Functionalized Au/Ag nanocages as a novel fluorescence and SERS dual probe for sensing


Abstract

We obtained chitosan-protected Au/Ag nanocages (NCs), i.e., hollow and porous metallic nanoparticles, by galvanic replacement reaction. Subsequently, we functionalized the NCs with a fluorescent derivative of 4-methoxy-1,8-naphthalimide (NAFTA6). The plasmonic properties of these structures, which exhibit an extinction maximum in the 700–800 nm range, allowed their use as SERS active substrates for excitation at 785 nm and an efficient identification of the vibrational bands of NAFTA6, in spite of the low ligand concentration (<10⁻⁵ M). Furthermore, NAFTA6 could also be identified from its fluorescence emission. The proposed functionalization with fluorescent compounds opens the way to the application of metal NCs using double-wavelength detection. Namely, Raman spectroscopy in the near infrared and fluorescence emission in the visible region, with considerable potential especially for in vivo medical applications, as the plasmonic band is centered in the visible light region where biological fluids and tissues are transparent.

1. Introduction

The increasing scientific effort to design and exploit novel materials down to the nanoscale has generated several types of metal/dielectric nanostructures. In particular, noble metal nanoparticles (NPs) represent an important extensively studied class of such materials, due to their possible applications in several fields, including medical diagnostics, sensing, drug delivery, and development of smart materials [1–5]. An interesting example is the case of Au/Ag nanocages (NCs), nanosized metal clusters with hollow interiors and ultrathin porous walls, which thus exhibit a low density and a high surface-to-volume ratio [6].

Gold/silver NCs can be prepared by a galvanic replacement reaction [7,8], where preformed Ag nanostructures serve as sacrificial templates, as they are oxidized in an aqueous HAuCl₄-containing environment. The electrochemical potential difference between the two metals drives the reaction, with one metal (Au) serving as the cathode and the other one (Ag) as the anode. The reduced Au grows epitaxially on the surface of the Ag nanostructure as long as Ag is oxidized and dissolves as Ag⁺. The process leads to hollow Au/Ag alloyed structures, with porous walls and smooth surfaces, the geometry of which (size, aspect ratio, and thickness of the shell) can be tuned by controlling the reaction parameters. Such geometrical parameters, as well as the chemical composition (the Au/Ag ratio), regulate the optical properties of the NCs, hence their extinction spectrum, which is characterized by a well resolved surface plasmon resonance (LSPR) [6,9]. In particular, the extinction of Au/Ag NCs can be tuned in the 650–900 nm spectral region, where the absorption by hemoglobin and, in general, biological tissues is negligible. Moreover, proper choice of the geometrical parameters also permits adjustment of the absorption to scattering ratio [10–12] and, hence, the ability to generate local heating of living tissues in applications of photothermal tumor therapy [13,14], or the surface enhanced Raman (SERS) activity in applications of Raman based sensing [15,16]. These properties and the availability of preparation procedures which allow the synthesis of novel composite structures [17] open the way to the development of innovative diagnostic and therapeutic methods. This has been elegantly demonstrated by recent work of Xia’s group that has exploited the NIR absorption properties of functionalized Au NCs to develop a sensitive contrast agent for in vivo photoacoustic tomography (PAT) and subsequent thermal treatment for melanoma [18]. In another application, Xia showed how the LSPR properties of Au NCs and surface functionalization with a fluorescent molecule could be exploited in a double-wavelength detection device for early diagnosis of cancer. In his experiment,
NIR absorption was used to localize the NCs in the biological tissues, while fluorescence recovery of the luminescent moiety, due to enzymatic cleavage in the presence of a specific biomarker associated with cancer cells, was used to detect tumor activity [19].

Besides, a number of reports have demonstrated the effective application of different types of structures as dual fluorescence and SERS probes, such as silica coated Au/Ag core shell nanorods [20], silica-encapsulated gold nanoparticles [21], and Ag coated dye embedded silica beads [22], all good candidates for disease detection and treatment. Despite the interesting results of these studies, the debate on nanosystem toxicity is still open, and only Au-based nanostructures have been approved by the FDA as drug carriers or therapeutic agents [18]. In this respect, Au nanocages represent, as mentioned above, an excellent candidate for serving in the therapeutic field due to their strong and tunable light absorption in the NIR region, ease of bioconjugation with specific ligands and noninvasiveness [18]. In addition to these properties, Au/Ag NCs exhibit a bright three-photon luminescence, already exploited in bioimaging [23]. Hence, also in view of future applications in the biomedical field, Au/Ag NCs have been here selected to test their capability of acting as multifunctional material.

In this paper, we present a model system consisting of fluorescence-functionalized Au/Ag NCs to illustrate its potential as a dual probe for the spectroscopic detection of analytes by means of two different spectroscopic investigations, namely fluorescence and Raman spectroscopy at different operating wavelengths. The Au/Ag NCs were synthesized using chitosan as a protecting agent. Chitosan is a basic polymer deriving from the partial deacetylation of chitin, the most abundant natural polysaccharide after cellulose. Its chemistry is largely determined by the amino and hydroxyl groups that act as potential sites for chelation of heavy metal ions. That being so, the polycationic nature of chitosan enables attachment of the polymer to the negatively charged Ag and Au nanoparticle surfaces through electrostatic interactions, facilitating its use as a stabilizer of such nanocomposites [24–26]. In addition, chitosan is a biocompatible, biodegradable, and non-toxic material [27–30], and it has been successfully used in the delivery of therapeutic drugs, proteins, and genes by intravenous, oral, and mucosal administration [31–33], which is expected to improve the biocompatibility of our nanohybrids.

To test the viability of our Au/Ag NCs as a dual probe, the nanostructures were conjugated with a derivative of 4-methoxy-1,8-naphtalimide (hereafter named NAFTA6) [34], which was selected for its high fluorescence cross-section. A spectroscopic characterization of our model system was then performed which showed that, beyond the well-known in vivo imaging properties of non-functionalized nanocages, it can behave as a double-channel diagnostic tool, based on the visible fluorescence emission of the organic moiety and the near infrared SERS activity of the metallic nanostructured substrate.

3. Results and discussion

3.1. Synthesis of Au/Ag nanocages

Chitosan-protected NCs (chit-NCs) were obtained in water by using the galvanic replacement-based procedure (Ref. [17]). Briefly, chitosan-capped Ag nanoparticles (chit-AgNPs) were synthesized through a classic reduction method, based on the reduction of AgNO3 by NaBH4 in the presence of chitosan, acting as both stabilizing and protecting agent. In particular, 8.6 ml of a 9.3 mM 1% acetic acid solution of chitosan were added to an aqueous solution of AgNO3 (10.2 ml, 6.2 mM). Then, an aqueous solution of NaBH4 (1.1 ml, 56 mM) was added drop by drop. The reaction was performed at ambient temperature under vigorous magnetic stirring. Under these experimental conditions, we obtained stable NPs exhibiting irregular shapes and a size distribution centered at 26 nm with σ = 24 nm and σ = 13 nm. Fig. 1 shows a typical TEM image and the statistical distribution of the particle size.

As for the synthesis of NCs, 9 ml of the as-prepared chit-AgNPs seeds was diluted with 75 ml of H2O and heated at 80 °C for 5 under vigorous stirring. To initiate the galvanic replacement reaction and subsequent formation of NCs (Scheme 1), 0.24 ml of 24 mM HAuCl4 was added to the solution. Stirring was maintained for 5, while the mixture was allowed to cool.

TEM inspection of the samples confirmed the formation of hollow structures with thin and porous walls. Several batches have been fabricated which resulted to have analogous characteristics. A typical TEM micrograph is reported in Fig. 2, with the corresponding statistical distribution of the particle size. Chit-NCs were purchased from Merck; NaBH4 = 24 nm and 0 (10.2 ml, 6.2 mM). Then, an aqueous solution of NaBH4 was added to the solution. Stirring was maintained for 5 and 0/C0 was added to the solution. Stirring was maintained for 5 and 0/C0 were evaluated by fitting the measured particle diameter histograms with Log-Normal functions.

Fluorescence emission was collected by using a Perkin–Elmer LS55 spectrophotofluorimeter equipped with a high-energy Pulsed Xenon source. To facilitate quantification, all spectra were recorded under the same experimental conditions (excitation wavelength, slit apertures, emission filter, scanning speed, and quartz cuvette).

Extinction spectra were measured at room temperature with a Cary 5 spectrometer using 1 cm pathlength quartz cuvettes.

Raman and SERS spectra were measured using a Renishaw RM2000 microRaman apparatus, equipped with two excitation sources, an argon-ion laser emitting at 514.5 nm, and a diode laser emitting at 785 nm. Sample irradiation was accomplished by using the 50 × microscope objective of a Leica Microscope DMLM. The backscattered Raman signal was filtered by a double holographic Notch filter system and detected by an air-cooled CCD (2.5 cm−1 per pixel). All spectra were calibrated with respect to a silicon wafer at 520 cm−1. The spectra of colloids were performed on samples obtained by depositing several drops on a gold-covered glass support and allowing them to dry at room temperature.

The determination of the silver and gold content in the NCs was performed by using a Varian 720-ES inductively coupled plasma atomic emission spectrometer (ICP-AES).

Theoretical 3D simulations were carried out by a commercially available FEM package (COMSOL Multiphysics), which allows to numerically solve the near and far fields of the tested nanostructures and eventually their extinction efficiencies in order to compare them with the experimental spectra [2].
maintained the overall shape of the core precursors with a single mode distribution corresponding to an average diameter of 67 nm ($r_+ = 31$ nm and $r_- = 21$ nm), i.e., a value almost double that of Ag templates (Fig. 1), and a shell thickness ranging from 3 to 15 nm, with an average value of 10 nm.

We quantified the overall amount of metallic Au and Ag in the NCs by performing ICP-AES analysis. For this purpose, a small amount of the NCs suspension was digested with an aqueous solution of aqua regia ($\approx 5\%$ v/v) at 75°C for 24 h. An Au/Ag molar ratio equal to 1.4 was determined ($C_{\text{Au}} = 0.045$ mM; $C_{\text{Ag}} = 0.032$ mM), corresponding to a percentage composition of about 60% Au and 40% Ag.

The progress of the galvanic replacement reaction was accompanied by a color change in the solution from yellow to blue, due to the quenching of the plasmon peak of chit-AgNPs at 416 nm (Fig. 3 solid gray line) and the 300 nm-redshift of the LSPR from the violet region to the NIR (Fig. 3 solid black line).

The next step has been verifying whether such spectral features could be consistent with the behavior of hollow Au/Ag nanostructures. So, on the basis of the experimental data, we calculated the extinction efficiency for both types of nanoparticles, AgNPs and NCs, by COMSOL software.

We treated AgNP as a truncated octahedron with sharp edges (Fig. 3) and a mean diameter $d_{\text{AgNP}} = 26$ nm, derived from the statistical analysis. On the contrary, modeling NCs was not a simple task, due to the large variability of shapes observed in our samples. We selected a simplified geometrical model based on an empty truncated octahedron with Au/Ag alloyed shell and regular holes on the surfaces (Fig. 3 top right). Also in this case, the structural parameters were deduced from the statistical analysis of TEM micrographs: external diameter $d_{\text{NC}} = 67$ nm and shell thickness $= 10$ nm. Besides, according to the ICP-AES measurements, the dielectric constant of the shell was set as the weighted average of the dielectric constants of gold and silver (60:40). In both cases, the exciting light was a plane wave traveling along the $z$ axis with the electric field $E$ directed along the $x$ axis (Fig. 3); the embedding medium was supposed to be water ($n = 1.33$), and the values of dielectric constants of gold and silver were taken from Johnson and Christy [35].

We performed calculations for...
different porosity levels and found that the model structure, whose spectral features are the closest to the experimental ones in terms of the maximum wavelength of the LSPR band, corresponds to a porosity degree of 18% (Fig. 3, dashed black line). The obtained results satisfactorily match the experimental data.

However, TEM images of the NCs show that the holes are usually not symmetrically positioned. Therefore, keeping the porosity constant, we also calculated the extinction spectrum in one of the other possible configurations as that reported in Fig. 3 (bottom right – dotted black line). Apart from the fact that the bandwidth for the non-symmetric case is wider, the position of the two plasmon peaks is essentially the same.

Finally, Fig. 4 shows the theoretical simulations of both a Ag core/alloyed Au:Ag nanoshell (solid line) and a hollow alloyed Au:Ag nanoshell (dotted line) as compared to that (dashed line) referring to the upper NC depicted in Fig. 3. These nanostructures, though with the same dimensions and alloy composition of the NC, exhibit maximum wavelength values about 200 and 100 nm lower than that of the NC, thus highlighting the crucial role played by the holes in redshifting the extinction spectrum.

3.2. Functionalization and purification of the nanocages

In order to validate the potential use of NCs as double-channel detection systems, we functionalized them with a test molecule, i.e., a fluorescent compound, which is a derivative of 4-methoxy-1,8-naphtalimide, bearing at the imide N-atom an aliphatic chain endowed with a SH terminal group (NAFTA6). The thiol terminal group enables covalent binding to noble metals, while the aliphatic chain, consisting of six methylene groups, limits the interaction between the fluorescent moiety and the metal surface and is expected to reduce the associated fluorescence quenching. The synthesis of NAFTA6, whose chemical structure is reported in Fig. 5, follows the two step procedure described in Ref. [34].

Before NC functionalization, we characterized NAFTA6 by absorption and fluorescence spectroscopy. The molecule absorbs in the near UV spectral region and emits in the bluish visible region. Excitation and emission spectra of NAFTA6 are reported in Fig. 6. They were measured by dissolving NAFTA6 in the same H₂O/acetone 5:1 mixture that was subsequently used for the NC functionalization procedure. The observed absorption and fluorescence maxima are far from the LSPR band of the NCs (Fig. 3), thus avoiding re-absorption of the fluorescence emission by the metal nanostructure.

The functionalization of chit-NCs (Scheme 2) was performed by means of prolonged ligand exchange-reaction, favored by the tendency of NAFTA6 molecules to assume a disposition on the metal surface characterized by binding through their thiol terminal groups. In particular, 0.5 ml of 0.23 mM NAFTA6 acetone-solution was added to 3 ml of chit-NCs; the H₂O and acetone volumes were corrected in order to achieve a 5:1 ratio.

After 24 h incubation, the sample was purified in order to eliminate excess NAFTA6. This was achieved by dialysis, which was preferred to centrifugation in order to preserve NC structure and morphology. Dialysis was carried out for 17 h and the external solvent mixture was replaced three times during this period. In this way, we continually extracted the free-fluorophore molecules from the colloid containing NAFTA6-functionalized-chitosan NCs (NAFTA6-NCs). NAFTA6 concentration in the extraction product was quantitatively evaluated by fluorescence spectroscopy, based on the calibration curve obtained by dissolving known quantities of NAFTA6 in a H₂O/acetone 5:1 mixture and reported in Fig. S1 in the Supporting Information. The amount of NAFTA6 in the dialysed sample, which was evaluated by comparison with the calibration curve, was estimated to be of the order of 10⁻⁵ M.

A destructive test was also performed by centrifugation of a small amount of the dialyzed NAFTA6-NCs colloid at 10,000 rpm...
for 30’ at 23 °C, in order to completely separate all the functionalized metallic nanostructures from free or chitosan-trapped NAFTA6 residues. At the end of this process, a comparison between the area of the absorption band of the dialyzed colloid (maximum at 372 nm) with that of the supernatant (Supporting Information Fig. SI2) gave a fluorophore concentration of 7 × 10⁻⁶ M for the purified NAFTA6-NCs sample.

3.3. Characterization and modeling of functionalized nanocomposites

NAFTA6-NCs were spectroscopically characterized after dialysis.

Fig 7a compares the absorption spectra of chit-NCs before (solid black line) and after functionalization with NAFTA6 (solid gray line) with that of NAFTA6 dissolved in a H₂O/acetone mixture (dashed black line). The absorption profile of NAFTA6-NCs contains the absorption signal of NAFTA6, with maximum at 372 nm (see also Fig. 6), as well as the typical NCs extinction band, which is 30 nm redshifted with respect to that of NCs before functionalization. We attribute this shift to dye chemisorption. Fig. 7b reports the emission intensity of NAFTA6-NCs compared to that of an equimolar NAFTA6 solution (7 × 10⁻⁶ M), measured under identical conditions. The emission of the colloid is 45% weaker than that of the free ligand solution. This effect is related to both the quenching and shielding effect deriving from the proximity of the molecule to the metal surface.

Further confirmation of the interaction between NAFTA6 and NCs is given by the SERS activity of NAFTA6-NCs, observed using Raman excitation at 514.5 and 785 nm (Fig. 8). The results are compared with the Raman spectrum of NAFTA6 powders obtained with 785 nm excitation [37]. In the case of the powder sample (gray line), the most prominent Raman bands closely correspond to those of naphthalene [38]. In particular, the bands at 510, 615, 1402, and 1586 cm⁻¹ are related to those of naphthalene at 514, 619, 1380, and 1578 cm⁻¹. The two bands at lower frequency (510 and 615 cm⁻¹) are attributable to ring bending modes, whereas the others (1402 and 1586 cm⁻¹) to ring stretching modes. Lastly, the Raman band at 1694 cm⁻¹ is assigned to C=O stretching mode.

In the case of NAFTA6-NCs, the Raman response obtained by 514.5 nm excitation was dominated by the dye fluorescence background, and no significant band structure could be detected (dashed black line). In contrast, excitation within the LSPR band of the NCs using the 785 nm laser line permitted observation of the characteristic vibrational bands of the fluorophore (solid black line). In particular, in the low frequency region, a weak spectroscopic feature near 300 cm⁻¹, which is not present in the Raman spectrum of the powder sample (gray line), can be assigned to a Au–S bond stretching mode [39,40], providing further confirmation of the chemisorption of the fluorophore onto the metal surface.

The difference in the two SERS responses was confirmed further by theoretical computations. Using the NC model of Fig. 4, we calculated the square of the near field and averaged it on the total external surface of the structure. We performed the calculation at two different wavelengths: that of the Raman excitation (i.e., 514.5 or 785 nm) and that of the Raman scattering, corresponding to the band at 1400 cm⁻¹. The field enhancement factors [41] were...
thus evaluated to be 15 and 419 for the 514.5 and 785 nm excitation wavelengths, respectively. Hence, excitation within the LSPR band of the metallic NCs structures is expected to provide a theoretical 30-fold intensification of the Raman response.

4. Conclusions

We have presented a novel nanostructure consisting of dye-protected Au/Ag NCs. Its double functionality offers considerable potential for applications in sensing devices, which exploit both the possibility of Raman monitoring of the chemical environment in the vicinity of the nanocages and selective sensing based on fluorescence spectroscopy.

The nanostructures exhibit a strong extinction in the 700–800 nm spectral range, related to the LSPR band of the metallic core. This feature makes them strong absorbers and, at the same time, efficient SERS substrates within the window of the maximum transparency of biological tissues. Furthermore, the interaction of the functionalized fluorescent moiety with the metallic substrate, which quenches considerably its emission, opens the way to the utilization of the nanostructures as biomarker sensors, which can cause fluorescence quenching or fluorescence recovery after quenching under cleavage of the Au-S bond [19].

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Appendix A. Supplementary material

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References