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Development of chitosan-coated gold nanoflowers as SERS-active probes

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Abstract

Surface-enhanced Raman scattering (SERS) has been intensely researched for many years as a potential technique for highly sensitive detection. This work, through the reduction of HAuCl$_4$ with pyrrole in aqueous solutions, investigated a facile one-pot synthesis of flower-like Au nanoparticles with rough surfaces. The formation process of the Au nanoflowers (AuNFs) was carefully studied, and a spontaneous assembly mechanism was proposed based on the time-course experimental results. The key synthesis strategy was to use pyrrole as a weak particle stabilizing and reducing agent to confine crystal growth in the limited ligand protection region. The nanometer-scale surface roughness of AuNFs provided several hot spots on a single particle, which significantly increased SERS enhancement. Good biocompatible stable Raman-active probes were synthesized by coating AuNFs with chitosan. The conservation of the SERS effects in living cells suggested that the chitosan-capped AuNFs could be suitable for highly sensitive detection and have potential for targeting of tumors in vivo.

Online supplementary data available from stacks.iop.org/Nano/21/375101/mmedia

(Some figures in this article are in colour only in the electronic version)

1. Introduction

In recent years, surface-enhanced Raman scattering (SERS) has been intensively researched as a new optical spectroscopic analysis technique with potential for highly sensitive detection of molecules [1–4]. There are several characteristics of SERS that suggest it may be of value as a readout method [5, 6]. First, Raman bands are generally 10–100 times narrower than most fluorescence bands, minimizing the possible overlap of different labels in a given spectral region. Second, the optimum excitation wavelength for SERS is not strongly dependent on the adsorbed molecule, allowing the use of a single excitation source for multiple species. Third, Raman scattering is not sensitive to humidity or affected by oxygen and other quenchers, facilitating applications in a variety of environments. These advantages render great promise for SERS-active probes to be widely applied in biomedical systems [7–10].

Based on the local electromagnetic field, experimental measurements and theoretical calculations have shown strong SERS enhancement of rough surfaces and periodic nanostructures [11–14]. Recently, periodic nanostructures have been fabricated by nanometer-scale lithography techniques, and a moderate enhancement ($10^5$–$10^6$) has been achieved [3]. But current techniques still have difficulty in setting controllable small gaps of a few nanometers between metal nanostructures. Currently, branched and flower-shaped nanometals with rough surfaces are capturing researchers’ interest [15–23]. There are three principal strategies for generating these complex nanostructures. Template-based synthesis is the first approach [17, 18]. The second approach is based on the phenomenon of oriented attachment of primary nanoparticles (NPs) when surfaces have similar atomic arrangements to each
other [19]. The third approach relies on the use of specific capping agents to induce anisotropic growth [20].

Peng et al reported the simple and convenient synthesis of nanoflowers of a few metal oxides by coupling 3D oriented attachment with anisotropic growth with limited ligand protection (LLP) [24, 25]. Compared to the sufficient ligand protection (SLP) reaction region, the LLP domain did not provide enough ligand protection for the crystal growth, which could induce crystal aggregation or oriented attachment. Generally, the nanometer-scale SERS-active probes consist of a layer of Raman reporter molecules coated on the surface of metal NPs, which act as the source of enhancement [6, 26]. The SERS-active metallic (Au) probes are usually synthesized in a two-step method [6, 23, 26–29]. Au nanoparticles (AuNPs) are first prepared, and then the as-synthesized AuNPs are coated by Raman reporter molecules. Pyrrole (Py) is a weak stabilizing agent, which could be used as LLP. At the same time, polypyrrole (PPy) has a strong Raman signal [30, 31]. In this work, a facile one-pot method, based on crystal growth in the LLP region, was used to produce Au nanoflowers (AuNFs) with intense SERS signals in high yield using HAuCl₄ and Py. The as-synthesized AuNFs were well dispersed as individual entities and appeared to be highly uniform in morphology with sizes in the range of 60–105 nm. The AuNFs displayed well-oriented 3D flower-like NPs consisting of a solid core with many (>10) short, irregular, and obtuse Au nanocrystals. The nanometer-scale surface roughness of AuNFs provided several hot spots on a single particle, which significantly increased SERS enhancement.

In addition, Raman reporters on metal NPs could easily be influenced by variations in the chemical or biological environment. A variety of encapsulation methods were, therefore, developed to enhance the stability of the Raman probes by coating the NPs with biomolecules (such as bovine serum albumin), polymers, or inorganic layers (such as SiO₂) [6, 23, 27–29, 32, 33]. Chitosan (CS) is the most abundant natural polysaccharide derived from chitin, which exhibits numerous interesting physicochemical and biological properties [34, 35]. Due to its biocompatibility, biodegradability, bioactivity and complexation with metal ions, it is considered more and more as a biomaterial suited for diverse applications [36–39]. Furthermore, CS has a specific ability to cross the cell membrane [40, 41]. In order to improve the Raman probes’ biocompatibility, stability and ability to enter into cells, the AuNFs were coated with CS. The application of these biocompatible Raman-active probes in living cells was then demonstrated by using the RAW macrophage cell line. The conservation of the SERS effects in living cells suggested that the CS-capped AuNFs could be suitable for the specific detection and have potential for targeting of tumors in vivo.

2. Experimental section

2.1. Materials

Chitosan (CS) with a 90% degree of deacetylation and average molecular weight of 3600 was purchased from Yuhuan Ocean Biochemical Co. (Taizhou, China). Sodium 1-dodecanesulfonate (SDS, 99%), pyrrole (Py) and hydrogen tetrachloroaurate (III) hydrate (HAuCl₄) were all purchased from Alfa Aesar (USA). Ethanol, glutaraldehyde (GA), sodium chloride (NaCl) and phosphate buffered saline (PBS) were obtained from Shanghai Chemical Co. (China). All chemicals were used as received. All the solutions were prepared with deionized water (18 MΩ cm) obtained from the Millipore system.

2.2. Synthesis of Au nanoflowers (AuNFs)

For the synthesis of NPs, glassware was well cleaned with freshly prepared aqua regia, then rinsed thoroughly with water and dried prior to use. In a typical synthesis, 10.0 μl of distilled Py monomer was mixed with 5 ml of SDS aqueous solution (8.27 mM, 1.0 CMC) and stirred for 2 h to obtain a uniform emulsion. Two milliliters of HAuCl₄ aqueous solution (14.8 mM) was added to the emulsion. The mixture was then allowed to react at 0–3 °C (in an ice bath) for 30 min while stirred. The product was washed with deionized water and ethanol several times to remove residual Py, and then dried under a vacuum for 24 h. A dark powder of AuNFs was obtained. The synthetic route of AuNFs was illustrated as follows:

\[
3n \text{N} + \frac{7}{n} \text{HAuCl}_4 \rightarrow \left(\begin{array}{c}
\text{N} \\
\text{H}
\end{array}\right)_{n} + \frac{7}{n} \text{Au} + 6n \text{HCl} + \frac{10}{n} \text{Cl}^{-}.
\]

2.3. Preparation of CS-coated Raman probes (CS-coated AuNFs)

96.4 mg of as-synthesized AuNFs was re-dispersed in 10 ml of deionized water with a sonicator to produce a stable suspension of AuNFs. The AuNFs solution was added to 10 ml of CS (4 g l⁻¹) aqueous solution and stirred for 2 h. Then, 0.1 ml of GA (30 wt%) aqueous solution was added to the above solution. The reaction was allowed to process at room temperature for 12 h. Residual CS and GA were removed by dialysis. CS-coated Raman probes were obtained.

2.4. Cell culture and morphology observation

50 μl of as-prepared CS-coated Raman probes was added to 1 ml of RAW264.7 cell suspension at a given density. The RAW cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C. For detection of CS-coated AuNFs, the fixed RAW cells were washed three times with PBS, and then viewed with an inverted fluorescence microscope (Nikon TE2000-U fluorescence photomicroscope) equipped with a B-1E filter set (λ = 470–490 nm) and a G-2B filter set (λ = 510–560 nm).

2.5. Characterization

Transmission electron microscopy (TEM) was conducted on a JEM-1005 instrument (JEOL Co.) at 80 kV. One drop of
the colloidal emulsion was placed on the sample grid, and the solvent was allowed to evaporate.

Scanning electron microscopy (SEM) was performed using a Hitachi S-4800 instrument. Samples were prepared by spin coating the emulsion directly onto the silicon slice. An accelerating voltage of 10 kV and Au coating of the sample was used to image these particles.

The mean particle sizes of the samples were determined by a BI-9000AT dynamic light scattering instrument (DLS). All measurements were repeated three times for each sample at room temperature.

The x-ray diffraction (XRD) pattern of AuNFs was recorded with a Shimadzu 600 (Japan) instrument using Cu Kα.

The ultraviolet–visible (UV–vis) spectra were recorded with a UV1800PC UV–vis spectrophotometer operating at 1 nm resolution.

Raman spectra were collected from the sample solution in a cuvette (pathlength = 1.00 cm) on a dispersive Raman spectroscopy system (Bruker Multi-RAM spectrometer) using an Nd light-emitting diode laser (λ = 1064 nm) at 3 mW.

3. Results and discussion

3.1. Characterization of AuNFs

The morphology and structure of the obtained AuNPs were characterized by SEM and TEM. The SEM images (figures 1(A) and (B)) obviously showed that the AuNPs were spherical. The low-magnification image also confirmed the production of large scale and narrow size distribution. The high-magnification image indicated that each Au nanoparticle was built up by tens of primary NPs. The low-magnification TEM image (figure 1(C)) clearly indicated that the as-synthesized AuNPs were well dispersed as individual entities and appeared to be flower-like, and highly uniform in morphology with sizes ranging from 60 to 105 nm (DLS data are shown in figure S1 available at stacks.iop.org/Nano/21/375101/mmedia). The high-magnification image (figure 1(D)) displayed well-oriented 3D flower-like NPs consisting of a solid core with many (>10) short, irregular, and obtuse Au nanocrystals. Figure 1(E) showed the nanocrystals as single-crystalline protrusions from the core of the nanoflower. Selected area electron diffraction (SAED, the inset in figure 1(E)) showed that the nanoflowers were crystalline and randomly oriented. In addition, the electron diffraction pattern, recorded from the end of the Au nanocrystals and indexed by the face-centered cubic (fcc) structure of Au, indicated that the nanocrystals grew along the ⟨111⟩ direction (XRD data are shown in figure S2 available at stacks.iop.org/Nano/21/375101/mmedia) [23, 42]. The lattice spacing between the ⟨111⟩ planes, 0.23 nm, was in agreement with that of the bulk crystal. It is worth noting that the AuNFs were not the simple accumulation of the small nanounit since the morphology of flower-like Au nanoparticles could not be destroyed by sonication for 30 min [42].

3.2. Influence of HAuCl₄/Py molar ratio on morphology

To investigate the influence of the reaction parameters on the size and shape of the resulting Au nanostructures, a series of control reactions were performed. Py was mainly considered as a ligand protection with a major role in protecting the product from agglomeration. The molar ratio of HAuCl₄ to Py (HAuCl₄/Py molar ratio) had a great effect on the morphology of the obtained Au nanostructures. Typical SEM images for Au–PPy NFs at HAuCl₄/Py molar ratios of 1/10, 1/5, 1/3, and 1/1 in the reaction solution are shown in figures 2(A)–(D), respectively. The diameter of the composite nanospheres increased from 70 to 500 nm with the increase in HAuCl₄/Py molar ratio, consistent with the expected increased conversion of HAuCl₄ into Au nanoparticles with a higher HAuCl₄ concentration. At the same time, more Py was oxidized into PPy with a higher oxidant concentration. When
the HAuCl₄/Py molar ratio was 1/1, there were separate PPy nanoparticles, which did not complex with nanogold. The inset TEM image (figure 2(D)) displays many separate PPy NPs with the dimension of around 30 nm.

3.3. Development of flower-like nanoparticles

In general, the formation of flower-like nanostructure is a complex process, which is affected by growth environment, capping agent, surface energy, and so on. In this reaction, Py not only acted as a reducing agent, but also a weak ligand protection. Herein, we present a Py reduction method to produce AuNPs with flower-like structures in high yield. By confining the growth of AuNPs in the LLP domain, the primary NPs could agglomerate to form intermediate particles which could then grow anisotropically into crystalline Au nanoflowers with ten or more tips per particle. The progress of the reaction and the evolution of AuNPs was followed by time-course measurements of TEM images (shown in figure 3). It displayed two steps in the formation of the AuNPs: (i) the reduction of Au(III) ions and the formation of Au–PPy nanocomposites; (ii) the subsequent self-assembly of nanocomposites into flower-like NPs. When the Py monomer was immersed into the HAuCl₄ aqueous solution, Au–PPy nanocomposites were obtained through the redox reaction of HAuCl₄ and Py monomer in the presence of SDS, which prevented the NPs aggregation when Py was oxidized and underwent seeded polymerization [31]. Then, in the process of Au nuclei growth, besides as a reductant, the oxidized PPy may also play a capping agent role to lower the surface energy of AuNPs though the interaction with the gold surface [43–45]. During the growth of AuNPs, the formation of flower-like aggregates should be thermodynamically favorable because of limited ligand protection. As a result, to reduce the total energy of the whole particle system, AuNPs with exposed facets were formed from the small AuNPs by anisotropic growth [24].

Figure 2. Representative SEM images of AuNFs with different HAuCl₄/Py molar ratio: (A) 1/10, (B) 1/5, (C) 1/3, (D) 1/1. The inset in (D) is the TEM image.

3.4. Raman properties of AuNFs

Raman spectroscopy is a highly specific technique that detects and identifies molecules based on their vibrational energy levels and corresponding Raman fingerprints [2, 23]. However, spontaneous Raman scattering is very weak. Colloidal Au nanoparticles have been used to increase the scattering efficiencies of Raman-active molecules [2]. Since AuNPs are particularly suitable for biological applications because of their good biocompatibility and low cytotoxicity, they may be used in the Raman spectroscopy of living cells for targeting and assaying species of interest.

Previous studies have shown that Au nanospheres (AuNSs) with sizes around 20–100 nm have the highest efficiency for SERS using red (633 nm) or near-infrared (785 nm) excitation [6, 46]. Because PPy has the intense Raman signal, PPy absorbed on the surface of AuNPs could act as a SERS-active molecule [30, 31]. Figure 4 exhibits the intense SERS signal of PPy on the surface of AuNFs (using approximately 80 m AuNFs as an example) in aqueous solution. The concentration of PPy on AuNFs was about $5.0 \times 10^{-5}$ mg ml⁻¹. Compared to AuNSs with smooth surfaces, the as-synthesized AuNFs with sizes around 40–100 nm may be a better candidate for fabricating SERS-active probes for a number of reasons (shown in figure 4): (i) tips of the nanoscale bumps or the tiny cavities on the AuNF surface are potential ‘hot’ spots for localized near-field enhancement effects [47, 48]; (ii) there is a larger total surface area because of the roughness of the AuNF surface; (iii) Py is a conductive polymer, so it could favor the electron transfer between Py and AuNFs.

It is well known that PPy has Raman vibrations around 1520–1620 cm⁻¹ (C=C stretching vibration), 1050–1080 cm⁻¹ (C–H deformation in plane), and 1320–1380 cm⁻¹ (Py ring stretching vibration) in the 600–2000 cm⁻¹ region [30]. In
order to better evaluate the SERS intensities observed in the system, the signals were normalized against the total surface area of the AuNPs, which could be estimated based on the total particle concentrations [6]. Using the Raman signals of pure PPy as a reference, the enhancement factor for AuNPs was estimated to be more than nine orders of magnitude of increase.

3.5. Characterization of CS-coated Raman probes (CS-coated AuNPs)

Recently, mainly three kinds of materials have been used in optical cell imaging: quantum dots (QDs), single-walled carbon nanotubes (SWNT) and near-infrared probes (e.g. SERS probes) [49–51]. Among them, nanogold is particularly suitable for biological applications because of its good biocompatibility and lower cytotoxicity, and it could be used in the Raman spectroscopy of living cells for targeting and assaying species [23]. PPy on AuNPs could easily be influenced by variations in the chemical or biological environment. CS is the most abundant naturally occurring polysaccharide and widely used for biotechnology applications. The as-synthesized AuNPs could be turned into stable compatible SERS-active probes by coating with CS (shown in figure 5). The average overall dimension of CS-coated AuNPs was 104.5 ± 8.9 nm (shown in figure S3 available at stacks.iop.org/Nano/21/375101/mmedia). DLS data indicated that most CS-coated AuNPs were still separate structures in suspension. Compared to the original AuNPs, the Raman probes protected by CS had a much better stability in solutions over a broad pH range (4–12) and in highly concentrated salt solutions (e.g., 1 M NaCl). As depicted in figure 6, UV–vis absorption spectra of the coated particles...
displayed few changes upon the addition of 1 M NaCl, whereas the uncoated colloids were completely aggregated and precipitated.

3.6. In vitro cell detection

The stability of CS-capped probes under various environmental conditions should improve their usability in both in vitro and in vivo applications. Moreover, the presence of an outer layer of CS molecules also offers a convenient platform for further bioconjugation reactions with suitable functional ligands.

The behavior of the CS-capped probes in living cells was investigated as a proof of concept for the potential application of these probes in in vivo applications. The delivery of NPs to the cellular interior, as well as the targeting of cellular compartments, can be achieved in various ways, depending on the cell type and the physiochemical properties of the particles [2, 23]. For example, macrophage cells can easily internalize structures smaller than 1 μm in size, with the highest efficiency shown for particles several tens of nanometers in size [52]. A common macrophage cell line, RAW264.7 (figures 7(A) and (B)), was used to explore the SERS activity of the as-prepared probes in living cells. The probes were delivered into cells grown on coverslips after 24 h of incubation, as shown in figures 7(C)–(F). At positions in the cells, the CS-capped probes displayed red fluorescence and green fluorescence with different laser excitation (figures 7(E) and (F)), and the images of cells without probes showed black. It was believed that the as-synthesized CS-capped probes entered into RAW cells. Compared with the microscope image of cells without probes (figure 7(A)), the morphology and amount of cells with probes (figure 7(B)) rarely changed. It indicated that the CS-capped probes had great biocompatibility and low cytotoxicity. At positions in the cells where the CS-capped probes were present, an intense SERS signal could be detected (dotted line, figure 8). Macrophage cells without internalization of probes showed a complete absence of the SERS signal (solid line, figure 8). The conservation of the SERS effects in living cells suggested that the CS-capped AuNFs could be suitable for in vitro detection and have potential for in vivo applications.

4. Conclusion

A simple one-pot synthesis, based on crystal growth in the LLP region, was used to produce AuNPs with flower-like
structures in high yield by HAuCl₄ and Py. The morphology and size of the Au nanoflowers were tunable by controlling the HAuCl₄/Py molar ratio. The as-synthesized AuNFs exhibited strong surface-enhanced Raman scattering effects. The AuNFs could also be developed into biocompatible Raman-active probes by packaging AuNFs with CS. The application of these Raman-active probes in living cells was then demonstrated by using the RAW264.7 macrophage cell line. The results suggested that the CS-capped AuNFs could be suitable for highly sensitive detection and have potential for targeting of tumors in vivo.

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