Type I collagen-mediated synthesis of noble metallic nanoparticles networks and the applications in Surface-Enhanced Raman Scattering and electrochemistry

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1. Introduction

Materials composed of multi-dimensional ensembles of nanoparticles (NPs) are becoming more and more important in material science and analytical science fields. Porous network nanostructures have attracted much attention due to their enhanced optical, catalytic, SERS and magnetic properties. For example, three-dimensional (3D), highly ordered porous gold and Ag films greatly enhanced SERS signals [1,2]. Ionic liquids-stabilized Pt NPs 2D networks showed good electrocatalytic ability to O2 [3]. Fe nanostructured magnetic networks revealed enhancement in the coercivity [4]. On the other hand, noble metallic NPs are unique supports for the study of biomolecules because of the size compatibility, chemical inertness and high dispersibility in aqueous solutions. It is necessary to investigate the synthesis and assembly of noble metallic NPs to form network-like nanostructures and the applications in analytical sciences.

There are two conventional strategies that can be used to fabricate the network-like nanostructures. The first strategy is the non-template synthesis. This strategy requires comparatively exotic processing technologies such as electron-beam lithography [5] or nanosphere lithography [6], sputtering technology [4], laser ablation technology [7]. So it was limited by the relatively high cost of fabrication and low surface area of the metal structure. The other strategy is the template synthesis, which is an elegant chemical approach for the fabrication of nanostructures, in particular for the preparation of diverse network-like nanomaterials.

Commonly used sacrificial template silica or polymer microspheres and porous anodic aluminum oxide (AAO) are called hard templates [1,8,9]. This kind of templates has several advantages, such as long-range periodic structures and ordered metal nanostructures with adjustable pore size. However, the defects also obviously exist. The diversity of template material is limited, the preparative process is complex due to the requirement of building blocks with superior quality and advanced features, and it is difficult to control the infiltration of the desired composite metals into the interstitial voids. The other kind of building blocks is called soft templates, such as biomacromolecules, polymers, ionic liquids, micelle systems, and so on. Among that, biomolecules as templates to synthesize network-like nanostructures have attracted increasing attention due to their easily assembly, controllable dimensions, large surface area, low cost and environmentally benign. Various biomolecules were used to synthesize and assemble porous or fibrous inorganic materials with unique pore or surface structures, like DNA [10–12], proteins or peptides [13,14], viruses [15,16] as well as carbohydrates [17].

In this work, for the first time we reported the preparation of 2D Au, Ag, Pt, and Pd NPs networks using type I collagen molecules.
2. Experimental section

2.1. Chemicals and materials

Type I collagen from calf skin (cell culture tested, acid soluble) and 4-aminothiophenol (4-ATP) were supplied by Sigma–Aldrich (U.S.A.). Acetic acid (CH₃COOH, A.R.), HAuCl₄, H₂PtCl₆ and PdCl₂ were obtained from Beijing Chemical Co. (Beijing, China). AgNO₃ (A.R.) and sodium borohydride (A.R.) were bought from Shanghai Sangon Biological Engineering Technology & Services CO., Ltd. (Shanghai, China). 100 ng/μL type I collagen was obtained by dissolving 1 mg collagen in 100 mL 0.2 M acetic acid. 56.4 mM H₂PdCl₄ (Shanghai, China). 100 ng/H₂PtCl₆ and AgNO₃ were obtained from Beijing Chemical Co. (Beijing, China). AgNO₃ was dissolved in 50 mL H₂O and brought to boiling, a solution of 1% sodium citrate (1 mL) was added rapidly. The solution was kept on boiling for approximately 1 h. A SERS substrate was prepared by dropping 30 μL of Ag colloid onto freshly cleaved mica.

2.2. Synthesis

1 mL type I collagen (100 ng/μL) was added into 10 mL of HAUCl₄ (1.0 mM and 0.5 mM, respectively), and vigorously stirred for 10 min. After that, 10 mM NaBH₄ solution was added drop-wise slowly under strongly stirring, until the color of colloid was unchanged. The final Au colloid was wine red. H₂PtCl₆ (0.5 mM), H₂PdCl₄ (0.5 mM) and AgNO₃ (0.5 mM) were used to prepare colloids by the same experimental steps.

2.2.1. Control experiment

10 mM NaBH₄ solution was added into 10 mL 0.5 mM HAUCl₄ dropwise slowly under strongly stirring until the color was unchanged, without adding collagen solution. 30 μL of each colloid was dropped onto the Si wafers immediately and dried in the air.

2.2.2. Comparison experiment

For comparison, the citric-capped Ag NPs were prepared as the typical method reported by Lee and Meisel [20]. In brief, 9 mg AgNO₃ was dissolved in 50 mL H₂O and brought to boiling, a solution of 1% sodium citrate (1 mL) was added rapidly. The solution was kept on boiling for approximately 1 h. A SERS substrate was prepared by dropping 30 μL of Ag colloid onto freshly cleaved mica.

3. Results and discussion

3.1. Preparation and characterization of the type I collagen-mediated Au nanostructures

3.1.1. The effect of HAUCl₄ concentration on the formation of collagen-mediated Au nanostructures

UV–vis absorption spectra have been proved to be very sensitive to the formation of Au and Ag NPs. Fig. 1 shows the UV–vis absorption spectra of type I collagen solution, HAUCl₄ solution, the mixture of collagen and HAUCl₄, and the obtained Au colloid.
loids from different ratios of the mixture. Type I collagen has no obvious adsorption in the measured band range (curve a). Two peaks at 216 and 284 nm exhibit the present of $\text{AuCl}_4^-$ ions in aqueous $\text{HAuCl}_4$ and in the mixture of collagen and $\text{HAuCl}_4$ solution (curve b and c in the inset image) [21]. When the collagen concentration was 1.0 mM, and the $\text{AuCl}_4^-$/collagen mixed solution was reduced by $\text{NaBH}_4$, the peak at 284 nm fell down, meanwhile the peak at around 533 nm occurred, which indicated the formation of Au NPs in the mixed solution (curve d) [20] and the corresponding SEM image was shown in Fig. 2A. It can be seen that inhomogeneous Au NPs appear on the substrate. When the collagen concentration was 0.5 mM, the UV–vis absorption peak occurred at 525 nm (curve e) with a large peak width at half height and a long tail. The flat absorption profile is the characteristic of the network-like Au nanostructures proved by Pei et al. [22] and Wang and co-workers [7]. Fig. 2B gives the corresponding SEM image, Au NPs assembled into networks and the networks were uniformly covered on the surface. The good agreement between SEM and UV–vis characterizations confirmed the formation of network-like structures of Au NPs. The chemical composition of the networks in Fig. 2B was determined by elemental analysis, and the SEM-EDS was shown in Fig. 2C. The Si signal is from the substrate of Si wafer, the strong peak indicates the existence of Au NPs, and the C, N, O signals come from collagen molecules, which presumably imply the formation of collagen–Au NPs composites.

The collagen–Au NPs composites were measured by TEM in order to observe the Au NPs networks structure. Fig. 3A presents the TEM image corresponding to the sample in Fig. 2B, and it can be further identified that the whole networks actually is the aggregates of NPs and the NPs connect each other to form the network-like nanostructures. Fig. 3B shows the histogram for the diameter of measured Au NPs shown in Fig. 3A, and a statistical analysis shows the diameter is about $8.3 \pm 1.5$ nm. The size of nanospheres on the networks was obviously smaller and more uniform than that in Fig. 2A. Previous studies revealed that the concentration of the capping agents have strong influence to the size and shape of metal NPs [22,23]. It is hypothesized that at low collagen concentration, growing Au NPs nuclei were adsorbed onto the energetically favorable sites of collagen molecules, which caused the assembly of Au NPs along the collagen chains. At relative high concentration, the specificity was lost when both specific and non-specific adsorptions of colla-
gen occurred. The more uniform coverage of collagen on the nuclei hided the intrinsic differences between sites, resulting in isotropic growth of the nuclei into spherical particles.

3.1.2. The influence of type I collagen molecules to the formation of Au NPs networks

A control experiment was done to understand the function of type I collagen molecules in the formation of Au NPs network. 0.5 mM HAuCl₄ solution was directly reduced by NaBH₄ solution, without adding collagen solution. The as-prepared colloids were unstable, and precipitation was formed rapidly in a few minutes. The colloids were immediately dropped onto a silicon wafer for SEM characterization, and the SEM image exhibits there are many small coagulated particles, and small and large particles coexisted (the SEM image was not shown). In the absence of collagen, uniform NPs network cannot form. We suggest that collagen molecules served as suitable templates to assemble Au NPs.

To further know the interaction between collagen molecules with Au NPs, FT-IR and XPS measurements were applied to study the Au NPs networks. Fig. 4a and b show the FT-IR spectrum of free collagen and collagen-templated Au NPs networks, respectively. The characteristic peaks of amide I band at 1650 cm⁻¹ and the amide II band at 1538 cm⁻¹ are present in pure collagen (curve a), owing to the abundant glycine, praline, and hydroxyproline in type I collagen molecules [19,24], while some slight shifts are observed in curve b. These changes were caused by the connection of the collagen molecules and Au NPs, according to the results reported by Bhattacharya et al., who proved that amines bind with Au NPs through electrostatic/covalent interaction [25].

XPS is proved to be a powerful tool for surface analysis that provides diverse information regarding the structure and chemical state of the type I collagen–Au NPs complex [26]. High-resolution XPS spectra presented in Fig. 5 reveal the existence of (A) Au 4f, (B) C 1s and (C) N 1s spectral regions. Fig. 5A shows the Au 4f₇/₂ and 4f₅/₂ peaks occur at 83.4 eV and 87.0 eV, respectively. These values were very comparable to those of pure Au [27]. The narrow widths of the bands indicate that the Au component is situated in a simi-
lar condition throughout the resulting protein–Au nanostructures [28]. Fig. 5B shows the C 1s region resulting from type I collagen–Au NPs networks, where three major peaks were observed at 284.5, 286.0 and 288.2 eV, corresponding to the existence of C–C, C–N and N–C=O of collagen molecules [29,30]. N 1s positioned at two peaks, 399.8 and 401.1 eV, as shown in Fig. 5C. We assigned the main peak at 399.8 eV to the nitrogen atoms of the collagen molecules, which revealed the interactions between the amino groups and the Au NPs. The minor peak at 401.1 eV suggests the presence of N as charged species, indicating electrostatic interaction between collagen and Au NPs. The NH group on the molecule chains combines with H+ to form NH2+, which makes the collagen molecules positively charged [31].

The XPS, IR, UV–vis, and SEM images confirmed our hypothesis that when collagen molecules were introduced into reaction solution as a template, an initial connection between the gold salt and the protein molecular chains took place and the collagen provided the special platforms for the assembly of prepared Au NPs to form the network-like nanostructures. The excellent assembly property of collagen has been confirmed in many literatures [29,32,33]. Based on the discussion above, we suggest that Au NPs assembled along the collagen molecules in suitable condition. After the Au colloids were dropped onto silicon wafer, the solvent evaporated slowly, and the hydrophobic interaction of peptide residues on collagen chains play a pivotal role, furthermore the inter- and intra-molecule hydrogen bonds reinforce and the collagen molecules self-assemble to widely linked networks [29,32,33]. It should be noted that the details of the interaction between collagen–Au composites and substrate requires further investigation.

Fig. 6. FE-SEM images of Pt NPs networks (A and B), Pd NPs networks (D and E), and the corresponding EDS spectra (C and F), respectively.
3.2. Preparation and characterization of the type I collagen-mediated Pt, Pd and Ag nanostructures

Based on the analysis above, we suppose that type I collagen molecules can be applied as an effective template to synthesize noble metallic nanostructures including Pt, Pd, Ag. Fig. 6A–E present the morphologies of Pt, Pd nanostructures prepared by the same strategy as that for Au NPs networks. It can be clearly observed that both of them formed comparatively uniform metal NPs networks. Ag colloids were prepared by the same experimental method, and the morphologies of as-prepared Ag nanostructures were shown in Fig. 7. Fig. 7A exhibits the Ag nanostructures, and the magnified image was shown in Fig. 7B. It can be observed that the networks consist of 20–40 nm nanospheres and their aggregations. Obviously, the diameter of Ag nanospheres was larger than that of the Au, Pt, Pd nanospheres on networks (around 10 nm).

EDS show the corresponding peaks of Pt, Pd and Ag (Fig. 6C, F and Fig. 7C), demonstrating that the main components of these three nanostructures are metallic Pt, Pd and Ag, respectively. The low-intensity peak of C proved the existence of the template collagen molecules.

The difference between morphologies of Ag NPs networks and other three metal NPs networks are ascribed to the effect of different noble metallic ions used at present study. The pH values of the mixture of the negatively charged metallic ions (AuCl$_4^-$, PtCl$_6^{2-}$, and PdCl$_4^{2-}$) and collagen solutions were all around 3.0, and that of Ag$^+$ and collagen solution was 3.2. As well known, type I collagen is a polyelectrolyte, whose isoelectric point is 7.6. Collagen molecules are positively charged and show good assemble properties in acidic solution [18,19,33]. To the negatively metal ions (take AuCl$_4^-$ as an example), the initial association of the positively charged protein with negatively charged AuCl$_4^-$ occurs because of electrostatic interactions. Subsequently, Au NPs on the collagen chains were reduced in situ, a covalent interaction between the gold and collagen takes place due to the presence of amines in the glycine, proline and hydroxyproline residues in type I collagen [25,34]. But the positively charged collagen molecules cannot adsorb Ag$^+$. After the addition of BH$_4^-$, negatively charged Ag NPs were produced in solution firstly, and then adsorbed onto collagen molecules by the electrostatic interaction. The “freely” Ag NPs produced in solution were larger and more inhomogeneous than the Au NPs formed in situ on the collagen chains.

3.3. The applications of type I collagen-templated Ag and Pt NPs networks in SERS and electocatalysis

Noble metal NPs have been widely applied in optics and catalysis due to their inherent properties such as SPR and high density. We choose type I collagen-templated Ag NPs networks and Pt NPs networks as representative examples for researching the basic applications in SERS and electocatalysis.

3.3.1. SERS spectra of 4-ATP on the type I collagen-templated Ag NPs networks

Herein, 4-ATP was selected as a probe molecule because it can form a self-assembled monolayer on metal surface and most of its distinct Raman bands have been assigned in literatures [35,36]. Fig. 8a and b illustrate the SERS spectrum of 10$^{-7}$ M 4-ATP on the substrates of citrate-capped Ag NPs and collagen-templated Ag NPs networks, respectively. It is obvious that collagen–Ag NPs networks show better enhancement ability than citrate-capped Ag NPs film. For instance, the intensity of the peak at 1078 cm$^{-1}$ in curve b are approximate four times as that in curve a. The main peaks at 1577, 1434, 1390 and 1143 cm$^{-1}$ belong to the b$_2$ vibration mode, which suggest that the enhancement via charge-transfer resonance mechanism is significant [18,35–37]. Two other main peaks, 1184 and 1078 cm$^{-1}$, are ascribed to the a$_1$ vibration mode, which imply that electromagnetic mechanism is also important [18,35–37].

3.3.2. SERS spectra of adenine on the type I collagen-templated Ag NPs networks

One of the potential applications for SERS in the biophysical/biochemical and biomedical fields is the rapid detection, quantification, and characterization of DNA and DNA fragments [38,39]. Compared with the fluorescence technique currently used for DNA analysis, SERS does not require any labeling step because it is a technique of vibrational spectroscopy that shows detailed fingerprint information of DNA bases. To explore the capability of the as-prepared collagen–Ag NPs networks for the detection of DNA
base, adenine was chosen as the probe molecule. Fig. 9 displays the SERS spectra of 10$^{-4}$ M adenine on Ag NPs networks and citrate-capped Ag NPs films. Two distinct Raman bands are observed at 730 and 1328 cm$^{-1}$, assigned to the purine ring breathing mode and the CN stretching mode, respectively [38,39]. In contrast, the citrate-capped Ag NPs represent extremely weak SERS enhancement (curve a). The SERS signal intensity at 730 cm$^{-1}$ from Ag NPs networks is approximately as five times as that obtained on normal Ag NPs films, indicating that type I collagen-templated Ag NPs networks is highly suitable for SERS detections.

SERS is known to be a very local phenomenon generally arising at the junction of adjacent Ag NPs and rough surfaces [40,41]. The obtained high SERS enhancement of 4-ATP and adenine on collagen-templated Ag NPs networks is possibly attributed to two main reasons. One factor is the formation of many nanoscale pores on uniform Ag NPs networks. Berlin et al. and Sailor et al. have obtained high SERS enhancements on their respectively prepared silver-coated silicon nanopores substrates [2,42]. Another successful research also get high SERS signals on ordered macroporous Au/Ag bimetallic nanostructures by Lu et al. [8]. The second factor is the formation of Ag NPs aggregations on collagen molecule chains, as shown in Fig. 7B. Indeed, the electric enhancement might occur at the junction of two or more colloids [43].

3.3.3. Electro catalytic property of O$_2$ on the type I collagen-templated Pt NPs networks

Regarding the use of Pt as the most efficient catalyst for O$_2$ reduction, we investigated the electro catalytic activity of the collagen-templated Pt NPs networks modified electrode for dioxygen reduction in detail. Fig. 10A gives the representative cyclic voltammograms (CVs) of the bare ITO (curve a) and Pt NPs network modified ITO (curve b) in 0.1 M H$_2$SO$_4$ saturated by ultrapurity nitrogen at a scan rate of 50 mV s$^{-1}$ from −0.2 V to 1.5 V. It can be observed that the CV of Pt NPs networks modified ITO electrode (curve b) is similar to that of a polycrystalline Pt electrode, indicating that the hydrogen adsorption/desorption peaks and the formation and removal of platinum surface oxide peaks [44,45]. No current peak appears at bare ITO electrode in this region. Fig. 9b shows the CVs for O$_2$ reduction at these two electrodes in N$_2$-saturated (curves a and c), and in air-saturated (curves b and d). These electrodes only give small background current in N$_2$-saturated 0.1 M H$_2$SO$_4$ (curves a and c). Compared to the bare ITO (curve b), a remarkable cathodic peak appears at 0.33 V (curve d) of CV obtained at Pt NPs network modified ITO electrode, which is attributed to the catalytic reduction of O$_2$ [3,44,45]. The good electro catalytic ability can be ascribed to the high surface area of

![Fig. 8. SERS spectra of 10$^{-6}$ M 4-ATP on prepared collagen–Ag NPs networks (curve b) and on citrate-capped Ag NPs film (curve a).](image1)

![Fig. 9. SERS spectra of 10$^{-4}$ M adenine on collagen–Ag NPs networks (curve b) and on citrate-capped Ag NPs film (curve a).](image2)

![Fig. 10. Cyclic voltammograms of (a) the bare ITO (curve a) and Pt NPs network modified ITO (curve b) in N$_2$-saturated 0.1 M H$_2$SO$_4$, and (b) these two electrodes in N$_2$-saturated (curve a (red) and c), and in air-saturated (curve b (blue) and d) 0.1 M H$_2$SO$_4$. Scan rate: 50 mV s$^{-1}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](image3)
the collagen-templated Pt NPs networks. From Fig. 6A, it can be observed that a great deal of small Pt NPs assemble to networks, and these small NPs supply high surface area to volume and high surface activity, both of them are in favor of good catalytic activity.

4. Conclusion

In summary, we demonstrated an effective and environmentally friendly template method to prepare 2D network nanostructures including Au, Ag, Pt and Pd NPs. The major advantage of the synthesis route is that type I collagen, an ordinary and excellent biocompatible protein molecule, can work as stabilizing agent and assembled template. The hydrophobic action and hydrogen bonds play important roles in the formation of collagen–Au NPs networks. As the representative collagen-templated noble nanomaterials, the Ag NPs networks can be used as a SERS substrate with high sensitivity and the Pt NPs networks show good electrocatalytic ability for O₂ reduction. It is also expected that these collagen-templated 2D porous noble metal nanostructures can find wider applications in biosensors, SPR, and so on.

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References