Fabrication, characterization, and application in surface-enhanced Raman spectrum of assembled type-I collagen-silver nanoparticle multilayered films

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In this paper, we report a facile method for the fabrication of type-I collagen-silver nanoparticles (Ag NPs) multilayered films by utilizing type-I collagen as a medium. These samples were characterized by UV-vis spectra photometer, atomic force microscopy, scanning electron microscopy, and Fourier transform IR spectrum. Experimental results show that collagen molecules serve as effective templates to assemble Ag NPs into multilayer films. These samples exhibit high surface-enhanced Raman scattering (SERS) enhancement abilities. For example, EF(νc) (EF means enhancement factor) at 1592 cm⁻¹ in the SERS spectrum of 4-aminophenol on seven-layered substrates was calculated to be 1.81 × 10⁵, which is larger than that reported in several literatures. The EFs increased as the layer number of multilayer films increases. © 2008 American Institute of Physics. [DOI: 10.1063/1.2832322]

I. INTRODUCTION

Metal nanostructures are of great interest for their potential applications in optoelectronics, catalysis, biological and chemical sensing, and surface-enhanced Raman scattering (SERS).1–4 New building blocks as well as new fabrication technology play important roles in fabricating these nanostructures. Biomacromolecules, such as DNA (Refs. 5–8) and protein,9–12 can be utilized as templates to build novel hybrid nanostructures. A biomolecule-templated method is facile, controllable, and reproducible for designing different Ag, Au, and other metal nanostructures. A successful example have recently demonstrated by Ongaro et al.3 to fabricate polycrystalline conductive gold nanowires with a dimension 20 nm high and 40 nm wide by a DNA-templated strategy. More recently, Pieczonka et al.9 fabricated assembled glycoprotein avidin/Ag nanoparticles (NPs) multilayer film, and these avidin/Ag NP layer-by-layer (LbL) films can act as chemically selective SERS substrates to detect the small molecule biotin by a biospecific interaction.

Natural collagen molecules as the structure framework are rich in animal connective issues and widely used in biomedical and biomaterial fields. It is a polyelectrolyte, whose isoelectric point is 7.6, and these molecules are positively charged in acidic solution. By adjusting the concentration of the collagen, the pH value, as well as the temperature of the solution, collagen exhibits a special self-assembly character and can form films, networks, and fibrils with fine strength and stability by self-aggregation and cross-linking.13–15 In the acidic solution, these three-dimensional biological molecule films are positively charged, which can anchor negatively charged metal ions or metal NPs. Accordingly, type-I collagen can be regarded as a versatile biological template to fabricate nanomaterials with a large surface area, porosity, and a well interconnected pore network.

There are several literatures referring to the collagen-mediated metal (with the exception of Ag) nanoparticles assembly and synthesis,16–18 but few reports about the preparation of collagen-Ag NPs composites. Besides, Ag or Au NPs with the diameter less than 10 nm are not easy to obtain good SERS signals when they act as substrates.19,20 Herein, we assemble 4 nm Ag NPs layer by layer via positively charged type-I collagen. By controlling the collagen concentration and the number of the fabrication layers, different substrates were obtained. SERS spectra were used to investigate the SERS activity of these collagen-Ag NP multilayer films, and the results exhibit that the probe 4-aminophenol (4-ATP) shows good SERS enhancement on these substrates.

II. EXPERIMENTAL SECTION

A. Chemicals

Silver nitrate (AgNO₃, A.R.), trisodium citrate dihydrate (A.R.), acetic acid (CH₃COOH, A.R.), and ethanol (G.R.) were obtained from Beijing Chemical Co. (Beijing, China). Sodium borohydride (A.R.) was bought from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd (Shanghai, China). Poly(dimethyldiallylammonium chloride) (PDDA) with medium molecular weight (5000–20 000) was purchased from Aldrich Chemical Co., Inc. 4-ATP was purchased from Aldrich. Type-I collagen from calf skin (cell culture tested, acid soluble) was supplied by

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Sigma-Aldrich (USA). 5 mg type-I collagen was dissolved in 0.2M acetic acid and diluted to 100, 50, and 10 ng/μl. All of these chemicals and materials were used without further purification. The water used in the whole experiment was ultrapure water (18 MΩ cm) from the Mill-Q system.

B. Small Ag NP preparation

The citrate-protected Ag NPs were prepared according to the reported method.21 10 ml of 0.5 mM trisodium citrate dehydrate was added in 10 ml of 0.5 mM AgNO3 aqueous solution. Under vigorous stirring, 0.6 ml of 10 mM NaBH4 was added all at once, then stirring was continued for 30 s. A bright-yellow colloid was obtained.

C. Fabrication of multilayer Ag NPs via LbL assembly method

The quartz slides were cleaned in a boiling “piranha” solution (30% H2O2: concentrated H2SO4 3:7 in volume) for 10 min, then rinsed with treated water by the Millipore system. The multilayers of (collagen/AgNPs)s on quartz slides were fabricated in the following steps (Scheme 1). First of all, a precursor film of PDDA was deposited on the quartz slide by immersing the slide in the 1 mg/ml PDDA solution for 2 h, then the substrate was immersed into the prepared Ag colloids for 40 min. Second, 500 μl and 5 ng/μl (2 ng/μl to the comparison sample) type-I collagen solution was dropped onto the substrate and dried in the air. Third, the substrate was immersed into Ag colloids for 40 min (20 min to the comparison sample). Then, the second and third steps were repeated for wanted layers. The resulting substrates of quartz/PDDA/AgNPs (collagen/AgNPs)s were used to record the UV-visible spectra to follow the assemble process.

D. Preparation of SERS-active substrates

The indium tin oxide (ITO) slides (1×1 cm2) were coated with ITO/PDDA/AgNPs (collagen/AgNPs)s (n = 0, 3, 6) multilayer. Then, these slides were characterized by SEM. Finally, a 50 μl 4-ATP (1×10−6M) solution was dropped onto each sample, then dried in the air, for SERS measurement.

E. Instruments

UV-vis spectra were collected with a UV-2450 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Tapping mode atomic force microscopy (AFM) imaging was performed on a Digital Instruments Multimode AFM controlled by a Nanoscope IIIa apparatus (Digital Instruments, Santa Barbara, CA) equipped with an E scanner. A standard silicon cantilever tip from Digital Instruments was used, and the scan rate was 1–1.5 Hz. Fourier transform infrared (FTIR) spectra were obtained on a Bruker IFS66V spectrometer equipped with a deturinated triglycine sulphate detector, recording with a resolution of 4 cm−1. The scanning electron microscope (SEM) images were produced by an XL30ESEM field-emission gun field emission SEM (FEI Company with 20 kV operating voltage). The SERS spectra were collected by a Renishaw 2000 model confocal microscopy Raman spectrometer with an air-cooled charge coupled device and a holographic notch filter (Renishaw Ltd., Gloucestershire, U.K.). The excitation line was 514.5 nm produced by an Ar ion laser.

III. RESULTS AND DISCUSSIONS

A. Characterization of the Ag NPs

Figure 1(a) shows the UV-vis spectrum of Ag colloids, in which a strong surface plasmon band for small Ag NPs appears at 394 nm, characteristic of Ag NPs. Figure 1(b) gives the AFM image of well-dispersed Ag NPs. The average diameter of NPs can be measured according to the height measurement. Figure 1(c) displays the result of height statistics from 300 Ag NPs in AFM images, indicating that the mean diameter of the silver NPs was about 3.75 ± 0.25 nm, which is similar to the results in the previous report.21

B. Characterization of the assembled process of the collagen-Ag multilayered films

The layer-by-layer technique, pioneered by Decher,22 provides a facile and versatile approach to construct ordered and well-defined nanostructures. Generally, the polyelectrolytes (both positively and negatively charged)23,24 act as linkers to achieve the assembly. In this case, the type-I collagen-silver NP multilayer films were fabricated by the LbL technique, as depicted in Scheme 1. First, the quartz slide was modified with a layer of positively charged PDDA, resulting in the formation of a positive surface. Ag NPs capped by anionic citrate can be easily adsorbed onto this positive surface via electrostatic interaction. Second, the positively charged type-I collagen was dropped onto the surface. Alternately repeating the above procedure enables the fabrication of nanoparticle films with the desired layer.

The LbL assembly process was monitored by the UV-vis spectrum online. Figure 2 gives the UV-vis spectra of such quartz/PDDA/AgNPs (collagen/AgNPs)s multi-layer substrates with the increase of n. As can be observed, the first layer of Ag NPs shows a peak at 396 nm, which approaches the peak of Ag colloids shown in Fig. 1(a). It demonstrates that most part of Ag NPs in the first layer were monodispersed. After the second layer of Ag NPs was assembled on...
the substrate, the UV-vis spectrum became stronger and some redshift. Then, with the increase of $n$, the intensity kept on increasing gradually and the maximal absorbance shifted to 416 nm until seven layers, which resulted from more NPs adsorbed onto the substrates, and more aggregations appeared. This trend further reveals that type-I collagen can act as an effective template to adsorb and assemble negatively charged Ag NPs.

C. The effect of collagen concentration

To investigate the effect of the concentration of the collagen on the quality of the multilayer films, the substrates of ITO/PDDA/AgNPs/collagen/AgNPs with different concentrations of collagen were prepared. Figure 3 shows the SEM images of these substrates: (a) 2 ng/µl and (b) 5 ng/µl.
type-I collagen. From these images, it can be seen that Ag NPs anchored on a collagen scaffold increase with the increase of the collagen concentration, and the Ag NPs tend to grow up simultaneously.

The IR spectrum has evolved as a unique tool for investigating the structure of proteins.\textsuperscript{24} To classify the interaction between template-type-I collagen and Ag NPs, FTIR was used to characterize these films. Figure 4 shows the FTIR spectra of (a) the pure type-I collagen assembled and (b) the Ag NPs assembled on the collagen film, both on CaF\textsubscript{2} slides. The prominent amide I band at 1650 cm\textsuperscript{-1} and the amide II band at 1538 cm\textsuperscript{-1}\textsuperscript{2} feature.\textsuperscript{29,30} 

FIG. 4. FTIR spectra of (a) the type-I collagen assembled on a CaF\textsubscript{2} slide and (b) Ag NPs adsorbed on the above sample.

The intensity of the SERS spectra from the films as-sembled on collagen films. The aggregations with 5 ng/\mu l collagen is twofold shift from 1592 to 1578 cm\textsuperscript{-1} occurred. These changes imply that the –SH bond in 4-ATP directly contacts with the Ag film surface by forming a strong Ag–S bond.\textsuperscript{29,30} It is generally observed that the SERS spectrum collected with 514.5 nm radiation was dominated by both \( b_2 \) modes at 1578, 1440, 1391, and 1142 and an \( a_1 \) mode at 1077 cm\textsuperscript{-1}.\textsuperscript{29,30} The intensity of the \( b_2 \) mode increases, which is related to the charge transfer between adsorbate molecule and metal. According to previous reports,\textsuperscript{29,31} the great enhancement of the \( b_2 \) mode in the visible originated from the charge transfer. However, the contribution of the electromagnetic (EM) effect should also be considered in this case because the \( a_1 \) (1077 cm\textsuperscript{-1}) mode obviously increased. We think that the electromagnetic effect may result from the localized surface plasmon resonance of the Ag NP aggregations assembled on collagen films. The aggregations with multiple particles can yield an enormous EM field at junction sites.\textsuperscript{32,33} The detailed assignment of the spectra of 4-ATP was given in Table I.

FIG. 5. The SERS spectra of 10\textsuperscript{-6}M 4-ATP obtained from the substrates shown in Figs. 3(a) and 3(b). Laser power: 25 mW, 25%; integration time: 10 s.

The intensity of the SERS spectra from the films assembled in the presence of 5 ng/\mu l collagen is twofold.

<table>
<thead>
<tr>
<th>Solid 4-ATP\textsuperscript{a} (cm\textsuperscript{-1})</th>
<th>4-ATP on the collagen-Ag NP multilayered substrate\textsuperscript{b} (cm\textsuperscript{-1})</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1592</td>
<td>( v_{CC}, 8a(a_1) )</td>
<td>( v_{CC}, 8a(b_2) )</td>
</tr>
<tr>
<td>1425</td>
<td>( v_{CC}, 1437 )</td>
<td>( v_{CC} + \delta_{CH}, 19b(b_2) )</td>
</tr>
<tr>
<td>1369</td>
<td>1390</td>
<td>( \delta_{CH} + \delta_{CC}, 3b(b_2) )</td>
</tr>
<tr>
<td>1179</td>
<td>1188</td>
<td>( \delta_{CH}, 9a(a_1) )</td>
</tr>
<tr>
<td>1126</td>
<td>1142</td>
<td>( \delta_{CH}, 9b(b_2) )</td>
</tr>
<tr>
<td>1084</td>
<td>1077</td>
<td>( v_{CS}, 7a(a_1) )</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Assignments for the Raman spectrum of solid 4-ATP from Refs. 20 and 28. 
\textsuperscript{b}Assignments for the SERS spectrum of 4-ATP on the collagen-Ag multilayer substrate from Refs. 20, 21, and 28.
higher than that from the 2 ng/µl collagen [in Figs. 5(a) and 5(b)]. These differences may be ascribed to positively charged NH$_3^+$ on collagen chains. On the one hand, more NPs can be adsorbed on the surface due to more positive charges brought by the 5 ng/µl collagen. On the other hand, the 2 ng/µl collagen molecule can form a thin film, while the 5 ng/µl collagen can supply a large surface area, porosity, and well-interconnected pore network to accommodate NPs; obviously, the latter is facile to build a better Ag film for the SERS test.13–15

**D. The effect of the layer number of Ag NPs**

Figure 6 shows the morphologies of quartz/PDDA/AgNPs/(collagen/AgNPs)$_n$, where (a) $n=0$, (b) $n=3$, and (c) $n=6$.

![Figure 6](image_url)

**FIG. 6.** The typical SEM images of substrates ITO/PDDA/AgNPs/(collagen/AgNPs)$_n$, where (a) $n=0$, (b) $n=3$, and (c) $n=6$.

AgNPs/(collagen/AgNPs)$_n$ ($n=0,3,6$) multilayer films. Obviously, the Ag NPs distribute fairly uniformly on the surface, and a few aggregates can be observed in Fig. 6(a). Consequently, the NP aggregations grow up uniformly with the increase of the number of NP layers [Figs. 6(b) and 6(c)]. One important reason is that the positive charges on collagen molecules spread uniformly on the surface; another reason is that the three-dimensional collagenlike scaffolds provide the space for the growth of the aggregations.16–18

Figures 7(a)–7(c) display the SERS spectra of 4-ATP obtained from samples given in Figs. 6(a)–6(c), and Figs. 7(d)–7(f) give the normal spectrum of solid 4-ATP, the Raman spectrum of 4-ATP adsorbed on collagen, and the solid type-I collagen. From comparison, the background of the collagen can be ignored. The collagen-Ag NP composites exhibit better SERS ability with the assembly of these nanostructures [Figs. 7(a)–7(c)].

To measure the enhancement effect of 4-ATP on the as-prepared collagen-Ag nanostructures quantitatively, the enhancement factors (EFs) of 4-ATP were calculated according to the following equation:34

$$EF = I_{SERS}N_{bulk}/I_{Raman}N_{surface}$$

where $I_{SERS}$ stands for the intensities of a vibration mode in the SERS spectrum and $I_{Raman}$ stands for the intensity of the same vibrational mode in the normal Raman spectrum of the target molecule. Both of these data can be obtained in the experimental data. $N_{bulk}$ is the density number of molecules in the bulk sample, solution or solid, and $N_{surface}$ is the density number of adsorbed molecules in the laser spot. Suppose the probe solution is dispersed on the film uniformly and then the density of the molecules on the film is assumed to be $10^{-6}M \times 50 \mu l \times N_A/cm^2$ (the surface area of the substrate is $1 \times 1 \text{ cm}^2$), namely, $3.01 \times 10^{13}/\text{cm}^2$.35 The laser spot has a 1 µm diameter, and it surface area is about $7.85 \times 10^{-15} \text{ cm}^2$; the number of adsorbed molecules on the substrate within the laser spot is $2.36 \times 10^5$. Taking the laser spot...
(1 μm in diameter), the penetration depth (about 2 μm), and the density of 4-ATP (1.17 g/ml) into account, N_{bulk} had a value of 8.9 × 10^{9} in the detected solid sample area.

The intensity of the vibrational mode (ν_{C}) at 1592 cm^{-1} was used to calculate the EF values. The EF was calculated to be 1.37 × 10^{4} from one layer of Ag NPs (n=0); with the increase of the Ag NP layer, the EF value increased to 3.35 × 10^{4} (n=3) and 1.81 × 10^{5} (n=6). The EF values from as-prepared collagen-Ag NP composite substrates were quite comparable to other Ag nanostructure substrates reported.38

It should be noted that the SERS-active substrates can greatly enhance the Raman signals of 4-ATP, but there are no Raman signals from the template type-I collagen or from the capping reagent citrate. This might be a result of the experimental conditions in our work, such as the concentration of collagen or citrate used, the wavelength and power of the laser, the integration time, and so on.7

IV. CONCLUSION

The type-I collagen-Ag NP multilayer films were fabricated faciely, which lie on two factors: (1) the excellent assembly properties of type-I collagen and (2) the attachment of the Ag NPs and collagen molecules via electrostatic interaction and weak covalent interaction. It is proven that this protein-Ag NP films can enhance Raman signals intensely. Furthermore, this type of new Ag-protein bioconjugates may have interesting physicochemical and optoelectronic properties, and may also have intriguing biological and medicinal properties because of the presence of protein.

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