

Article

Development of Biological-Window-Active Au Open-Shell Nanoparticles with High-Sensitivity Surface-Enhanced Raman Scattering Imaging Probe Properties

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Abstract

The development of anisotropic gold nanostructures supporting localized surface plasmon (LSP) resonances in the near-infrared (NIR) biological window is of great interest for diagnostic and therapeutic nanotechnologies. Here, we report gold open-shell nanoparticles (AuOSNs), a symmetry-broken nanoshell architecture exhibiting strong NIR surface-enhanced Raman scattering (SERS) activity. AuOSNs were fabricated via a surfactant-free strategy combining bottom-up silica sphere assembly with a simple top-down gold deposition process, without using highly cytotoxic surfactants such as cetyltrimethylammonium bromide (CTAB). Boundary element method (BEM) simulations revealed that the asymmetric open-shell geometry induces NIR LSP resonances with pronounced electromagnetic field localization near the opening edges, depending on excitation configuration. Consistent with these predictions, extinction spectra of AuOSNs dispersed in water showed an LSP resonance peak at ~793 nm, close to the 785 nm excitation wavelength for SERS. In aqueous dispersion, AuOSNs modified with 4-mercaptobenzoic acid (4-MBA) exhibited strong SERS activity with enhancement factors of ~10⁶. Furthermore, polyethylene glycol (PEG)-modified MBA/AuOSNs showed negligible cytotoxicity in vitro. SERS imaging confirmed that PEG/MBA/AuOSNs enable visualization of HeLa cells via characteristic MBA SERS signals. These results demonstrate that surfactant-free AuOSNs provide a biocompatible platform for NIR-excited SERS sensing and cellular imaging, highlighting their potential in plasmonic bioimaging applications.

Keywords: localized surface plasmon; surface-enhanced Raman scattering; bioimaging; open-shell nanostructures; nanoshells



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1. Introduction

Surface-enhanced Raman scattering (SERS) is generally defined as a phenomenon in which the Raman scattering signals of molecules or materials located in the vicinity of metal nanoparticles or nanostructures are dramatically enhanced [1,2]. The enhancement mechanisms of SERS are mainly classified into two categories [3]. One is the chemical effect, which arises from charge transfer and hybridization of electronic states induced by the chemical adsorption of molecules onto the metal surface, leading to a modulation of molecular polarizability. The other is the electromagnetic effect, in which metal nanoparticles or nanostructures resonate with incident light at specific wavelengths determined by their size, shape, and surrounding environment, thereby exciting localized surface plasmon (LSP) resonances. As a result, intense localized electromagnetic fields are generated near the nanostructure surface, enhancing both the incident and scattered light and consequently amplifying the Raman scattering signals. Through the synergistic contribution of these two effects, Raman signal enhancements of up to 10^{10} – 10^{11} times have been reported under appropriate conditions.

The SERS phenomenon has attracted considerable attention for applications in biomedical fields, including biosensing and bioimaging [4,5]. Because SERS provides spectra with high molecular fingerprint specificity that strongly reflect the structural and compositional characteristics of target molecules, it enables simultaneous highly sensitive detection and molecular identification of biomolecules. In addition, Raman scattering peaks are molecule-specific and intrinsically narrow, which allows the simultaneous discrimination and detection of multiple biomolecules. Furthermore, the Raman scattering of water is relatively weak, resulting in minimal interference from biological fluids. As a nondestructive analytical technique, and because it is less susceptible to photobleaching than fluorescence-based methods, SERS is well suited for long-term monitoring and observation [5]. Moreover, the linewidths of Raman scattering peaks are significantly narrower than those of fluorescence bands, which reduces interference from autofluorescence originating from biological materials [6]. Finally, by tuning the LSP resonance to wavelengths with high biological tissue transparency (the so-called biological window, 700–1300 nm), deep-tissue bioimaging can also be achieved [7].

Especially in biomedical imaging, two major SERS-based approaches have been established: direct SERS imaging, which directly amplifies the intrinsic Raman scattering signals of biomolecules to visualize the spatial distribution of target molecules within biological tissues, and indirect SERS imaging, which exploits the specific binding behavior of metal nanoparticles functionalized with known Raman reporter molecules toward target species [5]. In both approaches, the dominant factor governing sensitivity is the LSP resonance characteristics of the metal nanoparticles, as the enhancement of Raman scattering arising from the electromagnetic effect generally far exceeds the contribution from the chemical effect [8,9]. To achieve highly sensitive SERS imaging, rational design of metal nanoparticles—particularly in terms of material selection and morphological control—is essential to generate intense localized electromagnetic fields via LSP resonances. Among metal nanoparticles that generally exhibit LSP resonances in the visible to near-infrared region, silver nanoparticles are known to generate stronger localized electromagnetic fields than gold nanoparticles [10]. However, silver nanoparticles have been reported to exhibit relatively high cytotoxicity, necessitating careful consideration for in vivo applications [11]. Consequently, with a few exceptions [12], gold nanoparticles are predominantly employed for biomedical imaging purposes [13,14]. In addition, anisotropic gold nanoparticles are capable of generating stronger localized electromagnetic fields compared with spherical gold nanoparticles [15]. For example, gold nanoparticles possessing sharp tips or spike-like structures can produce extremely intense localized electromagnetic fields via the so-called

lightning rod effect [8]. Furthermore, whereas spherical gold nanoparticles typically exhibit LSP resonances around 520 nm, shape anisotropy is known to shift the resonance wavelength toward the near-infrared region, including the biological window [16,17]. Therefore, the utilization of anisotropic gold nanoparticles with appropriately engineered morphologies represents a highly effective strategy for the development of high-sensitivity SERS probes suitable for biomedical imaging.

To date, a wide variety of anisotropic gold nanoparticles that exploit strong localized electromagnetic fields arising from shape anisotropy have been experimentally demonstrated to exhibit high SERS activity. Representative examples include gold nanorods [18,19], multipod-shaped gold nanostars [20], triangular gold nanoplates [21,22], gold nanocubes [23,24], and gold nanoflowers [25,26]. However, many of these anisotropic gold nanoparticles require cetyltrimethylammonium bromide (CTAB), or its chloride analogue cetyltrimethylammonium chloride (CTAC), as a shape-directing agent during synthesis [18,20–23,25,26]. These surfactants are known to exhibit high cytotoxicity [27]. Moreover, even after synthesis, CTAB often remains strongly adsorbed on the nanoparticle surface as a stabilizing agent to maintain colloidal dispersion in solution. For some anisotropic gold nanoparticles, such as gold nanostars [28] and gold nanoplates [29], surfactant-free synthetic routes or synthesis methods employing surfactants with lower cytotoxicity have been reported. However, these approaches are generally limited in terms of applicable morphologies and synthetic conditions, and thus remain restricted as general strategies for simultaneously achieving high SERS activity and biocompatibility. To mitigate CTAB-induced toxicity, various post-synthetic surface modification strategies have been explored, including coating with polyethylene glycol (PEG) [30] or polydopamine [31], as well as electrostatic coating using negatively charged polymers [32]. In addition, ligand-exchange approaches using PAMAM dendrimers [33] or thiol-modified PEG [34–36] have been proposed to replace CTAB and remove it from the gold nanoparticle surface. Nevertheless, considering reports that even extremely low concentrations of CTAB can induce cytotoxic effects [37,38], the development of CTAB-free gold nanoparticles that retain strong SERS activity remains a fundamental and unresolved challenge for biological imaging applications.

Gold nanoshells are a promising class of metallic nanoparticles that meet these requirements [39,40]. They consist of a silica core coated with a gold shell [41] and can be synthesized without using highly cytotoxic surfactants such as CTAB [42]. Owing to plasmonic coupling between the inner and outer shell surfaces, gold nanoshells can generate strong localized electromagnetic fields, and their LSP resonance can be tuned into the biological window by controlling the coupling strength [40]. Despite these advantages, reports on in situ SERS imaging in biological tissues using gold nanoshells are still limited [43], likely because the SERS activity of individual nanoshells is not always sufficient, despite their high biocompatibility and design flexibility [44]. In this study, we designed and synthesized a new class of gold open-shell nanoparticles (AuOSNs) [45,46] with a hemispherical opening structure, as schematically illustrated in Figure 1, as an advanced form of conventional gold nanoshells, and systematically investigated their SERS imaging properties. These nanoparticles can be synthesized without the use of highly cytotoxic surfactants such as CTAB by combining a bottom-up approach with a simple top-down process. As a result, we demonstrate that the gold open-shell nanoparticles exhibit approximately one order of magnitude higher SERS enhancement than previously reported conventional gold nanoshells, while maintaining negligible cytotoxicity. The high SERS activity can be understood within the plasmon hybridization framework (Figure 2). Hybridization between the sphere plasmon mode at the outer medium/Au interface and the cavity plasmon mode at the Au/silica interface generates bonding and antibonding modes. In the open-shell geometry, the bonding mode further interacts with the dipolar

mode associated with the nanohole structure, leading to symmetry breaking and strong electromagnetic field localization at the rim region. This concentrated field enhancement is responsible for the increased SERS activity. Furthermore, through in vitro experiments, we confirm that these nanoparticles serve as effective probes for cellular SERS imaging.

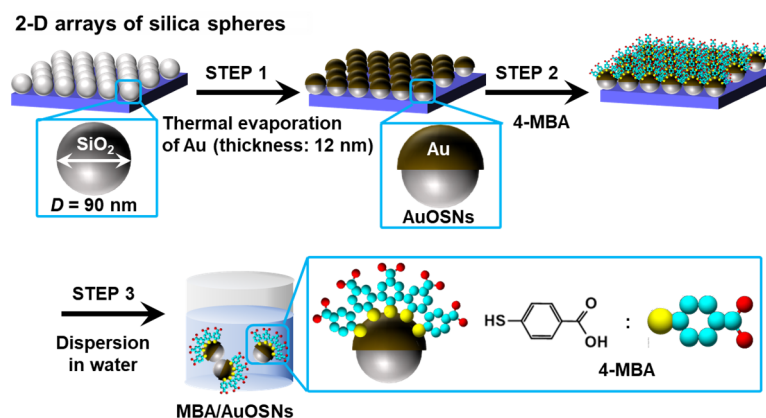


Figure 1. Schematic illustration of the preparation of AuOSNs and subsequent modification with 4-MBA monolayers.

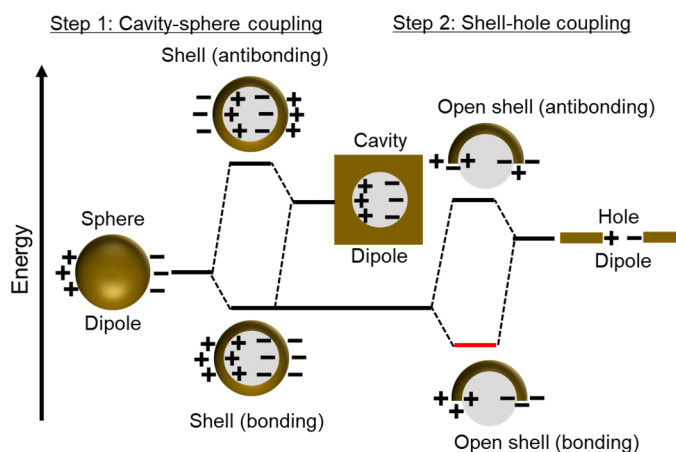


Figure 2. Plasmon hybridization models, which can be induced at AuOSNs.

2. Materials and Methods

2.1. Materials

Milli-Q-grade water (MilliporeSigma, Burlington, MA, USA, resistivity: 18.2 MΩ·cm) was used to prepare all aqueous solutions. Ethanol (EtOH), aqueous NH₃ (28%), aqueous H₂O₂ (30%), and 1-butanol (BuOH) were obtained from Kishida Chemical Co., Ltd., Osaka, Japan. Tetraethyl orthosilicate (TEOS) was obtained from Kanto Chemical Co., Inc., Tokyo, Japan. PEG (Mw: 20,000) was obtained from Sigma-Aldrich, St. Louis, MO, USA. Dulbecco's Modified Eagle's Medium (D-MEM, low glucose) with L-glutamine and phenol red, and phosphate-buffered saline (PBS) (−) were obtained from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. Penicillin streptomycin, fetal bovine serum (FBS), and trypsin-EDTA (0.25%) were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Polyethyleneimine (PEI) was obtained from Polysciences, Inc., Warrington, PA, USA. 4-Mercaptobenzoic acid (4-MBA) was obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Calcein AM and propidium iodide (PI) were obtained from DOJINDO Laboratories, Kumamoto, Japan. HeLa cell lines were purchased from JCRB Cell Bank, Osaka, Japan. The glass substrates (S1225) were obtained from Matsunami Glass Ind., Ltd., Osaka, Japan. Aluminum (wire cut) (99.999%) was obtained from High Purity Chemicals

Co., Ltd., Tokyo, Japan. Gold pellets were purchased from TANAKA Precious Metals, Tokyo, Japan and used as received.

2.2. Synthesis of AuOSNs

Monodisperse silica spheres were synthesized via a conventional Stöber-type sol–gel process [47]. In a typical preparation, EtOH (20.0 mL) and aqueous NH₃ (28 wt%, 1.47 mL) were mixed and agitated for 15 min to obtain a homogeneous reaction medium. TEOS (0.50 mL) was then introduced under continuous stirring, and the reaction was allowed to proceed for 3 h. As a result, silica spheres with an average diameter of 90 ± 4 nm were obtained. The as-prepared silica colloids were purified by centrifugation at 11,000 rpm for 10 min, first twice using ethanol as the dispersion medium and subsequently twice using BuOH. After purification, the silica particles were redispersed in 10 mL of BuOH.

Two-dimensional hexagonally packed monolayers of silica spheres were assembled on glass substrates (25 mm × 15 mm) following previously established protocols [48,49]. The resulting arrays were immersed in an aqueous solution of PEI (1.0 mM) for 1 min and then rinsed thoroughly with Milli-Q water. After drying under a nitrogen stream, a gold layer with a nominal thickness of 12 nm was deposited onto the silica arrays by thermal evaporation under high-vacuum conditions (STEP 1 in Figure 1, AuOSNs, $<5.3 \times 10^{-7}$ Torr) [50,51]. Subsequently, the Au-coated arrays, in which the upper hemispheres of the silica spheres were covered with gold shells, were immersed in an EtOH solution containing 4-MBA (0.1 mM, 4 mL) for 3 h. This treatment enabled the formation of a self-assembled monolayer of 4-MBA on the Au surface via Au–thiol interactions (STEP 2 in Figure 1). Finally, the arrays were dispersed in Milli-Q water (4 mL) to yield a stable colloidal suspension of 4-MBA-functionalized AuOSNs (STEP 3 in Figure 1, MBA/AuOSNs). Finally, to functionalize the MBA/AuOSNs with PEG (PEG/MBA/AuOSNs), an aqueous dispersion of the nanoparticles was mixed with a PEG solution (80 mg mL^{−1}, 5 mL) and stirred for 12 h. The resulting dispersion was then purified by centrifugation twice using Milli-Q water as the dispersion medium, followed by redispersion of the precipitate in 2 mL of Milli-Q water. Furthermore, for the cell viability assays and cell imaging experiments, when PEG/MBA/AuOSNs were added to the cells, the aqueous dispersion of PEG/MBA/AuOSNs was centrifuged and then redispersed in PBS.

2.3. In Vitro Cell Imaging Using MBA/AuOSNs

HeLa cell lines were cultivated in a 5% CO₂ environment at 37 °C by using D-MEM supplemented with 10% FBS and 1% penicillin/streptomycin. To evaluate the cell cytotoxicity and cell imaging ability of PEG/MBA/AuOSNs, HeLa cells in D-MEM (3.3×10^4 cells/mL, 300 µL) were plated in eight well plates and incubated for 48 h to facilitate cell adhesion to the well's bottom surface. Subsequently, the D-MEM was completely removed from the well plates, and PBS (100 µL) containing PEG/MBA/AuOSNs at the desired concentrations and D-MEM (200 µL) were added to each well to achieve final Au concentrations of 120, 180, and 200 µg/mL. The cells were further incubated for 3 h. For the cytotoxicity evaluation, after completely removing the PEG/MBA/AuOSNs solution from the well plates, 300 µL of D-MEM containing 1 µM calcein AM and 0.5 µM PI was added to the well plates. These dyes specifically label viable and nonviable cells by emitting green and red fluorescence, respectively. The plates were then incubated for 20 min at 37 °C under 5% CO₂. Finally, the solutions were removed from the plates. The cells were observed under a BX53 fluorescence microscope (Olympus, Tokyo, Japan). For cellular SERS imaging experiments, the PEG/MBA/AuOSNs solution was completely removed from the well plates, and the wells were rinsed with PBS, followed by removal of the PBS. The HeLa cells were then fixed by adding 4% paraformaldehyde solution (200 µL per well)

and incubating at room temperature for 20 min. After fixation, the paraformaldehyde solution was removed, and the cells were washed three times with PBS. Following removal of the PBS, SERS imaging measurements were performed.

2.4. Measurements and Calculations

Optical extinction spectra were recorded using a V-770 UV–vis–NIR spectrophotometer (JASCO Corporation, Tokyo, Japan). Transmission electron microscopy (TEM) observations were carried out on a JEM-2000EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 200 kV. Zeta potentials and hydrodynamic size distributions were determined by dynamic light scattering (DLS) measurements using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). The extinction, absorption, and scattering characteristics of the AuOSNs were numerically simulated by applying the boundary element method (BEM) with fully retarded electromagnetic potentials. These calculations were performed using the MNPBEM17 toolbox implemented in MATLAB R2024b (MathWorks, Natick, MA, USA), which provides solutions to the full set of Maxwell's equations. The dielectric function of Au employed in the simulations was taken from previously reported literature by Rakic et al. [52] Raman spectra of the dispersed aqueous solutions containing MBA/AuOSNs were acquired using a Micro-Raman spectrometer (NRS-4100, JASCO Corporation, Tokyo, Japan). A 785 nm continuous-wave laser was employed as the excitation source, and the laser power at the sample surface was adjusted to 16.4 mW using neutral density filters. The laser beam was focused onto the sample solution through a 100× objective lens, and Raman spectra were collected with an integration time of 10 s, averaged over two accumulations. SERS mapping of paraformaldehyde-fixed HeLa cells was performed using the same micro-Raman spectroscopic setup described above. The laser power at the sample was reduced to 3.3 mW to avoid photodamage, and SERS spectra were acquired with an integration time of 5 s per pixel. The mapping measurements were conducted over an area of $150 \times 150 \mu\text{m}^2$ with a step size of $5 \mu\text{m}$. SERS images were constructed based on the intensity of the MBA Raman band at 1077 cm^{-1} .

3. Results and Discussion

3.1. Preparation and Characterization of MBA/AuOSNs

To elucidate the excitation behavior of the LSP resonance in AuOSNs and the associated spatial distribution of localized electromagnetic fields, numerical simulations were carried out using the boundary element method (BEM). The calculations were based on the structural parameters of experimentally fabricated AuOSNs (described later in Figure 3A), consisting of a silica core with a diameter of 90 nm and a Au shell thickness of 12 nm. Owing to the pronounced structural asymmetry of the AuOSNs, their plasmonic response is expected to exhibit a strong dependence on both the incident light direction and polarization state. Accordingly, extinction spectra were evaluated under three representative excitation configurations, defined by distinct combinations of irradiation direction and polarization relative to the opening edge, as illustrated in Figure 3A. The averaged extinction spectrum of these calculated spectra (Figure 3C), which is assumed to be a random orientation in solution, exhibits a shoulder at 610 nm, and the peak wavelength of the dominant LSP resonance band is observed at 790 nm, which is close to the excitation wavelength (785 nm) used for SERS within the biological window. The LSP resonance band observed in this long-wavelength region can be explained by a plasmon hybridization model [40,50]. Owing to this characteristic, when the propagation direction of the incident light is along the z axis, strong localized electromagnetic fields are formed near the opening edge at 785 nm under specific polarization conditions where the opening edge is oriented along either the z or x axis (Figure 3B). A pronounced enhancement of Raman scattering from

MBA molecules immobilized on the AuOSNs is anticipated through the electromagnetic enhancement mechanism.

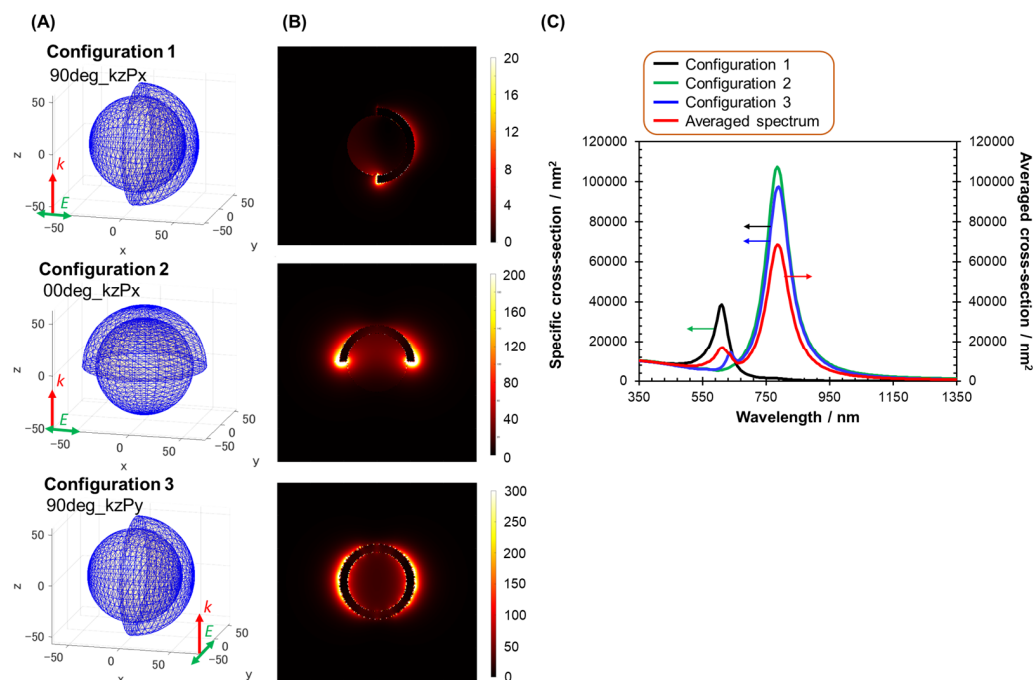


Figure 3. Theoretical optical properties of AuOSNs in water obtained by BEM calculations. (A) Geometrical models of AuOSNs corresponding to three excitation configurations. 90deg_kzPx (Configuration 1), 00deg_kzPx (Configuration 2), and 90deg_kzPy (Configuration 3) denote the orientation angle of the AuOSNs (0° defined as the configuration where the opening edge is parallel to the x axis), the propagation direction of the incident light (kz indicates propagation along the z axis), and the polarization direction (Px and Py represent x- and y-polarized light, respectively). (B) Local electromagnetic field distributions for each geometrical model shown in (A) at an excitation wavelength of 785 nm. (C) Extinction spectra calculated for each geometrical model and their averaged spectrum.

In this study, unlike conventional Au nanoshells that are synthesized exclusively through chemical processes, AuOSNs were fabricated by integrating a bottom-up process consisting of silica nanosphere synthesis and their self-assembly with a top-down process based on the thermal deposition of Au under high vacuum (Figure 1). Figure 4A shows a TEM image of nanoparticles sampled from a colloidal aqueous solution of MBA/AuOSNs, which were obtained by modifying the Au surface with a monolayer of 4-MBA as a Raman reporter. The image confirms the formation of nanoparticles with a characteristic morphology in which Au shells selectively cover only the hemispherical surface of silica spheres. DLS measurements were carried out to evaluate the colloidal dispersion state of the nanoparticles in aqueous media. The MBA/AuOSNs exhibited a narrow number-weighted size distribution with a polydispersity index (PDI) of 0.246, indicating a fairly monodisperse colloidal system, with the dominant population observed below 100 nm in diameter (Figure 4C). The Z-average hydrodynamic diameter (165.4 nm) obtained from DLS was larger than the particle size determined by TEM. This discrepancy is attributed to the presence of a minor fraction of larger particles or weakly aggregated species, which disproportionately contribute to the scattering intensity in DLS measurements. In addition, the zeta potential of the MBA/AuOSNs exhibited a negative value of -21.4 mV. This negative surface charge is attributed to the carboxyl groups of the 4-MBA functionalized on the Au surface, as well as to the hydroxyl groups present on the silica surface that is

not covered by Au. This moderate negative surface charge is consistent with the stable aqueous dispersion of the MBA/AuOSNs.

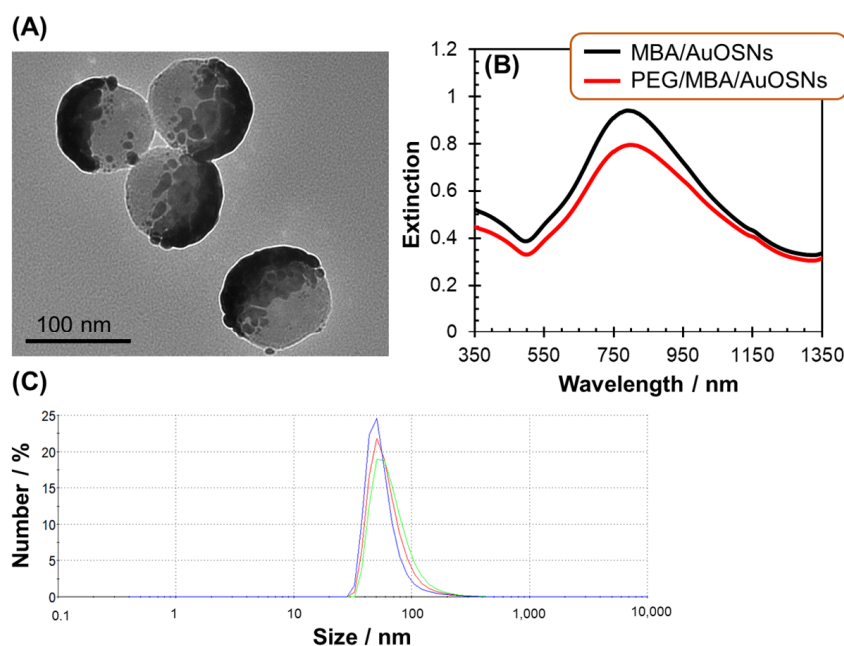


Figure 4. Morphological and spectroscopic characteristics of the synthesized AuOSNs. (A) TEM image of MBA-modified AuOSNs (MBA/AuOSNs). (B) Extinction spectra of MBA/AuOSNs and PEG/MBA/AuOSNs in colloidal aqueous solutions. (C) Size distribution of MBA/AuOSNs dispersed in water, obtained by DLS measurements.

The excitation wavelength employed for SERS measurements was 785 nm, which lies within the biological window. To achieve high SERS activity, the LSP resonance of the MBA/AuOSNs should be tuned to overlap with this excitation wavelength, since SERS enhancement requires spectral overlap of the LSP resonance band with both the excitation wavelength and the Stokes-shifted Raman-scattered wavelengths. BEM calculations revealed that the LSP resonance wavelength of AuOSNs can be systematically controlled by adjusting the diameter of the silica core and the thickness of the Au shell. In the present study, extinction spectrum of the colloidal aqueous solution of MBA/AuOSNs (Figure 4B) exhibits a pronounced LSP resonance peak at 793 nm, which is in good agreement with the BEM simulation results shown in Figure 2. The observed LSP resonance is spectrally close to the excitation wavelength of 785 nm and spans a broad wavelength range from 550 to 1100 nm.

3.2. SERS Enhancement Properties of MBA/AuOSNs

The Raman spectra of an aqueous dispersion of MBA/AuOSNs and a 300 mM EtOH solution of MBA used as a reference (excitation wavelength: 785 nm) are shown in Figure 5. In the SERS spectrum of the MBA/AuOSNs aqueous dispersion, multiple well-defined SERS peaks originating from 4-MBA molecules adsorbed on the Au surface were clearly observed. The two most intense peaks in the spectrum, located at 1077 and 1582 cm^{-1} , are assigned to the ν_{12} and ν_{8a} vibrational modes of the aromatic ring, respectively [53,54]. A weak peak observed at 1178 cm^{-1} is attributed to C–H bending vibrations ($\delta(\text{C–H})$) [55]. In addition, a weak band observed around 851 cm^{-1} is characteristic of 4-MBA with dissociated carboxyl groups and is attributed to the symmetric stretching vibration of the COO^- group [53,55]. The observation of this peak suggests that the carboxyl groups of 4-MBA adsorbed on the AuOSNs surface exist in a deprotonated state, indicating that 4-MBA contribute, at least in part, to the negative surface charge observed in the zeta

potential measurements. In contrast, in the Raman spectrum of the 4-MBA EtOH solution, the major peaks observed at 884, 1055, and 1096 cm^{-1} are all attributed to the EtOH solvent [56]. By comparison, only a weak Raman peak arising from the ν_{8a} vibrational mode of the aromatic ring of 4-MBA is observed at 1594 cm^{-1} [55]. Therefore, in this study, the SERS activity of 4-MBA immobilized on the AuOSNs was evaluated using the Raman scattering enhancement factor (EF) defined in Equation (1), based on the Raman peak corresponding to the ν_{8a} vibrational mode of the aromatic ring at around 1582 cm^{-1} [57,58].

$$EF = \frac{I_{SERS}/C_{SERS}}{I_{RS}/C_{RS}} \quad (1)$$

Here, I_{RS} denotes the Raman scattering peak intensity of an EtOH solution of 4-MBA used as a reference sample, and C_{RS} represents the 4-MBA concentration in the reference solution. In contrast, I_{SERS} corresponds to the SERS peak intensity of 4-MBA obtained from the aqueous dispersion of MBA/AuOSNs, while C_{SERS} represents the effective concentration of 4-MBA molecules immobilized on the surface of AuOSNs in the dispersion. The value of C_{SERS} was estimated to be 1.79 μM from the decrease in absorbance of the 4-MBA absorption peak at 269.5 nm (0.0163, Figure S1) before and after immersion of the AuOSNs array substrate in a 0.1 mM 4-MBA ethanol solution (4 mL), using the molar extinction coefficient of MBA at 269.5 nm ($9.14 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$). Notably, both the volume of the 4-MBA solution and the final volume of the MBA/AuOSNs aqueous dispersion were 4 mL. As a result, the Raman scattering signal of 4-MBA immobilized on the AuOSNs surface was found to be enhanced by 1.3×10^6 . In previous studies that systematically investigated the SERS properties of Au nanoshells, the enhancement factor obtained using thiobenzoic acid was reported to be at most on the order of 2×10^5 [44]. Although a direct quantitative comparison between Au nanoshells and AuOSNs is not straightforward due to differences in Raman reporter molecules and LSP resonance wavelengths, the high EF observed in this study is particularly noteworthy because it was achieved in a single-particle architecture without relying on aggregation-induced or interparticle hotspot effects. Reports of very large enhancement factors ($\sim 10^8$) do exist; however, these values are usually achieved under particular conditions and should not be taken as representative of the general SERS performance of single gold nanoshells [59]. This pronounced enhancement is attributed to the favorable overlap of both the excitation wavelength and the Stokes-shifted Raman scattering wavelengths with the LSP resonance band of the AuOSNs, as well as to the strongly localized electromagnetic fields at the edge regions of the open-shell structure originating from the hemispherical Au shell geometry (Figure 3B). To evaluate signal reliability and photostability, repetitive SERS measurements were performed on drop-cast and dried nanoparticle films under continuous laser irradiation (16.4 mW, 10 s per acquisition). The characteristic MBA peak at 1075 cm^{-1} exhibited a relative standard deviation of approximately 5.2% over five consecutive measurements (Figure S2). This low variation indicates that the AuOSNs provide stable and reproducible Raman enhancement under the applied experimental conditions. In addition to conventional gold nanoshells, more complex plasmonic architectures such as gold nanomatryoshkas have been reported to exhibit high SERS enhancement factors [60]. However, these multilayer structures require stepwise shell growth and precise interlayer control to achieve strong plasmonic coupling. In contrast, the AuOSNs developed in this study achieve comparable enhancement within a structurally simpler single-shell architecture fabricated via a straightforward bottom-up and top-down combination process.

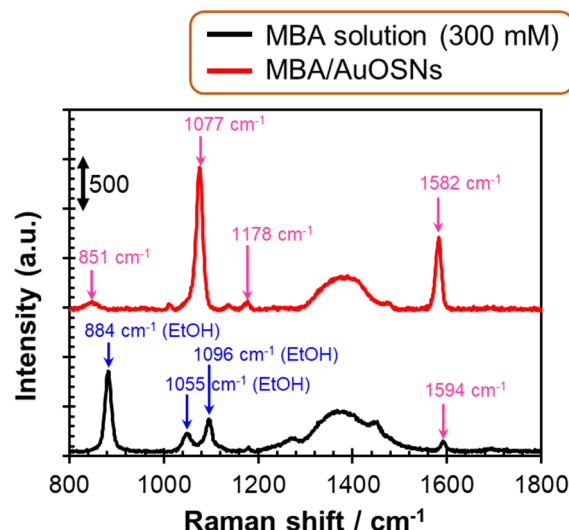


Figure 5. SERS spectrum of MBA/AuOSNs dispersed in water, and Raman spectrum of an ethanol solution of MBA (300 mM).

3.3. SERS Mapping of HeLa Cells Incubated with PEG/MBA/AuOSNs

To evaluate the functionality of MBA/AuOSNs as probes for cellular SERS mapping, SERS mapping measurements were performed using HeLa cells as a model system. To suppress nonspecific interactions with the cell membrane and to ensure dispersion stability in PBS, a high-ionic-strength medium, the surfaces of the MBA/AuOSNs were modified with polyethylene glycol (PEG/MBA/AuOSNs). DLS measurements of the PEG/MBA/AuOSNs revealed a PDI of 0.239, which was comparable to that of the unmodified MBA/AuOSNs described above, while the hydrodynamic diameter increased slightly to 197.2 nm relative to the unmodified particles. This increase is attributed to the expansion of the hydration layer surrounding the nanoparticles induced by the introduction of PEG chains. In addition, the zeta potential markedly decreased from that of the unmodified MBA/AuOSNs to -5.4 mV, suggesting that the surface negative charges were effectively masked by the neutral PEG chains coating the nanoparticle surface. These results confirm that PEG modification of the MBA/AuOSNs surface was successfully achieved.

Furthermore, the extinction spectrum of PEG-modified MBA/AuOSNs dispersed in PBS exhibited a spectral profile almost identical to that of MBA/AuOSNs dispersed in water (Figure 4B). These results indicate that PEG/MBA/AuOSNs remain colloidally stable in PBS under the experimental conditions relevant to the cellular assays, without inducing noticeable interparticle plasmon coupling. The LSP resonance peak exhibited a slight red shift of approximately 2 nm to 795 nm upon PEG modification. This shift is attributed to an increase in the local refractive index near the nanoparticle surface resulting from the introduction of PEG chains (refractive index of water: 1.33; refractive index of PEG: 1.46), as the LSP resonance wavelength is highly sensitive to changes in the local refractive index surrounding the nanoparticles [61].

To evaluate the cytotoxicity of the obtained PEG/MBA/AuOSNs, the cell viability of HeLa cells incubated with the nanoparticles at concentrations of 120, 180, and 200 $\mu\text{g/mL}$ for 3 h was investigated *in vitro*. Live and dead cells were distinguished by co-staining with calcein AM and PI, respectively, and the cell viability was calculated using the following equation:

$$\text{Cell viability} = \frac{\text{Number of cells stained with calcein AM}}{\text{Total number of cells stained with both calcein AM and PI}}$$

As shown in Figure 6, the control sample (i.e., in the absence of PEG/MBA/AuOSNs) exhibited a cell viability of $98 \pm 2\%$. Importantly, high cell viabilities exceeding 95% were consistently maintained under all incubation conditions across all PEG/MBA/AuOSNs concentrations. In contrast, anisotropic gold nanorods, which are typically stabilized by CTAB, have been reported to induce pronounced cytotoxicity even after short incubation times of approximately 1 h [62]. The PEG/MBA/AuOSNs developed in this study, synthesized via a combined bottom-up and top-down strategy under surfactant-free conditions, therefore demonstrate low cytotoxicity under the present experimental conditions, which can be primarily attributed to the surfactant-free synthesis and PEG surface modification.

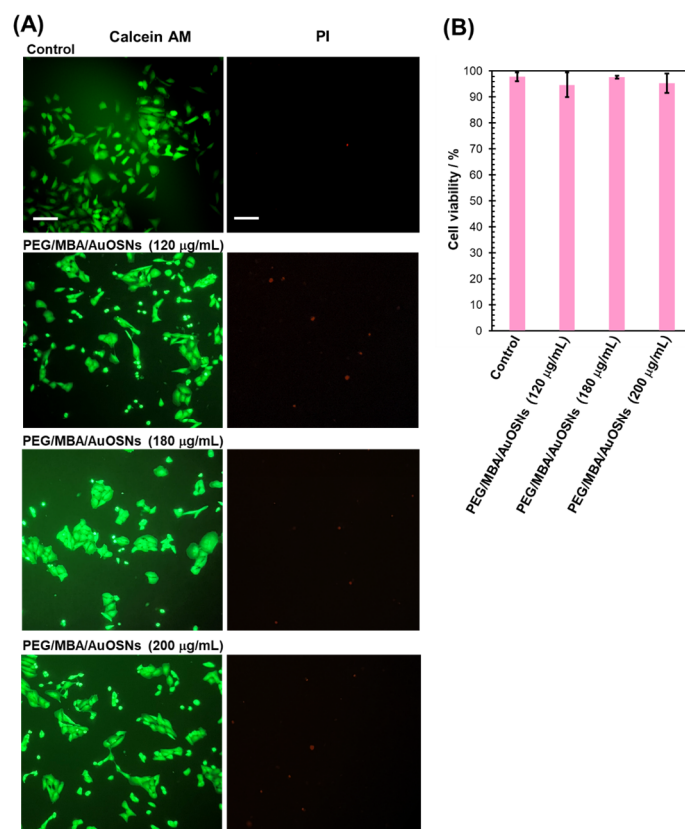


Figure 6. Cell viability of HeLa cells incubated with PEG/MBA/AuOSNs. (A) Fluorescence images of HeLa cells co-stained with calcein AM and PI after incubation without PEG/MBA/AuOSNs (control) or with PEG/MBA/AuOSNs at concentrations of 120, 180, and 200 µg/mL. The scale bar corresponds to 100 µm. (B) Quantitative cell viability obtained from the corresponding cell viability assays.

The SERS imaging results of HeLa cells incubated with PEG/MBA/AuOSNs at concentrations of 120, 180, and 200 µg/mL are shown in Figure 7A. The SERS images were constructed using the intensity of the strongest MBA-derived SERS peak at 1077 cm^{-1} . As a reference, no cellular features were visualized in the sample incubated without PEG/MBA/AuOSNs. In contrast, for samples incubated with PEG/MBA/AuOSNs, cells were clearly visualized by mapping the SERS peak intensity under all concentration conditions. Furthermore, the SERS peak intensity within the cell-populated regions increased with increasing PEG/MBA/AuOSNs concentration. This result suggests a good correlation between the amount of PEG/MBA/AuOSNs internalized by the cells and the nanoparticle concentration used during incubation. In the sample with the highest concentration (200 µg/mL), non-negligible SERS signals were partially detected even in regions without cells. This observation is attributed to the presence of excess PEG/MBA/AuOSNs that were not internalized by the cells and remained in the extracellular regions. Based on these

results, the optimal nanoparticle concentration under the present experimental conditions was determined to be 180 $\mu\text{g/mL}$. At this concentration, the highest SERS intensity was observed in the central region of the cells, while lower intensities were detected at the cell periphery. In regions without cells, the SERS signals were negligibly small (Figure 7B).

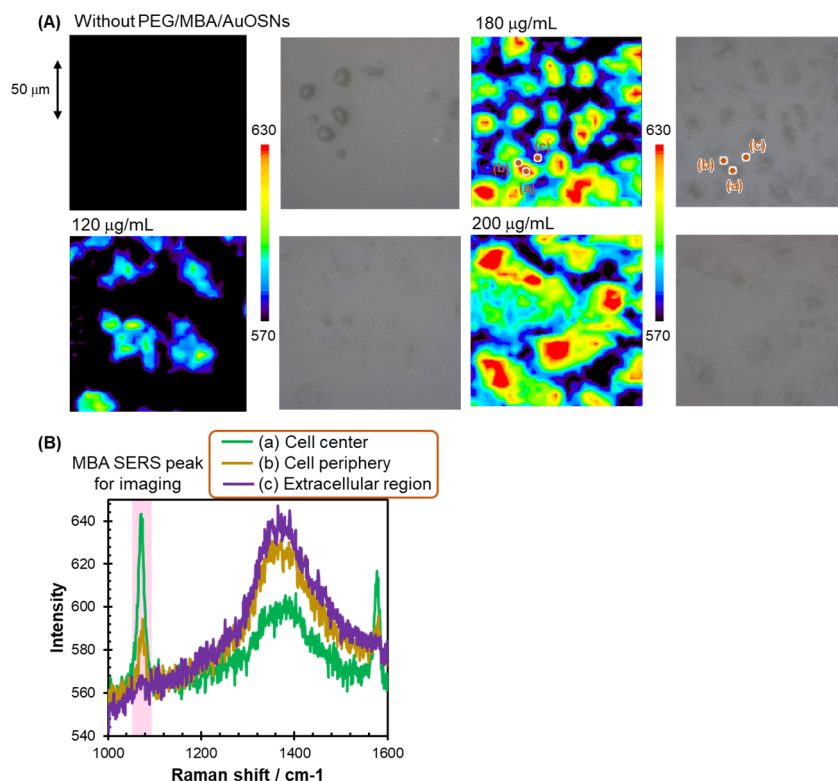


Figure 7. SERS imaging of cells using PEG/MBA/AuOSNs. (A) SERS imaging maps of HeLa cells incubated with PEG/MBA/AuOSNs at concentrations of 0, 120, 180, and 200 $\mu\text{g/mL}$. The SERS images were constructed using the characteristic MBA band at 1077 cm^{-1} on the AuOSNs. (B) SERS spectra acquired at positions (a) the cell center, (b) the cell periphery, and (c) outside the cell region in the SERS image of HeLa cells incubated with PEG/MBA/AuOSNs at a concentration of 180 $\mu\text{g/mL}$.

The spatially resolved SERS mapping revealed that the strongest SERS signals were localized in the central region of the cells. This observation suggests that the PEG-modified SERS nanoparticles were not merely adsorbed onto the cell membrane but were internalized into the cells. If the nanoparticles were confined to the cell surface, such a pronounced intensity contrast with a maximum at the cell center would not be expected. The preferential localization of SERS signals in the central cellular region is consistent with nanoparticle internalization followed by intracellular redistribution. However, pathway-specific inhibition or colocalization analyses were not performed in the present study, and therefore the precise endocytic mechanism and intracellular trafficking routes cannot be conclusively determined. Nevertheless, the observed perinuclear enrichment pattern is commonly associated with energy-dependent endocytic processes reported for nanoparticles of comparable size. Although PEGylation is widely regarded as a strategy to suppress nonspecific cellular uptake [63,64], our results demonstrate that PEG-modified MBA/AuOSNs can still be internalized by HeLa cells within a relatively short incubation time of 3 h. This observation highlights the strong dependence of PEG effects on surface composition, ligand heterogeneity, and nanoparticle architecture. The presence of 4-MBA alongside PEG likely provides residual interaction sites that facilitate cellular uptake, while PEG moderates excessive nonspecific adsorption, resulting in cellular internalization with good biocompatibility.

4. Conclusions

In this study, we developed AuOSNs with an intentionally symmetry-broken gold nanoshell structure and systematically investigated their SERS imaging properties. The AuOSNs were synthesized using a unique approach that does not require highly cytotoxic surfactants commonly employed for controlling morphological anisotropy. The resulting MBA/AuOSNs exhibited LSP resonance within the biological window, and evaluations using MBA as a Raman reporter molecule demonstrated high SERS activity under excitation in this wavelength region. Furthermore, SERS imaging using PEG-modified MBA/AuOSNs enabled clear visualization of cells, with HeLa cells serving as a model system. These results clearly demonstrate that PEG/MBA/AuOSNs are promising imaging probes that combine high SERS activity with good biocompatibility. Future evaluation of the photothermal conversion properties and reactive oxygen species generation capability of the AuOSNs is expected to extend this material platform beyond SERS imaging toward theranostic applications integrating diagnosis and therapy. In our laboratory, in vivo studies aimed at validating this potential are currently underway.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/nano16040271/s1>, Figure S1: Absorption spectra of 4-MBA before and after immersion of the AuOSNs array substrate; Figure S2: SERS spectra of AuOSNs drop-cast and dried on a glass substrate.

Author Contributions: K.S., J.O. and K.O. conceived the research theme and developed the strategic concept of the study. T.K. and Y.K. performed the biochemical and biological evaluations. H.T. conducted the theoretical analysis of the optical properties of the nanoparticles. Y.H., A.O., H.N., A.S. and T.A. carried out the synthesis of the nanoparticles and their physicochemical characterization. T.H. (Takeshi Hashimoto) and T.H. (Takashi Hayashita) evaluated the dispersion stability of the nanoparticles. All authors have read and agreed to the published version of the manuscript.

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