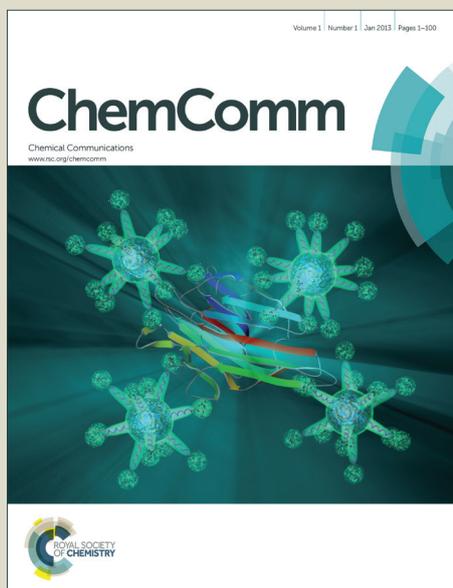


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COMMUNICATION

A Designer Peptide as a Template for Growing Au Nanoclusters

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A peptide was designed to generate a sub-nanometric template that guides the growth of fluorescent gold nanoclusters. The peptide was endorsed with nucleating moieties and a three-dimensional structure that arrests the growth of ultrasmall nanoparticles. The nanoclusters are not cytotoxic and can be found in the cytosol of cells.

The growth of inorganic crystals with controlled nanoscale dimensions is a well-documented phenomenon in biomineralization and bio-inspired crystal growth processes.¹ Among these, the formation of nanoparticles inside the protein ferritin has drawn much attention due to its potential for generating functional nanomaterials of controlled size for nanotechnology applications.² In this peptide template, amino acids with affinity for metal ions concentrate the precursor of the reaction and facilitate the nucleation of nanocrystals. Seed growth is then arrested by a peptide cage of well-defined dimensions to yield nanoparticles of controlled size. Although the protein can be engineered to grow different functional materials,³ the choice of nanoparticle sizes available using this biotemplate may not be adequate for some applications. For example, it would be desirable to find a peptide template for generating sub-nanometric metal nanoparticles, also known as metal nanoclusters. These nanoclusters possess intriguing catalytic, photoluminescent and magnetic properties that are intimately related to the number of constituent atoms.⁴ Although several proteins have been reported to enable the growth of metal nanoclusters,⁵ the role of protein composition and conformation in the growth process has not been fully described yet. In this context, the utilization of a small synthetic peptide with well-known composition and three-dimensional structure may unravel the key features of peptide templates for the growth of sub-nanometric nanoparticles.

Here, we report on the design of the peptide C(GRP)₃ (Cys-Gly-Arg-Pro-Gly-Arg-Pro-Gly-Arg-Pro) that mimics biological templates for crystal growth such as ferritin and guides the formation of Au nanoclusters with an exact number of constituent atoms. The Cys amino acid in C(GRP)₃ was added to concentrate

metal ion precursors and nucleate the growth of the nanocrystals. The conformation of minimum energy of the Pro-rich sequence Gly-Arg-Pro-Gly-Arg-Pro-Gly-Arg-Pro can adopt a helical structure whose groove defines a sub-nanometric cage that limits the growth of nanoclusters, similar to the templating effect of ferritin (Fig. 1). Furthermore, the utilization of a synthetic designer peptide allows for the easy incorporation of other functions within the template. For example, it has been reported that positively charged nanoparticles have cell-penetrating properties.⁶ Here, three Arg amino acids are added to facilitate the uptake of the resulting peptide-caged Au nanoclusters inside cells for potential nanomedicine applications. The growth of sub-nanometric Au nanoclusters can be achieved by simply triggering the reduction of Au ions with NaBH₄ in the presence of C(GRP)₃ (Fig.1). The resulting water-soluble Au nanoclusters emit in the near-infrared (NIR) region and are not toxic to cells, which are promising features for their use in vivo, in the context of bioimaging and drug delivery.

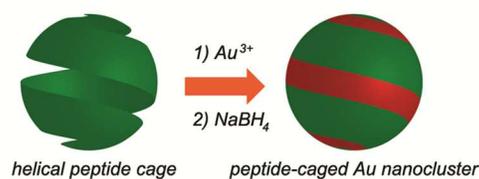


Figure 1. Schematic representation of the growth of Au nanoclusters with the peptide template C(GRP)₃; the three-dimensional structure of peptide can generate a cage that arrests the growth of Au nanoclusters.

It is well established that when the size of Au nanoparticles decreases below 2 nm their characteristic localized surface plasmon resonance (LSPR) disappears and the nanoparticles become fluorescent, a phenomenon that can be used to recognize the growth of Au nanoclusters.⁷ With this in mind, we tested the ability of C(GRP)₃ to template the growth of Au nanoclusters by studying the optical properties of nanoparticle solutions grown in the presence

and the absence of the peptide. In the absence of the peptide, red-coloured solutions were obtained that showed the typical LSPR of spherical Au nanoparticles with a diameter of ca. 5 nm (Fig. 2a (i) and Fig. S2 online). No fluorescence was observed in this solution. However, in the presence of the peptide template C(GRP)₃ the solution turned brown and no LSPR was observed (Fig. 2a (ii)). Fluorescence spectroscopy revealed an emission peak centred at 680 nm (Fig. 2b). The absence of LSPR and the observation of NIR emission demonstrate the growth of gold nanoclusters with the peptide template (see also Figure S1 online).⁷

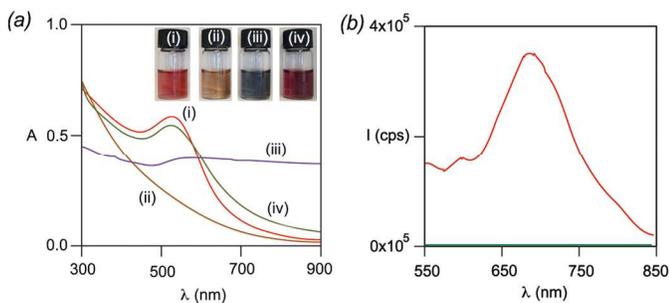


Figure 2. Optical characterization of nanoparticle solutions; (a) UV-Vis spectra and photographs of nanoparticle solutions grown (i) in the absence of peptide; (ii) with the peptide template C(GRP)₃; (iii) with Cys-free (GRP)₃; (iv) with Pro-free C(GRG)₃; (b) Fluorescence spectrum of Au nanoclusters grown with the template C(GRP)₃ (red) and of C(GRP)₃ alone (green) (excitation wavelength 440 nm).

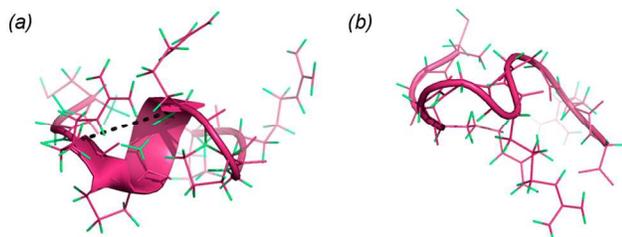


Figure 3. Molecular dynamics simulations of (a) the Pro-rich peptide template C(GRP)₃ and (b) the Pro-free control peptide C(GRG)₃. The dotted line (0.6 nm) shows the diameter of the groove of the helical conformation.

After demonstrating the growth of Au nanoclusters, the relevance of the different parts of the peptide design for obtaining sub-nanometric nanoparticles was explored. In the proposed design, the presence of a nucleating moiety was essential to mimic the templating effect of ferritin. In Fig. 2a (iii) the importance of the Cys amino acid in the nucleation of the nanoclusters was studied by repeating the growth process with the peptide (GRP)₃ (Gly-Arg-Pro-Gly-Arg-Pro-Gly-Arg-Pro), which contains the same sequence as C(GRP)₃ but without Cys. This experiment resulted in the generation of blue-coloured solutions containing nanoparticle aggregates that were not fluorescent (Fig. 2a (iii) and Fig. S3 online). The growth of aggregated gold nanoparticles and not nanoclusters can be attributed to nucleation and growth happening outside of the biotemplate. Under this condition the only role of the peptide is to act as a polycation that induces the aggregation of the negatively charged gold nanoparticles.⁸ Thus, these results prove that the presence of a nucleating amino acid is vital in the growth of Au nanoclusters.

Next, we studied the relevance of the three-dimensional structure of the peptide in creating a cage that limits the growth of nanocrystals and yields sub-nanometric nanoparticles of controlled size. Figure 3a shows a snapshot of a molecular dynamics simulation of the peptide template C(GRP)₃. The conformation of minimum energy is a helical structure that defines a groove with a diameter of ca. 0.6 nm (dotted lines in Fig. 3a). This groove could act as a cage inside which sub-nanometric nanoparticles could grow, as previously seen in ferritin. As a control, we also studied the conformation of the Pro-free peptide C(GRG)₃ (Cys-Gly-Arg-Gly-Gly-Arg-Gly-Arg-Gly). This peptide does not adopt a helical conformation and can exist in solution in multiple local free energy minima, one of which is shown in Fig. 3b. Nanoparticle growth in the presence of Pro-free C(GRG)₃ yielded red-coloured nanoparticle solutions that showed an LSPR centred at 523 nm and no fluorescence (Fig. 2a (iv)). The observation of plasmonic nanoparticles but not nanoclusters is attributed to the growth of peptide-decorated nanoparticles with the Pro-free peptide. In other words, this peptide acts as a mere ligand and not as a true template. The aforementioned results demonstrate that the presence of a Pro-rich conformation that can adopt a helical structure is essential to grow Au nanoclusters in the presence of the nucleating amino acid Cys.

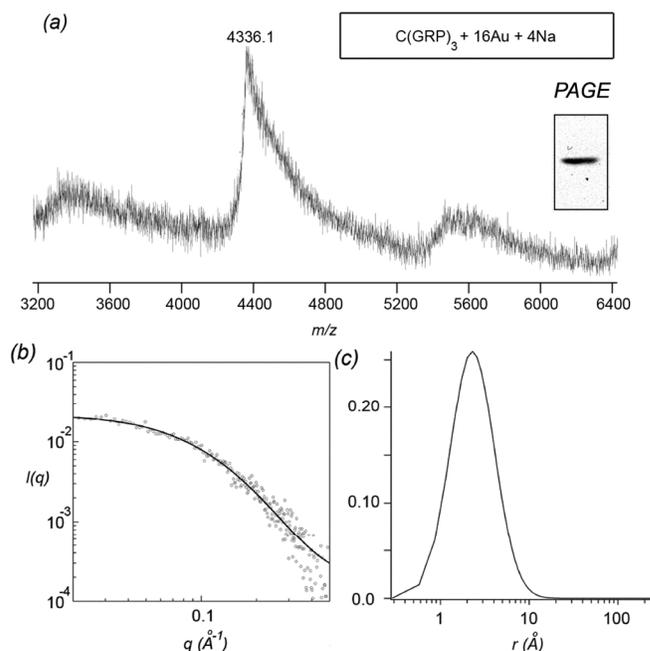


Figure 4. Analysis of the size of Au nanoclusters grown with C(GRP)₃; (a) MALDI MS reveals a single peak corresponding to 16 atoms of gold and one peptide; Inset: PAGE of the crude; (b) experimental scattering pattern and (c) lognormal size distribution of the nanoclusters grown with C(GRP)₃.

It has been reported that some thiolated peptides can generate Au nanoclusters by forming a stabilizing corona around sub-nanometric nanoparticles.⁹ This behavior deviates from the templating effect expected from C(GRP)₃, which limits the growth of nanoclusters inside the cage defined by its three-dimensional structure. When peptides act as stabilizing ligands, more than one peptide is found interacting with the nanocluster.⁹ However, if C(GRP)₃ is a true template, only one peptide should be encaging the nanoclusters. In Fig. 4a, analyses of the samples with matrix-assisted

laser desorption/ionization (MALDI) revealed a single peak attributable to Au nanoclusters that corresponded to the molecular weight of a single peptide and 16 atoms of gold. This result, along with the fact that the thiolated Pro-free peptide C(GRG)₃ does not generate Au nanoclusters, demonstrates that C(GRP)₃ acts as a peptide cage and not as a mere stabilizing ligand during the growth process. Another good experiment to prove the templating effect of the peptide is to compare the size of the nanoclusters with the size of the peptide cage in Fig. 3a, since both values should match when the peptide is acting as a template. With this in mind, we evaluated the size of the nanoclusters with small angle X-ray scattering (SAXS). Figure 4b shows the experimental scattering pattern of the nanoclusters grown with C(GRP)₃. The data was fitted to a model of spheres with a lognormal size distribution (Fig. 4c). The best fit to the model, represented by the solid line, yielded an average radius of 3.7 Å, which is in good agreement with the size of the peptide cage shown in Fig. 3a. This outcome further proves that the peptide is a real template and not a stabilizing ligand. Furthermore, it suggests that the 3D structure of the template is crucial to obtain nanoclusters with a particular number of constituent atoms. Finally, it is worth mentioning that the observation of a single peak with MALDI also suggests that the Au nanoclusters containing 16 atoms are the most abundant species in solution.¹⁰ This outcome was further demonstrated by polyacrylamide gel electrophoresis (PAGE, inset in Fig. 4a), which rendered a single band when the nanocluster solution was run in acidic conditions.¹¹ The nanoclusters did not show any electrophoretic mobility in basic media, which demonstrates that they are positively charged, as expected from the presence of Arg amino acids in the peptide template. Repulsion between positively charged nanoclusters is believed to be the main factor to avoid the aggregation of the nanoclusters in aqueous solutions.

After demonstrating that C(GRP)₃ encages Au nanoclusters, we studied the ability of the nanomaterials to penetrate cells for potential nanomedicine applications. To this end, we incubated the positively charged nanoclusters with human primary fibroblasts for 24 h and tested cell viability and biological distribution. To mimic physiological conditions, the nanoclusters were diluted with serum-containing medium, since serum proteins interact with nanoparticles to form a protein corona that determines their interaction with cells, potentially altering their cell-penetrating properties.¹² In Figure S3 online, the nanoclusters were detected by their intrinsic NIR emission with confocal microscopy (red color). They were found inside the cells diffused throughout the cytosol. The presence of nanoclusters in the cytosol is promising for their use as carriers in drug delivery, since most drugs must be present in active form in the cell cytosol in order to exert their therapeutic effect. The nanoclusters did not kill the cells, affect their metabolic activity or change their morphology even when incubated at a very high concentration (1 mg·mL⁻¹ peptide concentration), which all are essential requirements to translate these nanomaterials in vivo.

Conclusions

In conclusion, we designed the peptide C(GRP)₃ that acts as a template for growing Au nanoclusters with exact number of constituent atoms by drawing inspiration from naturally-occurring peptide cages such as ferritin. The key features of the proposed template are the presence of a nucleating amino acid, Cys, and a sequence that adopts a helical conformation whose groove determines the size of the sub-nanometric nanocrystals. In the future, nanoclusters of different sizes could be obtained by redesigning the template, for example, by using longer β- or γ-amino acids. Moreover, similar peptide templates could be used to obtain other

sub-nanometric materials such as semiconducting¹³ and magnetic¹⁴ nanoclusters. The resulting peptide-templated Au nanoclusters are internalized by cells and do not show any cytotoxicity, which is promising for the translation of these nanomaterials in vivo. Moreover, amino acids with functional groups could potentially be added to attach cargoes for intracellular delivery. These features, along with their intrinsic NIR emission, make these nanomaterials promising for next-generation nanomedicine and theranostic applications.

Notes and references

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