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Microstructure manipulation and guest release from cation responsive peptide microspheres

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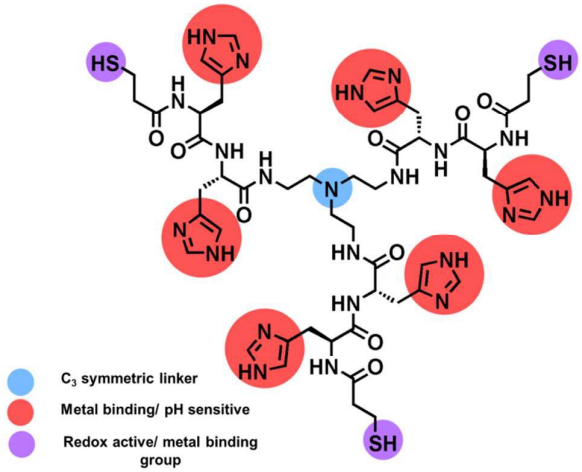
This communication presents a strategic design of a thiolated C_3-symmetric dihistidine conjugate and its self-assembly to yield nanospheres. Electrostatic interactions with ATP, coupled with the possibility of disulfide bond formation by thiolated termini of the conjugate, facilitate self-assembly resulting in the formation of doughnut shaped microspheres. Finally, cations present in biological environment, such as Mg(II) ions, could be used to disaggregate microspheres to release guest molecules.

Biomacromolecules such as amino acids and nucleotides show excellent ability to form self-assembled structures, with possible biomedical applications.[1] Side chain hydrophobic and hydrophilic functional groups in amino acids aid the organization of supramolecular ensembles suitable for assisted delivery of desired cargo.[2] Peptide-based arrangements provide an expeditious entry into design of tailored sequences to suit specific requirements.[3]

Cationic peptides like poly L-lysine, KK (lysine dipeptide) and $[FF]^+$ are reported to support formation of microstructures.[4] Poly L-lysine forms spherical coacervates due to dominant electrostatic interactions with anionic proteins,[4a] whereas, $[FF]^+$ induced formation of J-aggregates upon interaction with anionic porphyrin molecules.[4b,c] Notably, histidine despite having basic heterocyclic side chain, has not been explored much for the formation of supramolecular constructs. Imidazole ring present in histidine, with pKa of 6.5, interconverts between neutral to protonated form. In addition, imidazole ring also supports metal ion coordination,[5] which is crucial for the function of several metalloproteins, transcription factors containing Cys_2-His_2 motif[6a] and in nickel-based affinity purification strategies.[6b] In a recent example, coacervation of polyvinylimidazole cationic homopolymer at acidic pH (4-6.5) and an anionic polysaccharide, sodium alginate was studied.[7a] Oppositely charged poly(His)-poly(Asp) formed charge neutral



Scheme 1. Structure of the tripodal peptide $(Mpa\text{-}H\text{-}H)_3$ Tren (**1**), Mpa= 3-Mercaptopropanoic acid, H= Histidine.

complexes both in solution as well as in layer-by-layer adsorbed films.[7b] As a specific example, metal-histidine coordination has also been employed for the assembly of collagen related peptides into supramolecular structures[7c] and inhibition of amyloidogenic aggregates.[7d]

We have previously reported peptide-based soft spherical structures, which respond to external stimuli such as sunlight, metal ions, ultrasound and pH.[8] These structures involved various design paradigms to maximize non-covalent interactions in order to afford stable morphologies. In the present study, we decided to use dihistidine dipeptide around a tren scaffold to create a skeleton that will respond to pH variations and the positive charge so generated was projected to serve as a trigger for complex microstructure generation via interaction with an appropriate anionic species. Design of tripodal peptide conjugate **1** incorporated histidine and thiol functional groups, where the latter was used to further functionalize soft structures and facilitate formation of inter- and intra-molecular disulfide bonds (Scheme 1).

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Self-assembly of **1** at pH 6 and 1 mM concentration in methanol-water (1:9), revealed the formation of fused spherical nanostructures after 45 min incubation, as observed by FE-SEM (Fig. 1(a)). Their size distribution was also studied by dynamic light scattering (DLS) (Fig. S2) and it was found to be in coherence with the microscopy data. Strong T-shaped contact pairing between histidine residues, irrespective of their charge, could be responsible for this behaviour as confirmed by molecular dynamics simulations.[9] The nanospheres that tend to aggregate over time due to intermolecular disulfide bonds between thiol groups exposed on the surface of nanospheres, as described before for similar systems,[8d] could be reduced by DTT to furnish distinct nanospheres (Fig. 3(a)). They were also found stable upon exposure to heat (65 °C for 15 min) and ultrasound (15 min) (Fig. S1(a, b)).

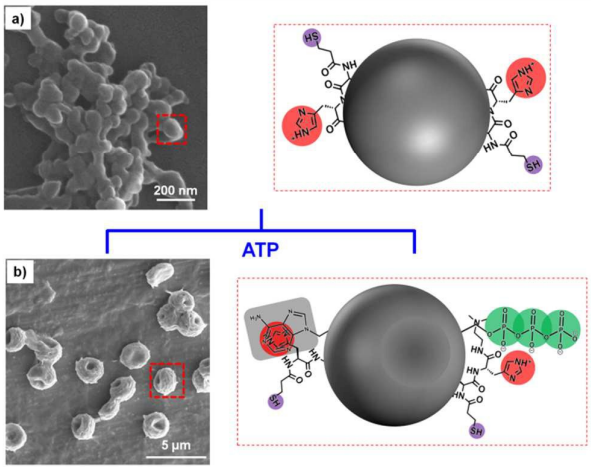

Fig. 1 a) Self-assembly of **1** at pH 6, a schematic representation of positively charged imidazole groups decorating the outer surface of the self-assembled structures (inset); b) Microspheres formed by electrostatic interaction of **1** with ATP, a schematic representation of possible electrostatic and π-π stacking interactions stabilizing these structures.

Electrostatic interactions dominate between protonated histidine residues and ATP during complex formation.[10] We decided to screen positive charges present in **1** at pH~6 by negatively charged ATP phosphate groups with a possibility of augmenting this interaction by stacking of ATP anions within the tripodal scaffold of peptide conjugate **1**. Microscopic analysis of samples prepared from a co-incubated solution of **1** (1 mM in 1:9 methanol- water) and ATP (0.1 mM in water) for 45 min, revealed formation of doughnut-shaped microspheres, as observed by FE-SEM (Fig. 1(b), AFM and TEM (Fig. S3). These microspheres were also characterised by UV-Vis and CD Spectroscopy (Fig. S4), which confirmed favourable interaction of peptide **1** with ATP.

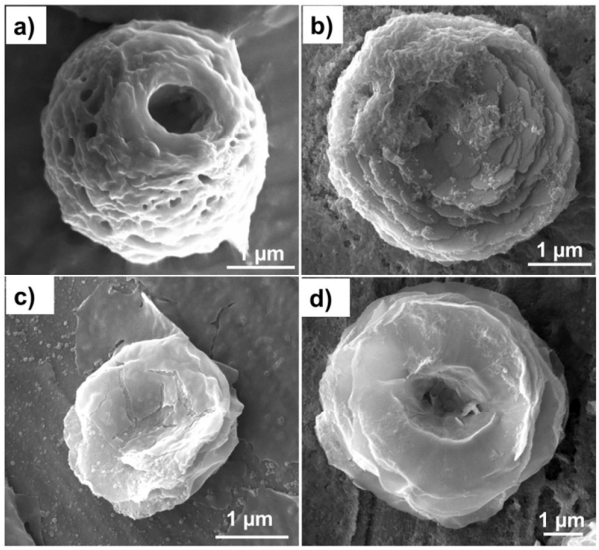

Fig. 2 FESEM Micrographs for **1** upon incubation with different phosphorylated molecules a) ATP; b) ADP; c) AMP; d) Na_3PO_4

The formation of self-assembled structures by **1**-ATP hybrids motivated us to further study the interaction of **1** with other phosphorylated nucleotides, such as ADP and AMP. It was observed that number of negative charges in the molecule had some effect on gross morphology as observed in FE-SEM micrographs (Fig. 2). The peptide conjugate afforded flaky microstructures with inorganic phosphate Na_3PO_4, despite the absence of hydrophobic stacking interactions (Fig. 2(d)). An overall positive surface charge of +7.8 mV, determined by zeta potential measurements (Fig. S5(a)), confirmed that peptide **1** is present in excess.

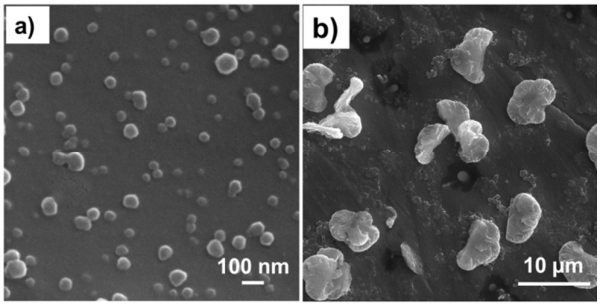

Fig. 3. FE-SEM Micrograph after DTT treatment a) **1** showing discrete nanospheres; b) **1**-ATP showing degradation.

Notably, microspheres showed signs of degradation in the presence of reducing DTT environment suggesting a possible role of disulfide bonds in formation of spherical morphology (Fig. 3(b), S6(b)). It is interesting to note that the microspheres were not resistant to heat and ultrasonication, and quickly degraded upon exposure to either one of them (Fig. S1(c,d)).

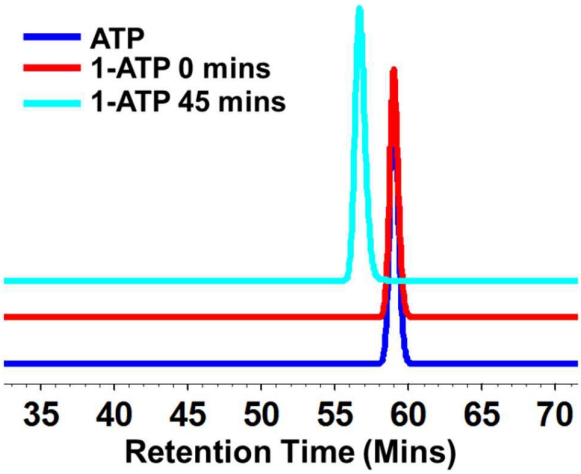

Fig. 4. HPLC profile of **1**-ATP complex over 45 min incubation, showing absence of ATP hydrolysis.

Use of imidazole derivatives for phosphate ester hydrolysis is of considerable interest.[11a-c] More recently, Gale and coworkers reported that histidine derivatives can hydrolyze organophosphate nerve agent model systems.[11d] As these structures involved interaction of histidine containing peptide and ATP, we decided to check the stability of **1**-ATP complex to ensure chemical homogeneity of these structures over a given time-period. HPLC studies indicated lack of by-products of ATP hydrolysis even after 45 mins of co-incubation, thus precluding the possibility of histidine aided degradation of ATP (Fig. 4, S9). However, a slight decrease in the retention time was observed, which could be ascribed to formation of aggregated structures of altered polarity.

It was of interest to further modify **1**-ATP structure, as there was residual net positive surface charge. Thus, we decided to engineer a layer of sodium phosphotungstate (PTA), a polyoxometalate anion of twelve tungsten oxyanions of octahedral geometry that surround a central phosphate group, to complement surface positive charge. Such transition metal oxyanions exhibit an extensive range of structures and diverse properties.[12]

It is known that colloidal peptide nanospheres are formed due to stabilization by electrostatic interactions and modulation of morphology occurs when treated with PTA.[13] We decided to examine whether morphology of peptide **1** nanospheres also responds to PTA treatment, but no significant change was observed (Fig S6 (a)). On the other hand, coating **1**-ATP microspheres with PTA resulted in large, flattened, disc shaped structures as observed through FE-SEM (Fig. 5), AFM and TEM (Fig. S7). An overall negative surface charge of -10.8 mV, through zeta potential measurements (Fig. S5(b)), confirmed the coating with a polyoxometalate. They were stable upon exposure to heat, but disintegrated in presence of ultrasound (Fig. S1 (e, f)). Thermogravimetric studies further reiterated that PTA-coated structures were more heat stable compared to unmodified **1**-ATP structures (Fig. S8). These results also confirm that polyoxometalate coating indeed renders the soft structures more resilient to high temperatures.

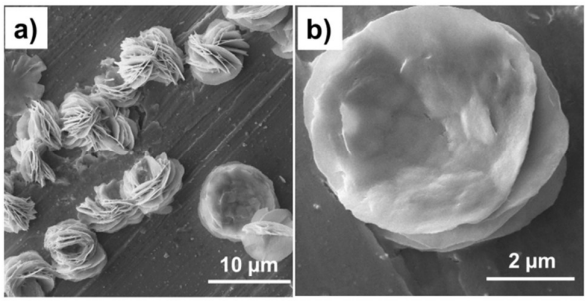

Fig. 5. Microstructural characterization of structures formed by electrostatic interaction of **1**-ATP hybrid with PTA. a) FE-SEM Micrograph; b) zoomed micrograph of a coated structure.

Although this process did successfully exploit electrostatically layered coating of microsphere, it caused structural deformation, making it unsuitable for entrapment applications. Thus, we decided to pursue unmodified **1**-ATP microspheres for further studies and study their core structure. Investigation to study interior of these structures was conducted using focused ion beam (FIB) milling technique, which involves a high energy Ga^{+2} ion beam (30 kV) to machine soft structures without compromising their integrity.[14] A porous interior was revealed upon exposure of microspheres to ion beam milling of 200 nm deep sections (Fig. 6). We believe that these compartments are formed due to a complex interplay between electrostatic and π-stacking interactions as has been discussed earlier for assemblies formed by oppositely charged molecules. [4b] This also suggested that porous confines of these structures could possibly allow entrapment and containment of guest molecules.

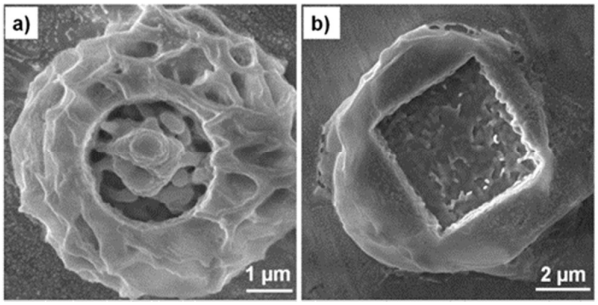

Fig. 6. Focused ion beam miling: a) **1**-ATP complex doughnuts showing their porous interior; b) PTA coated doughnuts with their flaky exterior.

ATP has multiple ionizable groups with varying acid dissociation constants and it exists mostly as ATP^{4-} under neutral conditions.[15a] The interaction of ATP with metal ions to form macrochelates is well documented in literature.[15b-d] Several metal ions like Mg^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Na^{+} etc. are present in the biological milieu as cofactors for enzymes. ATP exists mostly as its Mg^{2+} complex inside cells.[15e] This high affinity Mg^{2+}-ATP interaction was chosen as a test model to assess cationic release trigger due to breakdown of microstructure.

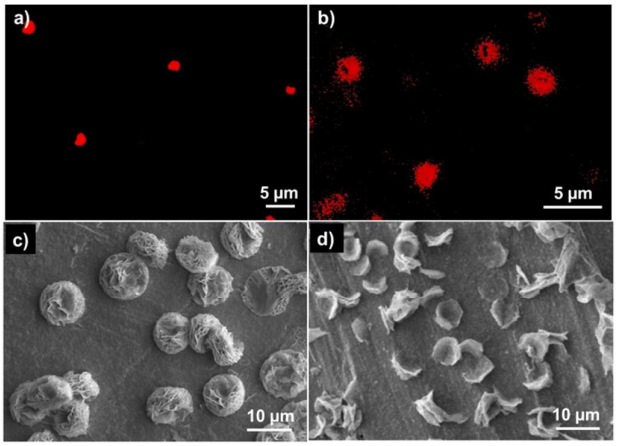

Fig. 7. Fluorescent microscopy of dye stained soft structures a) Dye stained microspheres of 1-ATP hybrids; b) Release of dye from them after incubation with Mg^{+2} ions for 6 h; c) FESEM micrograph of (a); d) FESEM micrograph of (b).

Rhodamine B was encapsulated in microspheres and its release was studied by fluorescence microscopy, following the addition of magnesium salt as an external stimulus (Fig. 7). Peptide **1** and ATP were co-incubated in a solution containing the dye for 45 mins till the self-assembly is formed, after which $MgCl_2$ was added to it and further incubated for 6 h. The doughnut shaped self-assembly disintegrated, resulting in release of the dye from it (Fig. 7 (b)). Similar observations were also made with another divalent (Ca^{2+}) and monovalent (K^{+}) cations (Fig. S10). On the other hand, in the control experiment, nanospheres of **1** were unaffected upon incubation with the dye and Mg^{2+} ions by a similar procedure (Fig. S11). This lends further credence to our premise that cations can be used to induce the delivery of cargo being transported by the microspheres.[8e]

In conclusion, we have elucidated the formation of self-assembled nanostructures by a tripodal peptide containing histidine residues arranged along the three arms of Tren molecule. We showed that the peptide can self-assemble into nanospheres at pH 6. We have also described the formation of porous doughnut shaped microspheres upon electrostatic interaction with ATP that can be used for transport of small molecules, as they disintegrate upon exposure to cations. These microspheres can be proposed as a prototypical delivery vehicle, containing biological building blocks. Further elaboration of microdroplet structure and cation concentration is in progress.

Notes and references

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