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Using the dendritic polymer PAMAM to form gold nanoparticles in the protein cage thermosome

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The chaperonin thermosome (THS) is a protein cage that lacks binding sites for metal ions and inorganic nanoparticles. However, when poly(amidoamine) (PAMAM) is encapsulated into THS, gold nanoparticles (AuNP) can be prepared in the THS. The polymer binds $HAuCl_4$. Subsequent reduction yields nanoparticles with narow size distribution in the protein-polymer conjugate.

The complexity and precision of protein-based nanostructures is impressive and often surpasses the possibilities of man-made nanomaterials. Of particular interest are protein cages, i.e. hollow spheres of high symmetry, ranging in diameter from 5 nm to several hundreds of nm.[1] Famous examples are ferritins, viral capsids, heat shock proteins and chaperonins. While the common feature of protein cages is their well-defined capsule-like structure, they differ widely in size, in function and in other features, such as the size of the pores that connect the interior of the cages with the surrounding solution. Thus, they are versatile functional scaffolds for the preparation of nanomaterials. Moreover, protein cages can be precisely modified at their interior and their exterior surfaces by genetic engineering methods and by chemical means, e.g. to attach polymers,[2] polymerization initiators,[3] catalysts,[4] enzymes,[5] molecular switches,[6] and contrast agents.[7] Thus, protein cages are a promising platform for applications ranging from drug-delivery,[1d, 2e, 8] to diagnostics,[3] and nanoreactors.[1b, 1c, 1e, 4, 9] Their scope of applications can be greatly enhanced by encapsulating inorganic nanoparticles (NPs) into their core.[1c, 1e, 1f, 10] Protein cages that contain NPs were used as catalysts, [11] contrast agents[12] and for bio-inspired material synthesis.[1f] Moreover, they allow the organization of NPs into ordered 2-D or 3-D arrays.[13] In order to prepare NP-containing protein cages, three main strategies have been followed. Protein cages that have natural binding sites for metal ions, e.g. ferritin, were loaded with metal ions. Subsequent reduction lead to the formation of NPs.[14] If a protein cage does not feature such binding sites, they can be genetically engineered into the protein,[11b, 13a, 15] or tether peptides can be used to bind metal ions and form metal NPs.[16] Alternatively, the protein subunits of some protein cages can be reassembled around a preformed metal NP.[17] However, protein cages that lack such features cannot be loaded with NPs. New strategies for the preparation of NP-protein cage hybrids are therefore of high interest.

Here we report the use of charged polymers for the templated synthesis of gold nanoparticles (AuNP) inside the protein cage thermosome, a group II chaperonin from the thermophilic archaea *Thermoplasma acidophilum* (Figure 1).[18] To this end, the dendritic polymer poly(amidoamine) (PAMAM) was conjugated into THS. PAMAM is well-known to facilitate the synthesis of AuNPs.[19] Here, it acted as an anchor to bind tetrachloroaurate anions in the protein cage. Upon subsequent reduction, AuNPs formed inside THS. Without the polymer, THS was not able to template the synthesis of AuNPs. Given the fact that polymers can be encapsulated into a broad variety of protein cages,[2a] e.g. during self-assembly of the cages,[20] by chemical conjugation[2e] or by polymerization reactions,[3-4] we believe that our approach represents a versatile and generic approach for the preparation of NP-containing protein cages.

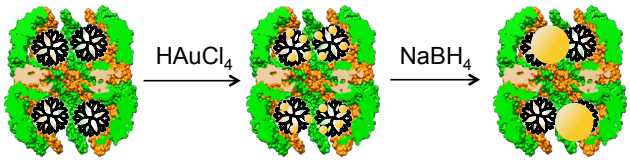

Fig. 1: Cross-sectional view of the structure of a group II chaperonin in an open conformation[21] conjugated with PAMAM and schematic representation of the formation of AuNP in THS-PAMAM.

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THS-PAMAM was synthesized as reported elsewhere by covalently binding fourth generation PAMAM into the cavities of THS, using a resonance stabilized bis-aryl hydrazone linker chemistry.[2e] The resulting bond between the polymer and the protein is stable in a wide pH range and at elevated temperatures.[22] A THS mutant was used that has cysteines solely exposed on the inside of the cavity. These residues act as anchor points for the conjugation of PAMAM to the interior of THS.[23] To form AuNPs in these protein-polymer conjugates, we incubated 1 eq. THS-PAMAM with 55 eq. chloroauric acid ($HAuCl_4$) for 45 min, added 275 eq. reducing agent sodium borohydride ($NaBH_4$) to reach a final concentration of 2.0 µM (2.8 mg ml^{-1}) THS-PAMAM, 550 µM $NaBH_4$, and 110 µM HAuCl4. This solution was incubated for another 45 min.[19a, 19b] The samples were filtered (cut off: 0.22 µm) to remove any bigger gold aggregates that formed. Clear solutions were obtained. UV/Vis spectra confirmed the formation of AuNP in the presence of THS-PAMAM. The spectrum in Figure 2 shows a broad peak around 520 nm, which is characteristic for AuNPs.[24] Additionally, the spectrum features the absorption of THS at 280 nm and a band with a maximum at 354 nm that corresponds to the bisaryl hydrazone linker between THS and PAMAM.[2e, 23]

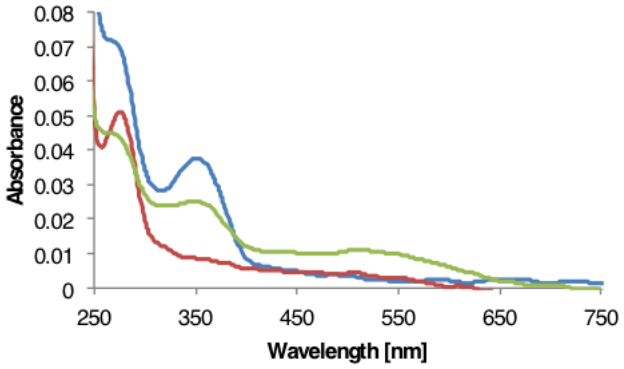
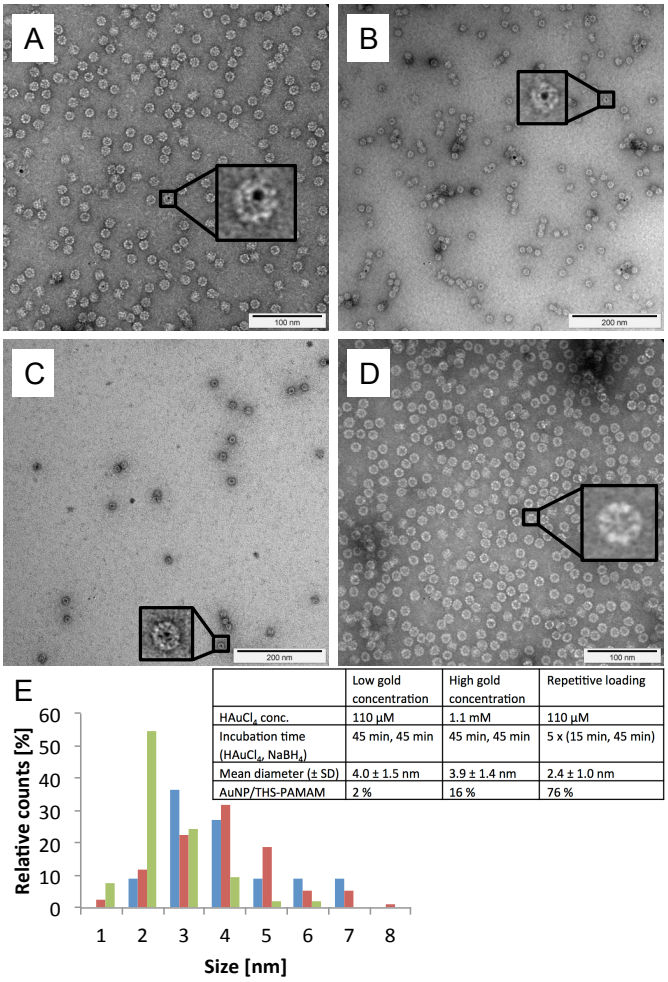


Fig. 2: UV/Vis spectra of AuNP-containing THS-PAMAM formed with 110 µM $HAuCl_4$ protocol (green), of THS-PAMAM (blue), and of THS after 110 µM $HAuCl_4$ loading protocol (red).

THS-PAMAM that contained AuNPs was investigated by transmission electron microscopy (TEM) (Figure 3). The micrographs show fully assembled protein cages, which prove that the highly reductive conditions during the synthesis of AuNPs did not harm the quaternary structure of the protein cage. 55 eq. $HAuCl_4$ to 1 eq. THS-PAMAM resulted in the formation of AuNPs with a diameter of 4.0 ± 1.5 nm. Only 2.3 % of the protein cages contained these NPs. Whether the other THS-PAMAM encapsulated gold particles with diameters below 1 nm or whether they were empty cannot be discerned, as this is below the resolution of the TEM images.

In order to increase the loading of THS-PAMAM with AuNPs, two strategies were explored. THS-PAMAM were loaded with gold ions at ten times higher concentration than in the previous experiment, and subsequently exposed to $NaBH_4$. The TEM images show AuNPs with a diameter of 3.9 ± 1.4 nm that populated 16 % of the THS-PAMAM (Figure 3F). Thus, a higher gold ion concentration resulted in more AuNPs-containing protein cages, but did not affect the size of the AuNPs. For the second approach, a repetitive loading and reduction protocol was adapted.[19c] THS-PAMAM was incubated with 55 eq. $HAuCl_4$ for 15 min and then reduced with $NaBH_4$ for 45 min. This process was repeated five times. On average, the resulting AuNPs had a diameter of 2.4 ± 1.0 nm (Figure 3F). The ratio of AuNP to THS-PAMAM was 0.76. Some protein cages hosted more than one AuNP. Thus, the repetitive method created smaller AuNP and produced significantly more AuNP per THS-PAMAM than the other methods. The smaller size of the AuNP could be caused by the shorter incubation time with $HAuCl_4$. The formation of more NPs per protein cage could be explained by creating more seeding points for AuNP through the repetitive ion binding and reduction steps[19c] and by the growth of very small nanoparticles to sizes that are visible in TEM.



	Low gold concentration	High gold concentration	Repetitive loading
$HAuCl_4$ conc.	110 µM	1.1 mM	110 µM
Incubation time ($HAuCl_4$, $NaBH_4$)	45 min, 45 min	45 min, 45 min	5 x (15 min, 45 min)
Mean diameter (± SD)	4.0 ± 1.5 nm	3.9 ± 1.4 nm	2.4 ± 1.0 nm
AuNP/THS-PAMAM	2 %	16 %	76 %

Fig. 3: TEM images of AuNP-containing THS-PAMAM formed with low gold concentration (110 µM $HAuCl_4$) (A), high gold concentration (1.1 mM $HAuCl_4$) (B), and repetitive gold loading (5 x 110 µM $HAuCl_4$) protocols (C). TEM image of THS after low gold concentration (110 µM $HAuCl_4$) loading protocol (D). Size distribution of the samples (110 µM $HAuCl_4$: blue, 1.1 mM $HAuCl_4$: red, 5 x 110 µM $HAuCl_4$: green) and statistical evaluation of the measured particle diameters (E). Inlets are in 4.4 x zoom. All TEM images were negatively stained with 1% uranyl acetate.

In order to assess whether the PAMAM is essential for the formation of AuNPs in the protein cage, we tried to synthesize gold

NPs with THS that lacked PAMAM. THS was incubated with 55 eq. (110 μM) $HAuCl_4$, followed by reduction with $NaBH_4$ and purification by filtration. The UV/Vis spectrum of this sample only shows THS's peak at 280 nm (Figure 2). Moreover, no AuNP were detected in TEM (Figure 3D). Thus, the protein cage alone is not able to template the synthesis of AuNPs and the cationic polymer is needed for the formation of NPs in the protein cage.

As a control and to show that the protein acted as a template in the reactions described above, we synthesized AuNP in the presence of fourth generation PAMAM alone. Instead of well-defined nanoobjects, polymer aggregates were obtained that hosted ill-defined numbers of AuNPs (Supporting Figure S1). This finding is in accordance with previous reports.[19b] The reason why no aggregates were observed with THS-PAMAM is that the dendritic polycations were sequestered into the confined volume of the protein's cavities.

In conclusion, we prepared AuNPs within THS-PAMAM, whereby the presence of PAMAM was crucial for the formation of AuNPs in THS. Out of the three experimental procedures explored, repetitive cycles of tetrachloroaurate-binding and reduction resulted in the highest loading of THS-PAMAM with AuNPs. NPs with a narrow size distribution were obtained. THS can be modified on its surface with cell targeting and cell penetrating moieties.[2e] Thus, the THS-PAMAM/AuNP hybrids could greatly improve the efficacy of AuNPs in biomedical applications, e.g. as contrast agents or in photothermal therapy for cancer treatment.[25] Finally, this report demonstrates that polymers can be used to facilitate the formation of inorganic NPs in protein cages that lack specific binding sites for metal ions.

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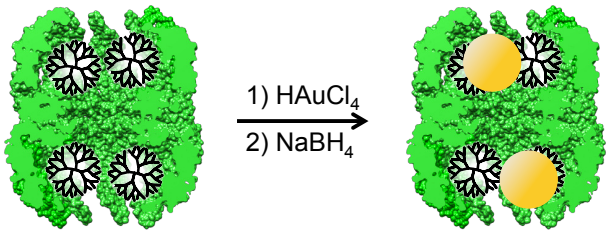

Many protein cages, including the chaperonin thermosome (THS), lack the ability to form inorganic nanoparticles. By conjugation of PAMAM into THS, metal ions could bind to the dendrimer and allowed the formation of gold nanoparticles in the protein cage.

