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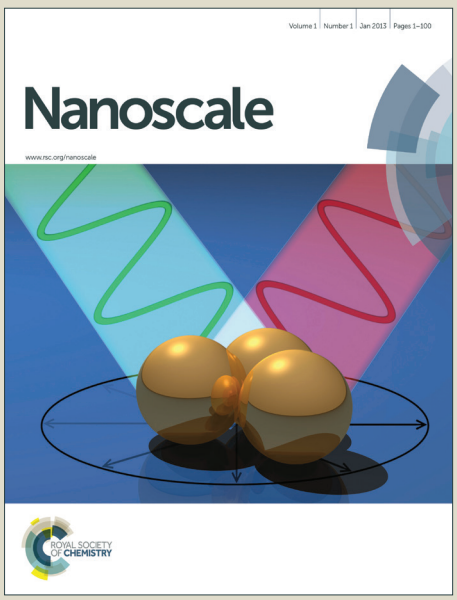

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Zwitterionic supramolecular nanoparticles: Self-assembly and responsive properties

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C. Stoffelen and J. Huskens*

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Supramolecular nanoparticles (SNPs) are of high interest in both nanoscience and molecular diagnostics and therapeutics, because of their reversible and designable properties. To ensure colloidal stabilization and biocompatibility, most reported strategies require the use of hydrophilic long-chain polymers such as poly(ethylene glycol). Here, we show the formation of zwitterionic supramolecular nanoparticles (ZSNPs) from appropriately functionalized mono- and multivalent components, based on the hetero-ternary host–guest complexation between cucurbit[8]uril (CB[8]), methyl viologen (MV), and azobenzene (Azo), while using the monovalent, small-molecule, non-fouling Azo-carboxybetaine analog (Azo-Zwit) as the shell-forming component. Even though steric shell stabilization is absent, the zwitterionic Azo-Zwit ensures stability of the ZSNPs in water, in PBS (pH 7.4) at ionic strengths ranging from 0-700 mM, and in PBS containing BSA. Size tuning by control over the stoichiometry of the components, as well as reversible assembly and diassembly by photoisomerization of the Azo moieties were observed. Surprisingly, the ZSNPs exhibited aggregation at the narrow pH range of 6.2-6.8.

Introduction

Nanoparticles (NPs) are of major interest because of their size-dependent chemical and physical properties. Next to catalytic, optical, sensor or electronic applications, the use of NPs in biomedicine has gained intense scientific interest over the past two decades.[1, 2] In oncology, for example, countless studies have been carried out to the targeted delivery of active compounds.[3-5]

A general problem of the administration of NPs to the human body is the rapid clearance of the NPs from the blood stream by the reticuloendothelial system (RES) or mononuclear phagocytic system (MPS).[6] This process is induced by opsonization, the non-specific binding of proteins to NP epitopes, resulting in NP aggregation and elimination of the NPs by the immune defense system.[6, 7] In general, these processes are strongly dependent on the surface properties of the NPs. To enhance the pharmokinetics and biodistibution of NPs in vivo, a poly(ethylene glycol) (PEG) coating is commonly used.[8, 9] However, PEG degradation by oxidation can result in loss of anti-fouling properties upon prolonged usage or storage.[10, 11]

Recently, a new class of non-fouling materials based on zwitterions has been put forward as an alternative for PEG in the functionalization of biomedically relevant materials.[12, 13] Zwitterions are molecules carrying equimolar amounts of negative and positive charges guaranteeing overall charge neutrality. Due to their ionic character, zwitterion-bearing compounds are ultra-hydrophilic. Various naturally occurring membrane lipids are zwitterionic origin and have shown to be strongly anti-fouling when assembled in a model bilayer.[14] Jiang and coworkers have used zwitterionic compounds for the coating of different types of NPs.[15-17] In these studies it was shown that, for example, a carboxybetaine shell around the NPs prevents nonspecific protein binding in undiluted blood serum.

Soft supramolecular nanoparticles (SNPs) are promising materials because their formation strategy results in stable, yet reversible nanovectors. A variety of biomedically relevant SNPs based on host-guest chemistry has been developed by including active compounds into the SNP formulation. Drug delivery SNPs have been formed by covalent modification of the supramolecular building blocks with chemotherapy drugs such as camptothecin[18] and doxorubicine,[19] whereas gene delivery vehicles have been based on electrostatic interactions of positively charged building blocks and negatively charged nucleic acids.[20] Despite the chemical flexibility of the SNP approaches, almost all SNP formulations require a PEG coating to ensure colloidal stabilization[21, 22] in aqueous media or to inhibit aggregation by interactions with proteins in the blood flow.[20] Not only the hydrophilic properties of PEG, but also steric stabilization by the long polymeric PEG chains is believed to be important for achieving these properties.

Here we show the formation, stability and responsive properties of zwitterionic supramolecular nanoparticles (ZSNPs). The zwitterionic motif is proposed here as an alternative, shell-forming moiety around the SNP core to provide colloidal stability and anti-fouling properties. Hereto, SNPs are designed based on the hetero-ternary host-guest interaction between azobenzene (Azo), methyl viologen (MV) and cucurbit[8]uril (CB[8]) (Fig. 1). The monovalent character

of the non-fouling Azo-Zwit is required to avoid uncontrolled growth of the SNP core by assembly of the multivalent components. An important research question is whether the relatively short zwitterionic molecule, which is much shorter than the typically used polymeric PEG molecules, provides sufficient colloidal stability in aqueous solution, in the absence or presence of BSA. Furthermore, stoichiometry-based size control, UV-initiated rupture by switching of the Azo moieties, and the stability of the SNPs in different pH media will be shown here as well.

Results and discussion

An Azo-carboxybetaine analog (Azo-Zwit) was synthesized (Fig. S1–S3, ESI†) and used as monovalent capping ligand in the formation of the ZSNPs. In the presence of the previously reported MV-substituted poly(ethylene imine) (MV-PEI, degree of substitution: 4.5 MV units per polymer chain, MW 10 kDa),[22] Azo-terminated poly(amidoamine) dendrimer generation 1 (Azo_8-PAMAM)[23] and CB[8], SNPs are formed by multiple hetero-ternary complexes between trans-Azo, MV and CB[8]. The core of these SNPs is assembled via multivalent intermolecular interactions between the MV-polymer and the Azo dendrimer in the presence of CB[8], whereas monovalent interactions involving Azo-Zwit constitute the shell.

The formation of ZSNPs was studied in water at an equimolar 1:1:1 ratio of the molecular recognition moieties CB[8], MV and Azo. Using 70% Azo derived from Azo-Zwit and 30% from Azo_8-PAMAM, ZSNPs with an average size of 78 ± 9 nm and an average hydrodynamic diameter of 133 ± 19 nm were observed by scanning electron microscopy (SEM) and dynamic light scattering (DLS), respectively (Fig. 2a-b). The ZSNP sizes observed by SEM are smaller than by DLS which is attributed to (i) ZSNP shrinkage during SEM sample preparation by the loss of water and (ii) DLS eflecting the hydrodynamic diameter of the ZSNPs.

To verify that the ternary host-guest complexation is essential for stable ZSNP formation, different control experiments were carried out. Congruently, ZSNP formation was detectable neither for samples prepared in the absence of CB[8] or MV-PEI nor for samples prepared with CB[7] instead of CB[8] (Fig. S4, ESI†). It is known, that the smaller CB[7] is too small to encapsulate both guest molecules. These results confirm that ZSNP formation is induced by ternary host-guest complexation between MV, Azo and CB[8].

The use of NPs for biomedically relevant applications requires NP stability at physiological ionic strength. Therefore, ZSNP self-assembly was evaluated in phosphate-buffered saline (PBS, 140 mM KCl pH 7.4). Using the same ZSNP composition, SEM and DLS showed negligible size and morphology differences in comparison to experiments carried out in water (Fig. 2c and S5, ESI†). This observation clearly shows that stable ZSNPs have been formed in PBS. In contrast, SNP formation experiments carried out with MV- PEI, Azo_8-PAMAM, CB[8] and Azo-functionalized tri(ethylene glycol) (Azo-TEG) in PBS, showed irreproducible results and uncontrolled aggregation by DLS. Similarly, SEM (Fig. 2d) showed indistinct structures, which are incomparable to the here reported ZSNP structures or to previously observed SNPs prepared by polymeric Azo-PEG (MW 5 kDa)[23] (Fig. S6, ESI†). This illustrates that the monovalent Azo-Zwit provides sufficient colloidal stability to the ZSNPs in PBS, whereas Azo-TEG with a comparable molecular length does not. Most likely, the ionic character of the Azo-Zwit renders the ZSNP particle shell ultra-hydrophilic, obviating the steric stabilization known for PEG-modified SNPs.

To investigate the effect of ionic strength on ZSNP formation and stability, self-assembly of the four supramolecular building blocks was carried out in PBS with

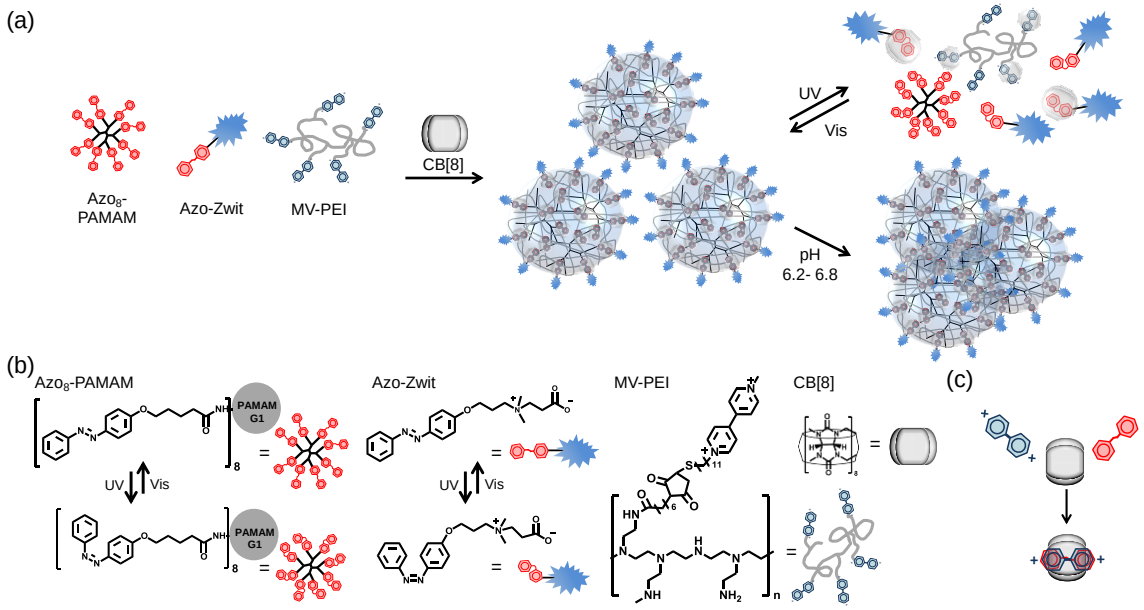

Fig. 1 (a) Schematic illustration of the supramolecular self-assembly of zwitterionic supramolecular nanoparticles (ZSNPs) mediated by ternary host-guest complexes between azobenzene (Azo), methyl viologen (MV) and cucurbit[8]uril (CB[8]), UV light-triggered SNP disassembly and pH-responsive SNP aggregation. (b) Supramolecular components involved in ZSNP assembly: azobenzene-functionalized poly(amidoamine) dendrimer (Azo_8-PAMAM), a monovalent Azo-carboxybetaine analog (Azo-Zwit), MV-functionalized poly(ethylene imine) (MV-PEI), and CB[8]. (c) Hetero-ternary host-guest complex of MV and Azo in CB[8].

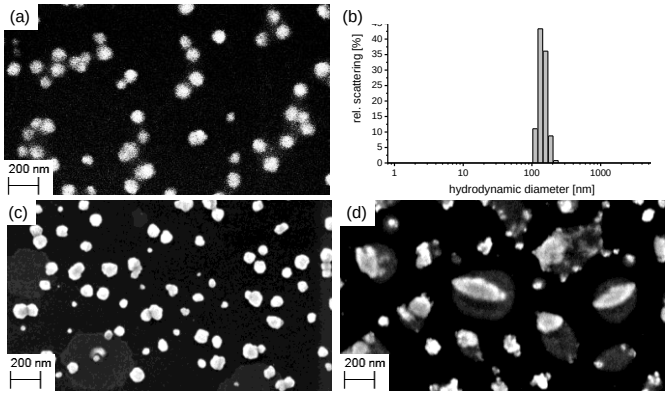

Fig. 2 Size determination of ZSNPs and SNPs prepared with Azo:MV:CB[8] = 1:1:1, with 30% Azo derived from Azo8-PAMAM prepared in different media: (a) SEM image of ZSNPs prepared with Azo-Zwit in water; (b) DLS of ZSNPs prepared with Azo-Zwit in water; (c) SEM image of ZSNPs prepared with Azo-Zwit in PBS (140 mM KCl, pH 7.4); (d) SEM of SNPs prepared with Azo-functionalized tri(ethylene glycol) in PBS

varying concentrations of KCl (50-700 mM) using 30% Azo from Azo_8-PAMAM. DLS and SEM revealed that ZSNPs are formed regardless of the ionic strength (Fig. 2c, 3 and S7, ESI†). The ZSNPs were stable for at least 5 days after self-assembly. Nevertheless, the ZSNP size and size distribution observed for the samples prepared in PBS with 700 mM KCl were higher (237 ± 42 nm by DLS and 117 ± 41 nm by SEM) as compared to the ZSNPs at 140 mM KCl (154 ± 13 nm by DLS and 72 ± 17 nm by SEM). We have recently shown that SNP destabilization at high ionic strength can be inhibited by a monovalent capping PEG (MW 5 kDa).[24] The results shown here indicate that also Azo-Zwit is capable of preserving colloidal stability at high ionic strength.

Importantly, ZSNPs were also stable after mixing 250 µL ZSNPs solution with 500 µL of a 5 wt% dispersion of BSA in PBS, which is the common concentration of serum proteins in blood. DLS gave inconclusive results because of the high protein concentration, but SEM images unambiguously revealed the stability of the ZSNPs in this medium: Fig 3f, acquired at the drying edge of the sample, shows individual ZSNPs not associated with protein and of similar size as the ZSNPs made in buffer. ZSNPs codeposited with BSA were observed (Fig. S8d, ESI†) in the center of the same sample, but again here the particles have a very similar size, and the occurrence of both particles and protein is therefore attributed to the large excess of BSA present during sample preparation.

Size tunability of the self-assembled ZSNPs was evaluated in aqueous solution by altering the ratio of the monovalent Azo-Zwit and the multivalent Azo_8-PAMAM while keeping the overall Azo:MV:CB[8] stoichiometry at 1:1:1. By increasing the amount of Azo from Azo_8-PAMAM from 10%to 30%, while decreasing the amount of Azo-Zwit from 90% to 70% correspondingly, an increase in ZSNP size was observed from 51 ± 11 nm to 78 ± 9 nm by SEM and from 62 ± 16 nm to 133 ± 19 nm by DLS (Fig. S9 and S10, ESI†). This size tuning is attributed to the competition between monovalent and multivalent hetero-ternary interactions, similar to the SNP formulations stabilized with monovalent PEG.

The reversibility of the Azo:MV:CB[8] host-guest complex, induced by photoswitching of the Azo moiety, has been used to trigger NP assembly and disassembly.[23, 25] Photoisomerization of trans into cis-Azo by UV light causes disassembly of the ternary complex because the bulkier cis-Azo and MV do not fit in the cavity of CB[8] simultaneously. To evaluate whether the photoswitching of Azo can be used for the rupture of ZSNPs, the particles in aqueous solution were irradiated with UV light. The effect of UV irradiation on the size of the ZSNPs measured by DLS is shown in Fig. S13 (ESI†). As observed for SNPs

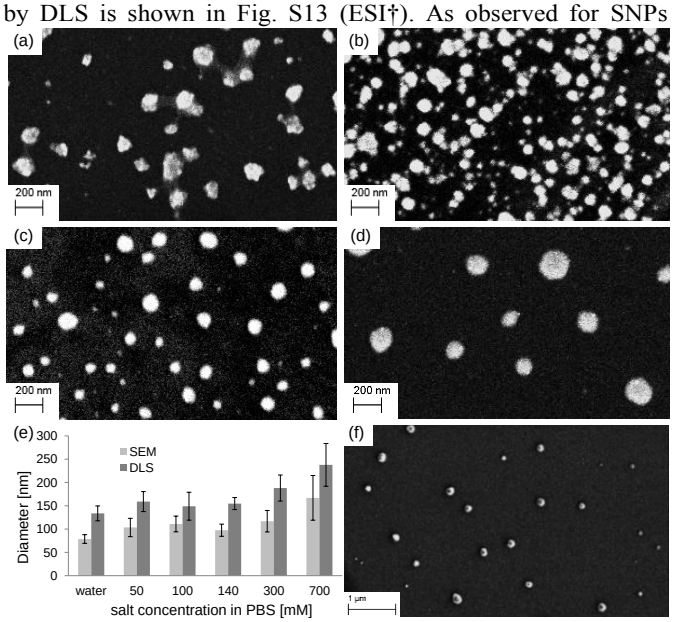

Fig. 3 Size determination of ZSNPs prepared in PBS (pH 7.4) with different KCl concentrations: a-d) SEM images at ionic strengths of: 50 mM, b) 100 mM, c) 300 mM, d) 700 mM, e) diameters of ZSNPs prepared in PBS at different ionic strengths as measured by SEM (■) and DLS (■); f) SEM image of ZSNPs prepared at 140 KCl and mixed subsequently with 5 wt% BSA (v:v 1:2), imaged at the drying edge

prepared with Azo-PEG (MW 5 kDa),[23] the ZSNPs started to aggregate after short UV irradiation. This effect continued until no ZSNPs were observed after prolonged UV exposure overnight. The relatively slow particle disassembly process is attributed to incomplete trans-cis isomerization (ca. 80%) and slow rearrangement of multivalently linked building blocks resulting in fast shell but slow core dissolution. In addition, ZSNP reassembly was achieved upon cis-trans isomerization of the Azo-bearing building blocks by irradiation with visible light for 8 h.

Whether the cis-Azo or the MV moiety stays in the cavity of the CB[8] after photoswitching, depends on the molecular structure of the components. If the Azo derivative contains a positive charge in close proximity to the Azo moiety, the cis-Azo isomer is stabilized in the CB[8] cavity by the interaction of the positively charged guest with the ring of carbonyls surrounding the CB[8] cavity, thus leading to release of MV upon trans-cis isomerization.[26] In contrast, in the absence of a cationic charge close to the Azo moiety, the MV stays in the CB[8] cavity and the cis-Azo is expelled.[27] As the positive charge in Azo-Zwit is not located in the immediate vicinity of the Azo moiety and the charge is partially compensated by the carboxylate anion at the tail of the molecule, it was not clear a priori which moiety would remain bound upon UV-induced trans-cis isomerization. UV/Vis spectroscopy (Fig. S11 and S12, ESI†) of cis-Azo-Zwit in absence and presence of CB[8] showed that the cis isomer can be encapsulated in the cavity as witnessed by the decrease of the absorbance of cis-Azo-Zwit at λ=432 nm characteristic for inclusion in CB[8]. However, in case MV was present in the solution as well, the absorbance

decrease was only half. Therefore we conclude that an equilibrium between CB[8]-bound MV and cis-Azo-Zwit exists after trans-cis photoswitching.

To study the behaviour of the ZSNPs as a function of pH, the supramolecular building blocks were dissolved in PBS at pH 5.6, 6.2, 6.8 and 7.4 prior to mixing. DLS and SEM images showed that individual ZSNPs were formed at pH 5.6 and 7.4 (Fig. 2c, 4 and S14 ESI†). In contrast, both techniques indicated the formation and simultaneous aggregation of ZSNPs at the intermediate pH values 6.2 and 6.8 (Fig. 4 and S14, ESI†) with the stronger aggregation at pH 6.8. The aggregates clearly contain individual particles of similar size as formed at pH 7.4, which indicates that the aggregates are hierarchical assemblies of ZSNPs. Notably, the pH regime of 6.2-6.8 corresponds with the extracellular pH observed in tumor tissue. Equally important to note, the pH regime corresponds also with the pKw (13.7) of the water equilibrium, which indicates a balance between hydronium cations and hydroxide anions at pH 6.8. A similar aggregation effect in this pH range was reported by Liu et al. for gold NPs modified with mixed monolayers of 11-mercaptoundecanoic acid and (10-mercaptodecyl)-trimethyl ammonium bromide.[28] Only when using equimolar fractions of the carboxylic acid and quaternary ammonium adsorbates, Au NP aggregation was observed in a window between pH 6.0 and 7.0, whereas individual Au NPs were observed above and below this regime. We assume that, at all pH values used here, the ZSNPs are surrounded by overall charge-neutral Azo-Zwit bearing unprotonated carboxylate ions. We therefore attribute aggregation of the ZSNPs to interaction between the shells in a perfectly charge-balanced environment, while a slight imbalance between hydronium and hydroxide ions is apparently sufficient to prevent aggregation.

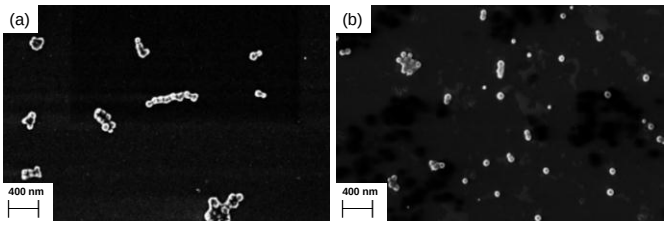

Fig. 4 SEM images of ZSNPs formed in PBS at: a) pH 6.8, b) pH 6.2

Conclusions

In conclusion, we have shown the formation of SNPs based on the ternary inclusion interaction between Azo, MV, and CB[8], using the small-molecule zwitterionic Azo-Zwit as the monovalent capping ligand. In contrast to more conventional SNP formulations requiring long PEGs for steric stabilization, colloidal stability of the ZSNPs, without this steric effect, was observed in different, biologically relevant media. The supramolecular assembly strategy enables size tuning by stoichiometric control of the ratio of multivalent and monovalent Azo components as well as light-triggered SNP disassembly. The ZSNPs show a variety of properties that make them potentially interesting for biomedical applications. First, the use of Azo-Zwit provides nonfouling properties also at high serum protein concentrations. Secondly, ZSNP aggregation is observed at pH values between 6.2 and 6.8. This aggregation mechanism may be more universal, and may potentially be used to design self-assembling materials that will show an enhanced EPR effect. All these properties combined underline the promise of ZSNPs as nanovectors in biomedical applications.

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Notes and references

Molecular Nanofabrication Group, MESA+ Institute for Nanotechnology, University of Twente, P.O. Box 217, 7500 AE Enschede, Netherlands

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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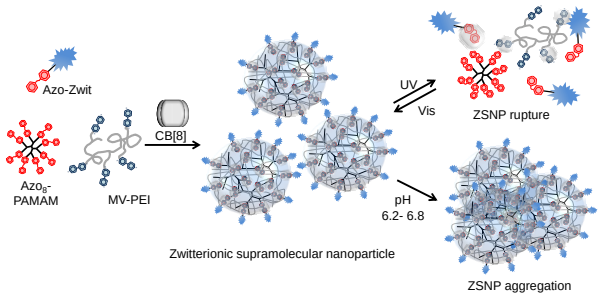

Zwitterionic supramolecular nanoparticles (ZSNPs) show photo- and pH- responsive properties and are stable in high-BSA solutions