to the visual appearance and heterogeneity of the formed surface structures. The structures formed with dLY-PEO(5000)₈ display high porosity and degree of structural variance, whereas the fractal seaweed structure formed with dLY-PEO(5000)₈-TA₈ exhibit a higher homogeneity and less structural variance due to the higher branching. The surface structures of the higher molecular weight polymer dcBSA-PEO(5000)₂₇ and its TA-modified derivative dcBSA-PEO(5000)₂₇-TA₂₆ display a relatively high D_F of 1.74 ± 0.07 and 1.72 ± 0.07 , indicating similar to the dLY derivatives weak intermolecular interactions. As observed for the dLY-derivatives, the structures of the unmodified and the TA-modified dcBSA derivatives show a decrease in λ of 0.38 ± 0.03 to 0.34 ± 0.02 , respectively.

The differences in the fractal structures are quite surprising considering that DLA is typically described as a universal process independent of size and chemical nature of the colloidal particles and mainly governed by the strength of intermolecular interactions.^{48,49} With similar D_F values as found for the polypeptide copolymers, one would expect similar surface structures, which is apparently not the case. Concentration fluctuations or “noise” can have a significant impact on the morphology of the DLA nanostructures.^{15,49} Strong noise as expected for systems with a strong tendency to form aggregates (large R_H) in solution should result in highly branched structures with low λ , whereas low noise promotes highly directional growth and the formation of fractals of rather low branching. Thus, macromolecules with a larger R_H should form surface structures with lower λ . However, the contrary was observed in the present case (Table 1).

Previously, it has been found that the particle shape has a considerable effect on the formed DLA morphologies and the fractal dimension D_F due to anisotropic intermolecular interactions.^{17–19,21} In particular, high aspect ratio rods form less dense morphologies than more compact particles, which has been explained by the particle shape controlling the distribution of intermolecular interactions.²⁰ Brener et al.²¹ calculated a kinetic phase diagram that relates the occurrence of different phases, seaweed and dendrite, to the anisotropy of interactions. They have shown that, for a given concentration, a decrease in anisotropy of interactions results in a transition from dendrite to seaweed morphology. Thus, isotropic interactions typically found for spherical particles result in a seaweed morphology, whereas anisotropic interactions as found in rodlike or elongated particles result in a dendrite morphology. This coincides with our finding that a decrease in random coil content $R_{S/H}$ results in a transition from

dendrite to seaweed. The conformation of the polypeptide is highly dependent on the content of secondary structures, as polypeptide chains with random coil conformation are less densely packed than those with helix or sheet conformations.⁴³ An increase in hard secondary structure elements due to less charges and higher hydrophobicity of the polypeptide backbone leads to the formation of a more compact polypeptide folding, which in turn results in more isotropic interactions and thus the formation of seaweed patterns. Upon TA modification, the zeta potential as well as the random coil content for all polypeptide copolymers is reduced, resulting in more compact folding and thus less intermolecular interactions. In the case of the cBSA and LY derivatives, smaller aggregates are formed in solution and a decrease in lacunarity of the surface structures is found.

Figure 5 summarizes our findings by correlating the physicochemical properties and the macromolecular conformation with the morphology of the formed surface structures.

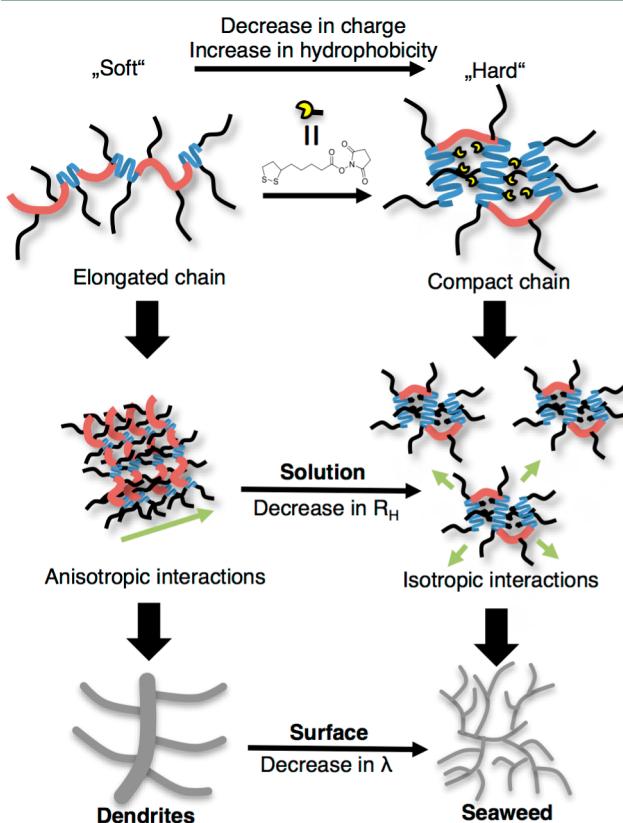


Figure 5. Schematic model correlating the changes in secondary structure to the observed self-assembly behavior. Increasing backbone hydrophobicity by conjugating TA to the copolymer peptide backbone is accompanied by an increase in hard secondary structure elements and a more compact polypeptide conformation. Depending on the compactness of the polypeptide folding, anisotropic and isotropic intermolecular interactions visualized by the green arrow direct aggregation in solution and on the surface.

The individual polypeptide conformation and weak non-covalent interactions between the PEO side chains can be considered the dominating parameters directing the DLA morphology of the polypeptide copolymers. High charges distributed along the polypeptide backbone direct the polypeptide chains into a less compact chain conformation. Thus, hydrophobic patches are exposed along the extended backbone and result in anisotropic intermolecular interactions

(green arrow in Figure 5), as manifested in the pronounced aggregation of dLY-PEO(5000)₈ in solution. The intermolecular interactions favor a side-by-side assembly, which in turn promotes a more directional growth and constrains the formation of branches on the surface. As a result, the formation of a dendrite morphology as found for dLY-PEO(5000)₈ and dcBSA-PEO(5000)₂₇ can be observed. The substitution of polar amines with lipophilic thioctic acid residues generates additional hydrophobicity along the polypeptide backbone. Accompanied by a decrease in charge density, this results in a more compact packing of the polypeptide chain, which promotes the formation of densely packed secondary structure elements such as α -helices and β -sheets. As a result, the polypeptide adopts a more compact globular conformation, which results in isotropic intermolecular interactions. Finally, this isotropy results in the formation of the seaweed morphology as found for dcBSA-PEO(5000)₂₇-TA₂₆ and dLY-PEO(5000)₈-TA₈.

Despite their similarity, the HSA derivatives behave significantly different from the BSA derivatives. The HSA-derived copolymers exhibit no apparent aggregation in solution and deposit as globular particles on the surface with no fractal self-assembly. This can be explained by the higher conformational flexibility and adaptability of the HSA polypeptide backbone compared to the polycationic cBSA.⁵⁰ Thus, for the more flexible HSA derivatives, folding into spherical conformations occurs as indicated by the low content of random coil ratio and the spherical particles observed with AFM. Consequently, hydrophobic patches are less exposed to the solution, impeding attractive intermolecular interactions and thus preventing aggregation in solution and on surfaces.

Fractal Structures Can Be Used for Hierarchical Nanopatterning. In nanotechnology, the bottom-up approach is considered a promising method to generate functional devices with hierarchical complexity. In this strategy, the self-assembly of molecules is exploited to generate complex architectures. In order to demonstrate that the polypeptide copolymers could serve as potential building blocks for this approach and to verify their stability toward postassembly modification, we investigated whether they are capable to template the hierarchical formation of functional surface supported architectures.

Tetramethylrhodamine-labeled HSA (HSA-TMRh) was added to the already formed fractals of dLY-PEO(5000)₈-TA₈ on mica. In native HSA, a single cysteine residue is solvent-accessible and therefore can be selectively modified.³⁷ Following this approach, HSA was site-selectively mono-functionalized using the cysteine-reactive tetramethylrhodamine-maleimide (TMRh-MI) reagent yielding HSA-TMRh with one TMRh attached to HSA.

After adding HSA-TMRh to a mica substrate coated with dLY-PEO(5000)₈-TA₈, the fractal superstructure of the polypeptide becomes visible in the fluorescence channel (Figure 6). The superstructures could hardly be visualized with the microscope in the brightfield mode or in the fluorescence mode in the absence of the dye-labeled protein, presumably due to the high optical transparency of the nanometer-thin polypeptide copolymer coating. Fluorescence microscopy visualizes only the stained main branches according to the apparent fractal morphology (Figure 6). The fractal arms remain unstained and become visible as a negative image due to fluorescence of HSA-Rho absorbed on the substrate areas that are not covered with the dLY-PEO(5000)₈-TA₈ fractals. This

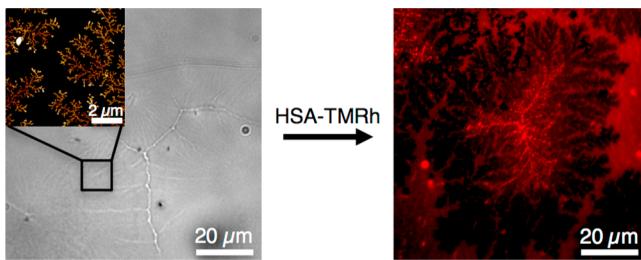


Figure 6. Brightfield image (left) of dLY-PEO(5000)₈-TA₈ and fluorescence image (right) of the same surface after staining with a HSA-TMRh conjugate, demonstrating the stability of the superstructures toward further noncovalent modification. The inset shows an AFM image of the surface.

nonspecific absorption on uncoated mica is not surprising because PEO chains are functional entities that are typically implemented on surfaces to inhibit protein absorption.⁵¹ Thus, the fractal structures form a “negative” imprint in the fluorescent HSA-Rho layer. In addition, this postassembly modification of the nanostructures demonstrates their high stability toward rewetting. We have shown that native albumin as well as albumin copolymer polypeptides can be modified with a large variety of functional groups, for example, folic acid groups³² or signaling RGD peptides⁵² that could be particularly attractive to achieve biocoatings for cell adhesion. Thus, the presented copolymer polypeptides are promising building blocks for the hierarchical fabrication of complex and orthofunctional surface structures with structural features spanning from the nano- to the mesoscale.

CONCLUSIONS

Six partially unfolded and high molecular weight polypeptide copolymers with different molecular weights, zeta potentials, and macromolecular conformations were investigated with respect to their nanostructure formation on mica. The polypeptides form aggregates in solution and fractal structures via diffusion-limited aggregation on the mica surface. The morphologies of the superstructures were investigated by means of fractal analysis and correlated with the physicochemical properties of the polypeptide backbones. Modification of the polypeptide backbone with thioctic acid increased the hydrophobic profile and resulted in a more compact macromolecular conformation, which had significant impact on the formed morphologies of the fractal structures. Consistently, the copolymers with a higher content of compact secondary structure elements and thus a denser polypeptide conformation form more homogeneous fractal structures than their analogues with higher random coil content. To our knowledge, this is the first report on the role of conformation compactness and thus isotropy of interactions on the aggregation of high molecular weight copolymers. In addition, we demonstrated that the fractal structures could be used as templates for the bottom-up construction of functional surface coatings using a Rhodamine-labeled HSA, which coassembles with the fractal patterns. This opens up various possibilities to explore these mesoscale patterns for patterning surfaces with biofunctional and hierarchically ordered coatings. The results reported herein reveal new insights into the aggregation of high molecular weight polypeptides and the assembly of partially unfolded proteins, which in nature often occur in their acylated, glycosylated, or phosphorylated form.

ASSOCIATED CONTENT

Supporting Information

DPS assay, SDS-PAGE, MALDI-ToF, and additional AFM images. This material is available free of charge via the Internet at <http://pubs.acs.org.org>.

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Notes

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