
This is an electronic reprint of the original article.
This reprint may differ from the original in pagination and typographic detail.

Chakraborti, Soumyananda; Korpi, Antti; Heddle, Jonathan G.; Kostianen, Mauri A.
Electrostatic Self-Assembly of Protein Cage Arrays

Published in:
Polypeptide materials : methods and protocols

DOI:
[10.1007/978-1-0716-0928-6_8](https://doi.org/10.1007/978-1-0716-0928-6_8)

Published: 01/01/2021

Document Version
Peer-reviewed accepted author manuscript, also known as Final accepted manuscript or Post-print

Please cite the original version:
Chakraborti, S., Korpi, A., Heddle, J. G., & Kostianen, M. A. (2021). Electrostatic Self-Assembly of Protein Cage Arrays. In M. G. Ryadnov (Ed.), *Polypeptide materials : methods and protocols* (Vol. 2208, pp. 123-133). (Methods in molecular biology ; Vol. 2208). Springer. https://doi.org/10.1007/978-1-0716-0928-6_8

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.

Electrostatic Self-Assembly of Protein Cage Arrays

Soumyananda Chakraborti¹, Antti Korpi², Jonathan G. Heddle^{1*}, Mauri A. Kostiainen^{2*}

¹Bionanoscience and Biochemistry Laboratory, Malopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7A, 30-387 Krakow, Poland. ²Biohybrid Materials, Department of Bioproducts and Biosystems, Aalto University, FI-00076 Aalto, Finland.

*mauri.kostiainen@aalto.fi *jonathan.heddle@uj.edu.pl

Abstract

Protein and peptide cages are nanoscale containers, which are of particular interest in nanoscience due to their well-defined dimensions and enclosed central cavities that can be filled with material that is protected from the outside environment. Ferritin is a typical example of protein cage, formed by 24 polypeptide chains that self-assemble into a hollow, roughly spherical protein cage with external and internal diameters of approximately 12 nm and 8 nm, respectively. The interior cavity of ferritin provides a unique reaction vessel to carry out reactions separated from the exterior environment. In nature, the cavity is utilized for sequestration and bio-mineralization to render iron inert and safe by shielding from the external environment. Materials scientists have been inspired by this system and exploited a range of ferritin superfamily proteins as supramolecular templates to encapsulate cargoes ranging from cancer drugs to therapeutic proteins. Interesting possibilities arise if such containers can themselves be arranged into even higher-order structures such as crystalline arrays. Here we describe how crystalline arrays of negatively charged ferritin protein cages can be built by taking advantage of electrostatic interactions with cationic gold nanoparticles.

Key Words: Protein cage, nanocrystals, protein engineering, protein design, nanocontainers

1. Introduction

Protein cages are typically spherical assemblies from under 10 to many 10s of nanometres in diameter and having a hollow central cavity. They are typified by viruses, which, in nature carry a genome cargo and demonstrate particular potential for gene delivery applications. Manipulation of natural and artificial protein cages is an area of growing interest¹⁻² and the idea that empty protein cages can be filled with different cargoes of choice and modified chemically as well as genetically has recently grown in prominence.³

The ability to organize protein cages into ordered arrays in three-dimensions may result in interesting physical properties or other useful characteristics depending on the nature of the encapsulated cargo.⁴ Such arrays can be achieved in a number of ways including: i) covalent “cross-linking” of protein cages e.g. by chemical crosslinkers between appropriate amino acid side chains⁵ ii) complementary binding interactions e.g. self-associating peptides can be recombinantly added to the surface of the protein cages which then interact. The disadvantage of covalent systems is the irreversibility meaning that “incorrect” crosslinks will quickly lead to lattice defects. Recombinantly produced modifications that introduce noncovalent interactions are time-consuming to produce and have the disadvantage that the modifications are introduced at every equivalent position on the protein cage which may not be desirable given that protein cages are typically constructed of many 10s of identical protein subunits.

An alternative approach is to use electrostatic effects, which allow self-assembly with dynamic and reversible rearrangements. This has been demonstrated using tobacco mosaic virus⁶, cowpea chlorotic mottle virus and ferritin protein cages that produce superlattices when mixed with appropriately modified gold nanoparticles.⁷⁻⁸ Subsequently many other linker molecules, which are able to electrostatically self-assemble with protein cages have been developed in the past few years.⁹⁻¹¹ Recently, 3D protein cage arrays have been found effective in, for example, enzyme cascade reactions and other applications.^{3,12} Ferritin is of interest as it

is a widely characterised bionanotechnological tool and can be easily modified.¹³⁻¹⁴ Here we give a detailed method for the preparation of electrostatically self-assembled binary lattices consisting of ferritin protein cages and cationic gold nanoparticles (**Figure 1**), based on our recently published research.¹⁵

2. Materials

All the reagents used in this study are analytical grade they were mostly purchased from major suppliers (Sigma, Fisher Scientific and VWR) and used as received unless otherwise specified. All samples were prepared using ultrapure water produced by a Nanopure® water purification system (Thermo Fisher Scientific). Glassware was purchased from Fisher Scientific.

1. Ferritin preparation buffer: 0.02 M HEPES, pH 7.4: Weigh 4.77 g of HEPES (Sigma catalogue number: H3375) and transfer to a glass beaker. Add water to a volume of 900 mL. Mix and adjust pH with NaOH (see **Note 1,2**). Make up to 1000 mL with water. Filter the prepared buffer through 0.45 µM Whatman filter paper and then autoclave. Store at 4 °C.
2. 0.05 M Tris-HCl, pH 8.5: Weigh 7.88 g of Tris-HCl (Sigma catalogue number: T3252) and transfer to a glass beaker. Add water to a volume of 900 mL. Mix and adjust pH with NaOH. Make up to 1000 mL with water. Filter the prepared buffer through 0.45 µM Whatman filter paper and then autoclave. Store at 4 °C.
3. Ferritin cage assembling buffer: 0.1 M MgCl₂ in Tris-HCl, pH 8.5: Weigh 20.33 g of MgCl₂, 6 H₂O (Sigma catalogue number: M2670) and transfer to a glass beaker. Add Tris-HCl, pH 8.5 to a volume of 900 mL. Filter the prepared buffer through 0.45 µM Whatman filter paper and then autoclave. Adjust pH with NaOH. Make up to 1000 mL with Tris-HCl. Store at 4 °C (see **Note 3**).

4. Prepare 1 M NaCl by dissolving 0.584 g of NaCl (Sigma catalogue number S7653) in 10 mL filtered MilliQ water. Store at room temperature.
5. Gold nanoparticles can be prepared according to the method developed by Brust and Schiffrin.^{16,17}
6. Expression plasmids: ferritin expression plasmid: Ferritins in general are facile to produce and purify. The gene sequence of ferritin from *Thermotoga maritima* is available from GenBank (Genbank ID: GenBank: AAD36204.1) and can be codon optimised against model organism *E.coli*. After optimisation, the predicted, translated protein sequence should be checked against the protein sequence database UniProt (UniProt ID: Q9X0L2). Gene synthesis along with cloning of the gene to pET21a (+) plasmid (see **Note 4**) can be performed using a commercial service (we typically use GenScript, <https://www.genscript.com>). For our studies, the ferritin from *Thermotoga maritima* ferritin was most appropriate because of its specific characteristics (see **Note 5**).
7. Ferritin protein: Highly pure (> 95%) recombinant *Thermotoga maritima* ferritin protein can be produced and purified as reported earlier.^{15,18} Protein is stored in 20 mM HEPES buffer (pH 7.4) at 4 °C (See **Note 6**). For long term storage 15 % glycerol is added and sample is flash frozen using liquid nitrogen (-180 °C) and finally stored in a -80 °C freezer (see **Note 7**).
8. Amicon Ultra- 4 Centrifugal Filter Unit (molecular weight cutoff 10,000 Da, Millipore).
9. Eppendorf MiniSpin Centrifuge, Rotor F-45-12-11.
10. 200-mesh copper grids with lacey carbon support film (Electron Microscopy Sciences).
11. Leica EM GP2 automatic plunge freezer.

3. Methods

3.1 AuNP Surface Modification

Gold nanoparticles prepared using the Brust-Schiffrin method are stabilised by alkanethiols, which are not effective for generating superlattices through electrostatic self-assembly. Therefore, the AuNP surface was further modified with a cationic ligand (**Figure 2**). This is first synthesized by slight modification of the method developed by Rotello *et al.*¹⁹ (**Figure 3**) as follows:

1. Weigh 30 mg of AuNPs using an electrotonic balance.
2. Dissolve AuNPs in 5 mL of dichloromethane.
3. Transfer the AuNP solution to a 50 mL glass beaker (see **Note 8**), add a magnetic stirrer bar, place on a magnetic stirrer and stir (e.g. at 500 rpm).
4. Weigh 120 mg (0.28 mmol) of cationic ligand and transfer it into a 25 mL glass beaker.
5. Filter 5 ml of MilliQ water through 0.45 μ M Whatman paper, and to the beaker containing cationic ligand. Dissolve by simple shaking.
6. Transfer the solution to the glass beaker containing the AuNPs. Incubate for 1 h at room temperature (RT) with continuous stirring. During the process of mixing, gold nanoparticles will spontaneously transfer from the dichloromethane phase to the aqueous phase.
7. Carefully transfer the AuNP-containing the aqueous phase to a 50 mL round bottom flask. Discard dichloromethane. For handling dichloromethane follow the link (<http://www.sciencemadness.org/smwiki/index.php/Dichloromethane>).

8. Fix the round bottom flask to a rotary evaporator and evaporate remaining organic solvent until AuNP is completely dry (see **Note 9, 10**).
9. Prepare a dialysis tank by filling a 1 L glass beaker with 1 L of filtered MilliQ water and a magnetic stirrer bar.
10. Collect Dried AuNP and further dissolve in 1 mL filtered MilliQ water and transfer to a cellulose membrane dialysis tube (e.g. Sigma-Aldrich, molecular weight cut-off = 14,000, see **Note 11**). Seal the bag using dialysis clips. Carefully check the bag for any leaks.
11. Transfer the dialysis bag to the dialysis tank. For better rotational control, a floating device can be added to one end of the dialysis bag. Cover the top of the beaker with aluminium foil to prevent contamination with dust. Place on a magnetic stirrer and stir at a constant speed of 200 rpm at room temperature for 48 h. During dialysis replace the water in the tank at 6 h 12 h and 24 h.
12. After the dialysis is complete, remove the dialysis bag from the beaker and dismantle by removing dialysis clip. Carefully transfer the AuNP solution to a 10 mL round bottom flask.
13. Fit the 10 mL round bottom flask to a lyophilizer and operate in high vacuum mode. After 30 minutes stop the instrument and collect the lyophilized AuNPs (brown solid; see **Note 12**).
14. The isolated AuNP can be further characterised for size and shape by dynamic light scattering (DLS), transmission electron microscopy TEM and for the presence of cationic ligand on the surface by nuclear magnetic resonance (NMR). These methods are beyond the scope of this article but are explained elsewhere.⁸ The synthesized gold nanoparticle should have a core size of approx. 2.5 nm in diameter, with total diameter for the particle being approx. 8.5 nm once the ligand is taken into account.

15. Prepare AuNP stocks by dissolving dried particles in Milli-Q water to give a final concentration of ~10 mg/mL and store at 4 °C (See **Note 13**).

3.2 Preparation of Ferritin

1. TmFtn requires buffer exchange into buffer of appropriate pH (50 mM Tris-HCl pH 8.5) for superlattice formation. Exchange can be achieved by a standard protocol such as follows: Take 100 μL of ferritin and place into a 5 mL Amicon Ultra- 4 Centrifugal Filter Unit (molecular weight cut off 10000 Da, Millipore). Add appropriate buffer to make up to a total volume to 5 mL and centrifuge at 3381 rcf (Eppendorf MiniSpin Centrifuge, Rotor F-45-12-11) for 30 mins at 4 °C, with a proper balance. This should result in a decrease of volume of the solution retained in the filter unit to approx. one fifth, though this can change on a case-by case basis so caution should be taken in choosing centrifugation time. The centrifugation unit should be disassembled and the flow through discarded. Take care not to discard the retained solution.
2. After the 5th wash, the protein sample solution should be carefully collected from the filter unit using a 200 μL pipette and transferred to a 1.5 mL microcentrifuge tube, adjust the volume of protein sample to 100 μL by adding fresh buffer.
3. Determine protein concentration using a UV-vis spectrometer and the theoretical extinction coefficient of $29910 \text{ M}^{-1} \text{ cm}^{-1}$ for TmFtn. Remember to measure the buffer alone as a blank and subtract it from the reading obtained with the protein-containing sample.
4. The protein is expected to be a dimer in solution in these conditions. Assess homogeneity by DLS. In our case, we placed 30 μL of protein sample into a black quartz cuvette and measured with a Malvern Zetasizer Nano ZSP (see **Note 14**). Perform

measurements at 298 K and make each measurement in triplicate. A single peak corresponding to the size of ferritin dimer (approx. 5 nm diameter) should be obtained.

5. For generating cage, mix 100 μL of (20 mg/mL) of ferritin dimer with an equal volume of 100 mM MgCl_2 , Tris-HCl (50 mM, pH 8.5) and incubate for approx. 12 h at RT in a 1.5 mL microcentrifuge tube. Then centrifuge at 16863 rcf (Eppendorf MiniSpin Centrifuge, Rotor F-45-12-11) for 10 min at RT to remove any large aggregates. After centrifugation carefully collect the supernatant and transfer to a new 1.5 mL microcentrifuge tube.

6. After cage formation, assess sample quality by again using DLS using parameters as described above. A single peak corresponding to the size of ferritin (approx. 12 nm diameter) should be obtained (see **Note 15**).

7. TEM is used to confirm cage formation. For this, add four microliters of sample (0.1 mg/mL protein cage) on to a copper-Formvar grid (QUANTIFOIL, catalogue number: Q35406) prepared by glow-discharging (see **Note 16**). Incubate for 1 min and remove excess solution by blotting with filter paper. Shortly after soaking, add 4 μL of staining solution 2 % uranyl acetate (see **Note 17**), incubate for 1 min and remove excess stain by blotting with filter paper. Full details of TEM sample preparation and sample acquisitions are beyond the scope of this chapter and should be carried out in conjunction with a TEM expert.

3.3 Superlattice Formation

1. For superlattice formation, add 10 μL of 8 mg/mL ferritin to a 250 μL microcentrifuge tube and mix with 5 μL of Tris-HCl buffer (pH 8.5).

2. Adjust electrolyte concentration so that the final concentration is in the range 0-100 mM NaCl by adding 1 μL of NaCl to the reaction mixture from the appropriate serially

diluted NaCl stock (See **Note 18**). Optimal NaCl concentration for superlattice formation is ~20 mM for *Thermotoga maritima* ferritin.

3. Finally, add 4 μL of 20 mg/mL AuNP to the ferritin and mix of AuNP with ferritin by pipetting. Final volume of reaction should be kept at 20 μL .
4. Keep ferritin to AuNP ratio at 1:1 (w/w) for the final reaction mixture to avoid excess AuNP. Final Mg^{2+} concentration in the reaction should be 25 mM.
5. Incubate reaction mixture for 10–15 minutes in room temperature (see **Note 19**).
6. Characterisation of superlattice should be first carried out by DLS analysis. Further characterization of superlattice can be made by small angle X-ray scattering (SAXS) and Cryo-TEM.

3.4. Further Characterisation

Cryo-TEM images can be obtained. For example, using a JEM 3200FSC field emission microscope (JEOL) operated at 300 kV in bright field mode with Omega-type Zero-loss energy filter. Cryo TEM sample preparation and data collection is complex and should be carried out with an expert in the field. A brief overview of the method as applied to this work is as follows:

1. Prepare 200 mesh copper grids with lacey carbon support film (Electron Microscopy Sciences) by plasma cleaning. For example, treating in a Gatan Solarus (Model 950) plasma cleaner for 30 seconds.
2. Place 3 μl of freshly prepared aqueous sample dispersion was onto the grids.
3. Plunge-freeze in a $-170\text{ }^{\circ}\text{C}$ ethane/propane mixture e.g. using a Leica EM GP2 automatic plunge freezer at 100 % humidity.
4. Cryo-transfer glassy specimens to the microscope.

5. Capture images, e.g. with Gatan Digital Micrograph[®] software while maintaining the specimen temperature at -187 °C.

SAXS can be used to verify superlattice formation. The details of this method are beyond the scope of this article but can be found elsewhere.⁸⁻¹¹ SAXS results should show that ferritin-AuNP complexes arrange into crystal lattices with interpenetrating face-centred cubic (fcc) structure.

4. Notes

1. Concentrated NaOH (10 M) can be used at first to achieve a pH close to 8. Then use a lower concentration of NaOH to avoid a sudden increase in pH above the required pH. Before using pH meter, it should be calibrated against standard pH solutions available commercially.
2. Use a magnetic stirrer for mixing.
3. It is advisable to use freshly prepared buffer, however if necessary, buffer can be stored at 4 °C for limited periods.
4. *Thermotoga maritima* ferritin (without any modification) are commercially available from MoLiRom (<http://www.molirom.com>).
5. The ferritin from *Thermotoga maritima* is extremely thermostable,^{15,18} there is no apparent denaturation of protein was observed when its boiled at 100 °C; furthermore, *Thermotoga maritima* ferritin shows divalent metal (Mg⁺², Ca⁺²) mediated assembly behaviour, which is unusual among the ferritin family of proteins.
6. All the purified ferritin samples were filtered through a 0.22 µm filter to avoid bacterial contamination. Ferritin aliquots were always prepared under laminar flow in completely sterile conditions.

7. For long term protein storage, we used 15 % glycerol, this is particularly useful if your sample is to be flash frozen, as it prevents ice crystal formation. The exact percent glycerol required may need to be optimised depending on the protein. Handling of liquid N₂ must be performed with caution, as contact can cause severe burns. The use of appropriate protective clothing, gloves laboratory goggles is advised.
8. Glassware for gold nanoparticle reactions should be thoroughly cleaned before use. In our case, we first cleaned with chromic acid, followed by water (at least 3-4 times). Glassware was then baked at 200 °C for 1 h. Try to avoid cleaning of glassware using detergent (to avoid any contamination), trace amounts of which may interfere with the reaction.
9. Rotary evaporation should be carried out using a 35-40 °C water bath and rotation speed greater than 100 rpm to assure stable boiling. To avoid loss of material by splashing of the solvent out of the sample flask, slowly approaching the target pressure is recommended. Boiling point of dichloromethane is approximately 40 °C at atmospheric pressure, so no or very weak vacuum is needed for evaporation. However, it is recommended to keep the solution at 500-750 mbar and 40 °C for 5-10 minutes to completely remove the organic solvent, as these conditions exceed the boiling point of dichloromethane but are insufficient for evaporating water.
10. Rotary evaporators can house high vacuums inside glass components, which may break catastrophically if they are not initially intact, and rapid pressure changes can occur if sample flask is detached incorrectly, which may cause splashing of solvents. Therefore, appropriate safety gear should always be worn when handling the instrument, including safety goggles, laboratory coat and gloves.
11. Before starting dialysis, the dialysis bag should be cleaned properly, in our case we cleaned the dialysis bag extensively with filtered MilliQ water (3-4 times) before use.

According to our observations, full recovery of samples after dialysis cannot be achieved.

12. It was found in many studies that AuNP, especially those smaller than 5 nm, are highly toxic to cells,²⁰ so care must be taken during handling them, using personal protection is advisable.

13. Pipette up and down to make sure complete mixing of protein in the solution.

14. The cuvette for DLS should be thoroughly cleaned before use. First with 70 % ethanol (3 times), followed by Milli Q water (4 times), and finally dried by N₂ purging. DLS measurements should be performed in a clean and dust free environment, as atmospheric dust can influence the results heavily. DLS can be measured with very low concentration of protein, in our experimental setup 0.1 mg/mL ferritin protein cage is sufficient.

15. *Thermotoga maritima* ferritin cage has an octahedral symmetry and can store iron in its 8 nm long cavity, the external diameter of ferritin is ~12 nm when measured under TEM and 12-16 nm when measured using DLS.

16. Glow discharge was performed to make TEM grids (copper-Formvar) more hydrophilic. Normally copper-Formvar grids are highly hydrophobic in nature. By performing glow discharge, the grids become more hydrophilic, surface of grids also becomes more negatively charged on glow discharging, which helps aqueous solutions to spread on the grid surface.

17. For negative staining in TEM we use uranyl acetate which is radioactive. In addition, uranium is a toxic heavy metal. Appropriate safety cautions and local regulations must be followed when using it. As an alternative to uranyl acetate one can use phosphotungstic acid (PTA) or platinum blue. These compounds are less hazardous compared to uranyl acetate but, on a case-by-case basis may not be as effective at staining samples.

18. Self-assembly of ferritin into superlattices required fine adjustment of the interparticle interactions. Normally very strong interaction between the particles leads to the formation of amorphous aggregates. On the other hand, weak interparticle interaction fail to produce any kind of assemblies.⁸ Changing electrolyte concentration of the reaction mixture is the most common way to finely tune interparticle interaction, another way is changing pH. Both ways were found very effective, however through optimization is needed in order to get the best result.

19. We found freshly prepared ferritin was more effective in superlattice formation, compared to old (aged) protein, especially those protein which were frozen several times. We also observed that protein stored at -80 °C for a month is suitable for superlattice formation.

Acknowledgements

SC was supported from the Homing programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund, grant No. Homing/2017-3/22 awarded to SC. JGH was funded by the Team Programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund (TEAM/2016-3/23) awarded to J.G.H. A.K. and M.K. acknowledge the financial support from the Academy of Finland (Grants 308578, 303804, 273645, 267497, 272579) and Sigrid Juselius Foundation. The research made use of the instrumentation of Aalto University Nano-microscopy Center.

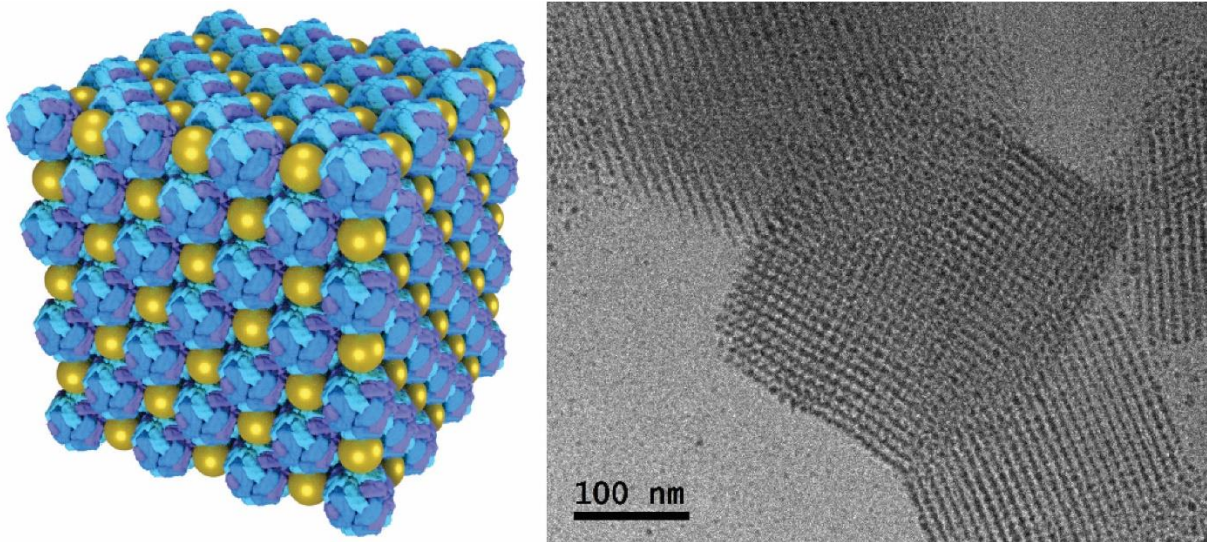


Figure 1. Schematic representation of superlattice (left) and cryo-TEM image (right) of the crystalline structures formed by the ferritin and AuNPs. In the schematic, ferritins are coloured blue, AuNP yellow.

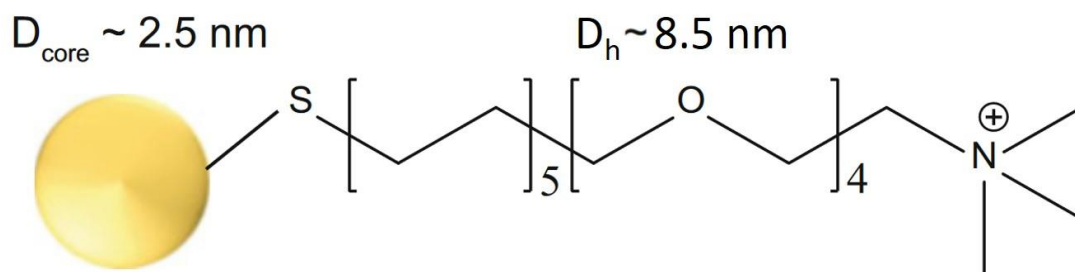


Figure 2. Cartoon showing gold nanoparticle with cationic ligand used in the described method. Diameter of gold nanoparticle (D_{core}) was determined using TEM, and hydrodynamic radius (D_{h}) of gold capped with cationic ligand is measured using DLS. The respective sizes of gold nanoparticles with and without capping agents are 2.5 and 8.5 nm respectively.

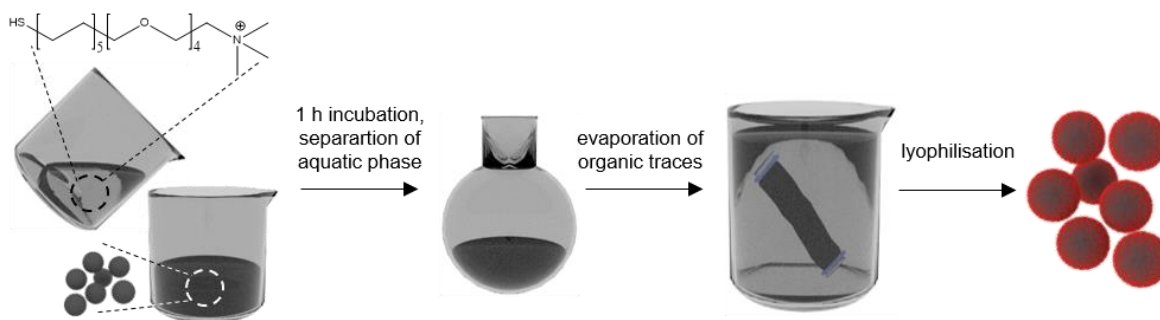


Figure 3. Schematic showing modification of the AuNPs. Mix Brust-Schiffirin AuNPs in organic solvent and cationic ligand in aqueous solvent, incubate together, separate the aquatic phase and evaporate traces of organic solvent. Purify the cationic AuNPs by dialysis against water and collect by lyophilisation.

5. References

- Heddle, J. G. *et al.* (2017) Natural and artificial protein cages: design, structure and therapeutic applications. *Curr Opin Struct Biol.* 43, 148-15.
<https://doi:10.1016/j.sbi.2017.03.007>.
- Heddle, J. G. Tame J.R.H. (2012) Protein nanotubes, channels and cages. The Royal Society of Chemistry, Cambridge.
- Pulsipher, K.W. *et al.* (2016) Ferritin: versatile host, nanoreactor, and delivery agent. *Isr. J. Chem.* 56, 660-670.
<https://doi.org/10.1002/ijch.201600017>.
- Aumiller, W.M. *et al.* (2018) Protein cage assembly across multiple length scales. *Chem. Soc. Rev.* 47, 3433–3469.
[https://doi: 10.1039/c7cs00818j](https://doi:10.1039/c7cs00818j) (2018).
- Abe S *et al.* (2016) Design of a confined environment using protein cages and crystals for the development of biohybrid materials. *Chem. Commun.* 52, 6496-6512.
[https://doi: 10.1039/c6cc01355d](https://doi:10.1039/c6cc01355d).
- Liljeström, V *et al.* (2017) Cooperative colloidal self-assembly of metal-protein superlattice wires. *Nat Commun.* 8, 671.
[https://doi: 10.1038/s41467-017-00697-z](https://doi:10.1038/s41467-017-00697-z).
- Hassinen, J *et al.* (2015) Rapid cationization of gold nanoparticles by two-step phase transfer. *Angew Chem Int Ed Engl.* 54, 7990-7993.
[https://doi: 10.1002/anie.201503655](https://doi:10.1002/anie.201503655).

8. Kostiainen, M. A. *et al.* (2013) Electrostatic assembly of binary nanoparticle superlattices using protein cages. *Nat Nanotechnol.* 8, 52-56.
<https://doi:10.1038/nnano.2012.220>.
9. Liljeström, V *et al.* (2015) Electrostatic self-assembly of soft matter nanoparticle cocrystals with tunable lattice parameters. *ACS Nano.* 9, 11278-11285.
<https://doi:10.1021/acsnano.5b04912> (2015).
10. Liljeström L *et al.* (2014) Self-assembly and modular functionalization of three-dimensional crystals from oppositely charged proteins. *Nat Commun.* 5, 4445.
<https://doi:10.1038/ncomms5445>.
11. Korpi A *et al.* (2018) Self-assembly of electrostatic cocrystals from supercharged fusion peptides and protein Cages. *ACS Macro Lett.* 7, 318-323.
<https://doi:10.1021/acsmacrolett.8b00023>.
12. Uchida M *et al.* (2018) Modular self-assembly of protein cage lattices for multistep catalysis. *ACS Nano.* 12, 942-953.
<https://doi:10.1021/acsnano.7b06049>.
13. Georgiev, I. S. *et al.* (2018) Two-component ferritin nanoparticles for multimerization of diverse trimeric antigens. *ACS Infect. Dis.* 4, 788-796.
<https://doi:10.1021/acsinfecdis.7b00192>.
14. Tsukamoto, R. *et al.* (2013) Effect of PEGylation on controllably spaced adsorption of ferritin molecules. *Langmuir.* 29, 12737-12743.
<https://doi:10.1021/la4029595>.
15. Chakraborti, S *et al.* (2019). Three-dimensional protein cage array capable of active enzyme capture and artificial chaperone activity. *Nano Lett.* 19, 3918-3924.
<https://doi:10.1021/acs.nanolett.9b01148>.
16. Kanaras, A.G. *et al.* (2002) Thioalkylated tetraethylene glycol: a new ligand for water soluble monolayer protected gold clusters. *Chem Commun*, 20, 2294-2295.
<https://doi:10.1039/B207838B> (2002).
17. Brust, M. *et al.* (1994) Synthesis of thiol-derivatised gold nanoparticles in a two-phase liquid-liquid system. *Chem Commun.* 0, 801-802.
<https://doi:10.1039/C39940000801>.
18. Ceci, P *et al.* (2011) The characterization of *Thermotoga maritima* ferritin reveals an unusual subunit dissociation behavior and efficient DNA protection from iron-mediated oxidative stress. *Extremophiles* 15, 431-439.
<https://doi:10.1007/s00792-011-0374-3>.
19. Miranda, O.R. *et al.* (2010) Enzyme-amplified array sensing of proteins in solution and in biofluids. *J. Am. Chem. Soc.* 132, 5285-5289.
<https://doi:10.1021/ja1006756>.

20. Carnovale C *et al.* (2019) Identifying trends in gold nanoparticle toxicity and uptake: size, shape, capping ligand, and biological corona. ACS Omega 4, 242–256.
<https://doi:10.1021/acsomega.8b03227>.