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Tunable length of cyclic peptide-polymer conjugate self-assemblies in water

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ABSTRACT: Polymers conjugated to cyclic peptides capable of forming strong hydrogen bonds can self-assemble into supramolecular bottlebrushes even in aqueous solutions. However controlling the aggregation of these supramolecular assemblies remains an obstacle that is yet to be overcome. By introducing pH-responsive poly(dimethylamino ethylmethacrylate) (pDMAEMA) arms, the repulsive forces were tuned by adjusting the degree of protonation on the polymer arms. Neutron scattering experiments demonstrated that conjugates in an uncharged state will self-assemble into supramolecular bottlebrushes. Reducing the pH in the system led to a decrease in the number of aggregation, which was reversible by addition of base. Potentiometric titration showed a correlation between the number of aggregation and the degree of ionization of the pDMAEMA arms. Hence, a balance between the strength of the hydrogen bonds and the repulsive electrostatic interactions determine the number of aggregation and extent of self-assembly. The presented work demonstrates that conjugate self-association can be controlled by tuning the charge density on the conjugated polymer arms, paving the way for the use of responsive cyclic peptide conjugates in pharmaceutical applications.

Stimuli responsive materials that react to changes in their environment such as temperature or pH have attracted considerable attention in material science. These responsive materials have found applications in sensors, surface modifications, actuators, or scaffolds, but most interestingly they play a key role in efficient pharmaceutical delivery systems. Upon a change of environment such as pH, temperature, or light they undergo a modification in their structure or disassemble into smaller parts. Prominent examples of pH sensitive materials are peptides with chargeable groups or polymers such as poly(acrylic acid) (PAA), poly(ethylene imine) (PEI) or poly(dimethylamino ethylmethacrylate) (pDMAEMA), which can be reversibly protonated or deprotonated with changes of pH. The resulting variation in charge density can be utilized to control solubility in aqueous media and/or electrostatic repulsion, which can affect the integrity of self-assembled structures such as polymer micelles or vesicles, thereby allowing effective loading and release of drugs encapsulated within these nanostructures.

A relatively new class of self-assembled nanostructures are supramolecular polymer brushes, which are formed either by strong hydrogen bonds or π-π interactions. These materials combine flexible polymer chains with a supramolecular backbone to create rigid anisotropic hairy nanoparticles, often having a rod-like shape. In particular, polymers conjugated to self-assembling cyclic peptides have attracted considerable attention for designing functional supramolecular nanotubes. These peptides are made of alternating L- and D-amino acids that result in a planar configuration with hydrogen bonding sites oriented perpendicularly from the peptidic backbone plane. The side chains of residues on the peptide allow for selective modification with functional groups or polymers. Under the appropriate conditions these materials form strong nanotubes via hydrogen bond self-assembly, even when modified with large polymer chains. Exploring their potential, we recently demonstrated their use as drug delivery agents or membrane channels.

Nevertheless, controlling the size of this self-assembly still remains a challenge. Here we investigate the influence of charge density on this self-assembly process to create a responsive material. Thus, the aforementioned pH responsive polymer pDMAEMA, was conjugated to cyclic peptides and the self-assembly of these materials was thoroughly studied in aqueous solution. Small angle neutron scattering (SANS) of solutions at different pH provided comprehensive information on the effect of protonation on the shape and size of the self-assembly. Alternative approaches such as size exclusion methods or light scattering techniques were not considered, as they require additives such as salts to provide reliable results, which might interfere with the assembly. In addition to SANS, the degree of ionization β of the conjugate was determined for the pH range of interest, pH 4-10, and compared to the polymer without CP.

The conjugation of pDMAEMA to peptides or other materials can be quite demanding due to the strong basicity of the tertiary amine groups on the polymer. Only recently were we able to establish a synthetic protocol to create well defined cyclic peptide (CP)-polymer conjugates with various polymers including pDMAEMA. Polymer chains of narrow distribution were grown from a CP modified with two diametrically opposed pendant chain transfer agents via the reversible addition–fragmentation chain transfer (RAFT) process (Scheme 1).
The CP consists of alternating D- and L-amino acids (sequence: L-Lys-D-Leu-L-Trp-D-Leu-L-Lys-D-Leu-L-Trp-D-Leu) that ensure the required planar configuration for hydrogen bond formation. The chain transfer agents for RAFT polymerization were attached to the free amine groups of the lysine residues. After polymerization, a theoretical average molecular weight of 9800 g/mol, corresponding to a degree of polymerization of 26 for each polymer arm was calculated from the conversion (93%, determined by NMR) and the ratio of monomer to CTA moiety ([M]/[CTA] = 28). A narrow distribution of 1.19 and a $M_{n, SEC}$ of 13600 g/mol of the CP conjugate was observed by SEC analysis (for details, see supporting information). The difference between theoretical molecular weight and that determined by SEC for CP-(pDMAEMA$_{26}$)$_2$ is related to the unknown influence of the CP on the hydrodynamic volume or potential interactions with the column material in SEC.

The modification of the CP with polymer arms of pDMAEMA significantly improves their solubility in water. The tertiary amine groups on the polymer behave as a base, and when directly solubilized in D$_2$O, a pH of 9 was observed. The self-assembly of the conjugates into nanotubes was analyzed by SANS, in order to obtain information on both the morphology, from the $q$ dependency of the scattered intensity, and the size of the aggregates, which is related to the intensity of the plateau at low $q$ values. The intensity at low $q$ values (Figure 1) of the conjugates dissolved in pure D$_2$O revealed that self-assemblies are formed, since the observed values are higher than expected for single conjugates at this concentration (3.5 cm$^{-1}$ instead of 0.15 cm$^{-1}$ for unimers at this concentration). However, in contrast to previously analyzed CP conjugates, we do not observe an extended $q^2$ dependency, which would indicate the formation of long nanotubes. To obtain more detailed information on the structure, a fit of the data was attempted with the form factor for a short cylinder (see Figure S1 in Supporting information). Indeed, at low $q$ this form factor fit the data well, proving that the conjugates still form short nanotubes. However, at high $q$ values, a $q^2$ dependence of the scattered intensity is observed which is characteristic of Gaussian chains. The combination of the observed rod-like character at low $q$ values and flexible chain character at high $q$ values suggests the presence of hairy rod-like objects. The fit obtained using a form factor for a hairy rod-like micelle (see Supporting Information) is represented in Figure 1. Details on the fitting parameters are provided in the Supporting Information. The estimated length ($L$) of the self-assemblies at the initial pH 9 is 68 Å. The distance between two cyclic peptides (LC) has been previously determined to be 4.7 Å. It is thus possible to calculate a number of aggregation $N_{agg} = L/LC$ of 14.6.

In order to assess the effect of the charge density of the polymer arms on this aggregation, the solution was acidified by addition of small amounts of DCI. Decreasing the pH of the solution is expected to result in deuteration (addition of D$_-$, in contrast to protonation with H$^+$) of the amine groups of the pDMAEMA arms, and thus, to introduce a positive net charge on the polymer arms, which should cause an electrostatic repulsion between individual conjugates. Reducing the pH to 8, the scattering intensity at low $q$ dropped considerably, confirming the anticipated reduction in size of the aggregates. Moreover, a peak appears at around 0.02 Å$^{-1}$, which is typical of a structure factor due to polyelectrolyte interactions. As the amine groups are protonated and become positively charged, these interactions are expected. To take this effect into account for our fit, an empirical approach was used to fit the structure factor (see Supporting Information). The fit gave a length of the self-assembly of 37 Å corresponding to $N_{agg} = 7.8$, which is almost half the initial size. At pH 7 the length is further reduced to 22 Å, corresponding to $N_{agg} = 5$. Such a small structure can only partially be considered a rigid-rod and is at the limit of use of this model. The calculated fit does not fit as well as for the two previous measurements. In acidic conditions (pH 2) the scattering intensity in the medium $q$ range (0.01 – 0.1 Å$^{-1}$) is in the range of the aforementioned single conjugate. A Gaussian chain form factor, which is characteristic of polymer chains in solution, fits the data well at this intermediate and high $q$ range. However, at low $q$ an upturn in scattering intensity is observed, which is unrelated to a Gaussian chain. This increase has been fitted with a power law of 2 (details in Supporting Information) and is similar to previously reported measurements on polyelectrolytes. While the origin of this upturn is still the subject of discussion, we hypothesise that our conjugates become highly charged at pH 2. Nevertheless, we conclude that the gradual increase of the repulsive electrostatic interactions, due to the protonation of the amine groups of pDMAEMA polymer chains, is competing with inter-conjugate hydrogen bonding and progressively disrupting the self-assemblies. After this stepwise reduction to pH 2, the pH was increased to 9.7 by addition of NaOD to determine whether the self-association of the cyclic-peptide core is a reversible mechanism. The observed scattering intensity returned to a value similar to the initial scattering intensity at pH 9, which gives a comparable length of the self-assembly of 59 Å ($N_{agg} = 13$), demonstrating that the self-assembly of this system is reversible.
The degree of ionization ($\beta$) was determined using equation 1 and fitted using equation 2:

$$\beta = \frac{[R-(\text{CH}_3)_2\text{NH}]^+}{[R-(\text{CH}_3)_2\text{NH}]^+ + [R-(\text{CH}_3)_2\text{N}^-]}$$  \hspace{1cm} (1)$$

Where $[R-(\text{CH}_3)_2\text{NH}]^+$ is the concentration of charged DMAEMA units, $[R-(\text{CH}_3)_2\text{N}]$ is the concentration of non-charged DMAEMA units and $[R-(\text{CH}_3)_2\text{N}] = [R-(\text{CH}_3)_2\text{N}]^+ + [R-(\text{CH}_3)_2\text{NH}]^-$ the total concentration of DMAEMA units.

$$\beta = \frac{1}{1+10^{(p\text{H}-pK_a^{\text{app}})}}$$  \hspace{1cm} (2)$$

Assuming a constant $pK_a^{\text{app}}$, we were able to fit the data in Figure 3 giving an average $pK_a^{\text{app}}$ of 7.84 for the conjugate and a slightly higher value of 7.95 for the polymer, which matches well with previously observed values for pDMAEMA. However, at low pH values, the fit deviates from calculated values for $\beta$ in the case of the conjugate. It has been reported that for polybases and polyacids the $pK_a$ can depend on the charge density of the polymer, thus, it is dependent on the degree of ionization and can be calculated as function of $\beta$ using a modification of equation 2:

$$pK_a^{\text{app}} = pH - \log \frac{1-\beta}{\beta}$$  \hspace{1cm} (3)$$

The resulting values as a function of $\beta$ are given in Figure 3.

Figure 1: SANS measurement of CP(pDMAEMA₃₅)₂ in solution in D_2O at 5 g/L at pH < 9, > 8, > 7, > 2 and > 9.7. Fit of the data using a hairy rod-like micelle form factor and  a Gaussian chain form factor.

The SANS experiments clearly demonstrate that the electrostatic repulsion due to a high charge density at low pH can compete with the strong hydrogen bond formation of the cyclic peptide core. To elucidate this relationship in more detail, we determined the degree of ionization ($\beta$) as a function of pH (Figure 2) (the method is given in the SI). For comparison this relationship was also evaluated for a comparable polymer representing the two arms without the CP core (the synthesis is given in the SI).

Figure 2: Degree of ionization $\beta$ as a function of pH of CP(pDMAEMA₃₅)₂ and its corresponding polymer. The values were determined from the titration of an acidified solution with NaOH (see SI for details). The line represents a fit according to eq. 2 assuming a constant $pK_a$ of 7.84 for A, and a constant $pK_a$ of 7.92 for B. Insert: Magnification of the section at high charge density.

Figure 3: $pK_a^{\text{app}}$ as a function of degree of ionization $\beta$ of CP(pDMAEMA₃₅)₂ and its comparable pure polymer. The values were calculated according to equation 3.

The obtained $pK_a$ remains constant for the polymer and in a wide range of $\beta$ (0.1 – 0.8) for the CP conjugate. At high degrees of ionization, however, a significant drop is observed for the conjugate. The increased charge density at this stage usually causes an increase of energy required to add another charge to the polymer arm, and as a consequence the protonated base becomes more acidic, i.e. the $pK_a$ is reduced. However, at a relatively low degree of polymerization ($DP \leq 50$) this effect is limited, as can be seen for the small change for the comparable polymer. In the case of CP(pDMAEMA₃₅)₂ the cyclic peptide core allows self-assembly of the polymer into larger aggregates and thus it behaves like a higher molecular weight polymer. Consequently, the local charge density increases in comparison to loose polymer chains. The steeper decrease of $pK_a$ at very high $\beta$ (> 0.8) can be explained by a relatively large change in molecular weight considering that the number of aggregation decreases from 5 at pH = 7 ($\beta = 0.8$) to 1 at pH = 2 ($\beta = 1$). This is a 5-fold decrease of overall molecular weight, while between pH = 9 and pH = 7 it is only 3-fold. Another explanation for this behavior is found in the overall energy in the system. In the neutral state (pH > 9), aggregation of the conjugates is energetically favorable due to the strong hydrogen bonds formed. With increasing degree of ionization $\beta$, not only must the increased charge density be overcome, but also the extra energy for breaking the hydrogen bonds is required. Consequently, this additional protonation becomes increasingly difficult, i.e. the basicity is diminished or the $pK_a$ decreases, respectively.
In conclusion, by combining a pH responsive polymer pDMAEMA with a cyclic peptide that forms nanotubular structures via hydrogen bonding, we have created a water-soluble supramolecular brush whose self-assembly can be controlled by variation of pH, and is fully reversible. SANS experiments revealed that the conjugate in solution forms cylindrical polymer brushes with a number of aggregation of 15. Due to the basic amine groups on the polymer an initial pH of 9 was observed. Subsequent addition of acid to this solution resulted in a steady decrease in the number of aggregation, to 8 at pH 8, 5 at pH 7, and finally 1 at pH 2. Parallel titration experiments allowed the determination of the degree of ionization $\beta$ as a function of pH, and from these values the pKa at different $\beta$ was calculated. A greater drop in pKa was observed at high $\beta$ in the case of the conjugates, when compared with the corresponding linear polymer. This large variation is based on an increased charge density within the self-assembled structure and related to the additional energy required to break the hydrogen bonds. It seems that a linear inverse proportionality is observed between the number of aggregation and the degree of ionization. Considering that the repulsive Coulomb energy scales quadratically with the number of charges, and a full disassembly of the conjugates is only observed at a very high degree of ionization, the hydrogen bond energy counteracting this repulsive force must be significant.\footnote{A more advanced study is required to elucidate a detailed correlation.} Nevertheless, the presented work indicates that control over the supramolecular structure can be obtained by adjusting the charge density on the conjugated polymer arms. This knowledge will aid in the design of tailor-made, responsive cyclic peptide conjugates for pharmaceutical applications such as gene delivery vectors or antimicrobials.

ASSOCIATED CONTENT

Supporting Information. Experimental section including material, synthesis protocol, NMR, potentiometric titration, SANS data and fit.

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Notes
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