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Optical Probes for Biological Applications Based on Surface-Enhanced Raman Scattering from Indocyanine Green on Gold Nanoparticles

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We report surface-enhanced Raman scattering (SERS) studies on indocyanine green (ICG) on colloidal silver and gold and demonstrate a novel optical probe for applications in living cells. In addition to its own detection by the characteristic ICG SERS signatures, the ICG gold nanoprobe delivers spatially localized chemical information from its biological environment by employing SERS in the local optical fields of the gold nanoparticles. The probe offers the potential to increase the spectral specificity and selectivity of current chemical characterization approaches of living cells and biomaterials based on vibrational information.

The concept of optical probes for highlighting specific molecules or cellular structures has been very successful.[1] Current biochemical and cell biology research generates a strong need for improving optical probes regarding sensitivity, specificity, molecular structural information content, and spatial localization. In particular, probes that can deliver chemical structure information from a biological environment would be of enormous advantage. At the same time, such probes need to be chemically inert, stable, and identifiable in multilabel experiments, which implies that they need to deliver their own optical signature. In the following, we report on the feasibility of an optical probe fulfilling these requirements.

Metal nanostructures open exciting new ways to create efficient optical probes, based on the strongly enhanced spectroscopic signals that can occur in their local optical fields. One of the most impressive effects associated with local optical fields is surface-enhanced Raman scattering (SERS).[2−5] Total SERS enhancement factors can reach 14 orders of magnitude, which brings nonresonant surface-enhanced Raman signals to a level comparably to or even better than fluorescence. Unlike fluorescence, which produces relatively broad bands, Raman scattering as a vibrational, i.e., structure-specific method yields a unique spectrum composed of several narrow spectral lines, resulting in well-distinguishable spectra even for similar molecules.[5] Recently developed SERS probes provide high spectral specificity, multiplex capabilities, and photostability.[6,7]

Here we study the SERS effect of the dye indocyanine green (ICG) on silver and gold nanoparticles and propose a probe based on the SERS signal of the dye ICG on gold nanoparticles. We demonstrate the application of this biocompatible probe in living cells and discuss the potential of this kind of SERS probe for controlled targeted vibrational characterization of biological systems.

EXPERIMENTAL SECTION

Preparation of ICG SERS Samples. Colloidal silver solution for SERS studies was prepared by the reduction of silver nitrate using sodium citrate with the method published by Lee and Meisel.[8] Gold colloidal solution containing 60-nm gold spheres was purchased from Polysciences Inc. (Eppelheim, Germany/Warrington, PA). ICG (Sigma, St. Louis, MO; Acorn Inc., Buffalo Grove, IL) was dissolved in water and in 5 mg/mL aqueous solution of human serum albumin (HSA; Sigma). These solutions were prepared in concentrations between 10^{-5} and 10^{-8} M and subsequently added to the silver and gold colloidal solutions to result in different concentrations of the dye in the SERS samples between 10^{-7} and 10^{-10} M. To determine the influence of HSA on the SERS spectrum, 10^{-5} M HSA was complexed with 60-nm gold particles.

Incorporation of ICG Gold Probes by Cells. Cells of a metastatic Dunning R3327 rat prostate carcinoma line (MLL) (donated by Dr. W. Heston, Memorial Sloan-Kettering Cancer Center, New York, NY) were plated onto flamed glass microscope coverslips (Fisher Scientific) and grown for 24 h in RPMI 1640 culture medium with 10% fetal calf serum and 600 mg/mL G418 antibiotic in six-well tissue culture plates (Fisher) at a density of ∼100 000 cells/well in a humidified incubator (37 °C, 5% CO_2). At 20 h prior to the Raman experiments, the culture medium was

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replaced by a culture medium diluted in gold colloidal solution (1:5) containing 5×10^{-11} M gold nanoparticles loaded with 10^{-7} M ICG bound to HSA. Internalization of the gold nanoparticles occurred by fluid phase uptake from the culture medium. Before the experiments, the medium/gold colloid solutions were removed, and the coverslips were rinsed thoroughly with PBS containing Ca^{2+} and Mg^{2+} (Mediatech). The cells were kept in this buffer during the Raman measurements. Controls were grown in culture medium diluted in 0.1 M NaCl aqueous solution (1:5), culture medium diluted with 10^{-6} M ICG in 0.1 M NaCl aqueous solution (1:5), and in undiluted culture medium.

Raman Measurements. SERS spectra were measured using a 60× microscope water immersion objective for focusing the excitation laser. The same microscope objective was also used to collect the Raman scattered light. A single-stage spectrograph with holographic edge or notch filters in front of the entrance slit and a liquid nitrogen-cooled CCD detector were used for spectral dispersion and collection of the scattered light. Spectra of silver and gold colloidal sample solutions were measured in 25-μL droplets from a probed volume of ~50 fL. Excitation at different wavelengths (680, 786, and 830 nm) was applied.

Raster scans over single living cells were carried out with a computer-controlled x,y stage in 2-μm steps at a laser spot size of $\sim 4 \times 10^{-8}$ cm^2. All experiments on the cell culture were carried out at 830-nm excitation. Excitation intensities in all experiments were between 10^5 and 10^6 W/cm^2, not exceeding 1×10^5 W/cm^2 for measurements on the cell culture corresponding to <4 mW at the $\sim 4 \times 10^{-8}$ cm^2 spot size. Collection times for each spectrum were 1 s or less.

RESULTS AND DISCUSSION

SERS Experiments on ICG in Silver and Gold Colloidal Solutions. Figure 1 shows electron micrographs of silver and gold nanoparticles used in the SERS experiments and displays extinction curves of silver and of gold colloidal sample solutions (A and B in Figure 1b, respectively) containing 5×10^{-6} M indocyanine green bound to HSA. The spectra show the plasmon resonance peaks for silver and gold nanoparticles at 420 and 520 nm, respectively. The slight broadening of the bands to the near-infrared indicates the presence of some small 100−200-nm silver and gold colloidal clusters in addition to mostly isolated particles.

The extinction spectra (Figure 1b) show an absorption maximum of ICG around 800 nm. This is an indication that the dye mainly exists as monomer. For comparison, the inset in Figure 1b shows the same absorption maximum of 5×10^{-6} M ICG bound to HSA in aqueous solution in the absence of colloid. ICG is known to form aggregates under certain conditions.[9−11] Although the absolute dye concentration in our solutions is too low ($\leq 10^{-6}$ M) for ICG to form aggregates,[9] the local concentration on the surface of the gold and silver particles may be higher, and the presence of ions could increase the rate of aggregation.[9] Serum albumin prevents aggregation of the ICG dye.[12] In addition, it stabilizes

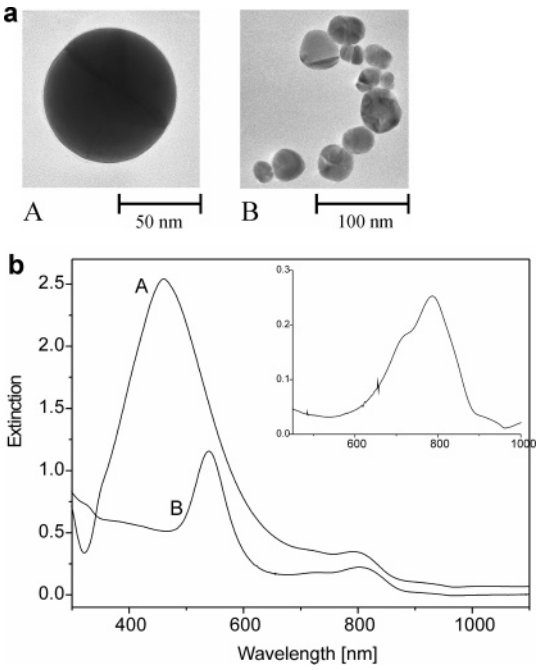

Figure 1. (a) Electron micrographs of gold (A) and silver (B) nanoparticles used in the SERS experiments. (b) Extinction spectra of silver and gold colloidal sample solutions (spectra A and B, respectively) containing 5×10^{-6} M ICG bound to HSA. The inset shows the absorption of 5×10^{-6} M ICG bound to HSA in water.

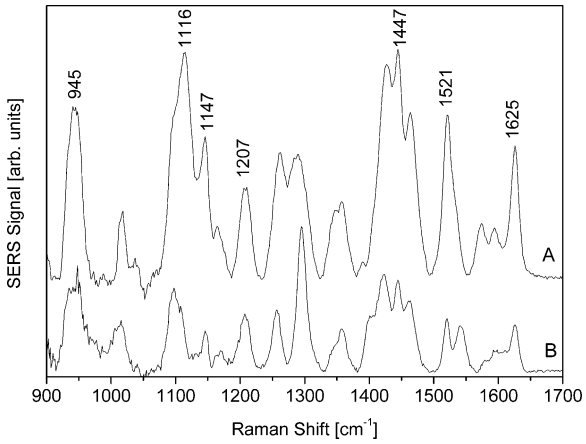

Figure 2. SERS spectra measured from ICG (10^{-7} M) bound to HSA (A) and from the pure dye (B) in gold colloidal solutions (excitation wavelength 680 nm).

the gold nanoparticles and is therefore commonly used in cell biology experiments employing such particles.[13−15]

We measured SERS spectra of 10^{-7} M pure ICG and ICG bound to HSA in gold colloidal solution (Figure 2). Both spectra show very similar SERS features. A control, consisting of 10^{-5} M HSA complexed with gold nanoparticles, did not produce a SERS signal. These findings are in good agreement with recent observations that serum albumin does not interfere with sensitive SERS

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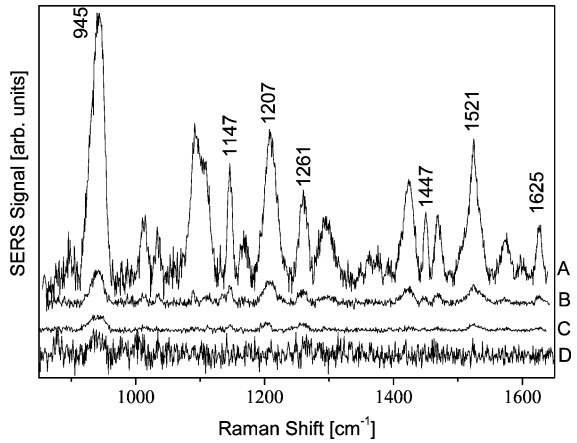

Figure 3. SERS spectra of ICG bound to HSA in gold colloidal solution at different concentrations (excitation wavelength 830 nm). Spectra A−D were collected from gold colloidal solutions containing ICG in 10^{-7}−10^{-10} M concentrations, respectively.

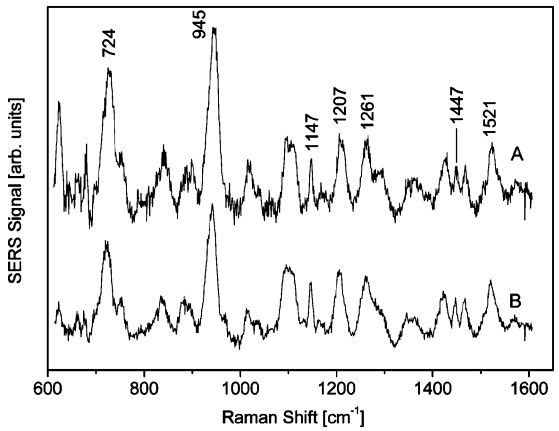

Figure 4. SERS spectra of 10^{-7} M ICG in silver (A) and gold (B) colloidal solutions (excitation wavelength 786 nm).

detection of glucose.[16] The small differences between spectra A and B are very likely associated with formation of dye aggregates in the HSA-free samples. The high similarity between the spectra measured in the presence and absence of HSA (Figure 2) indicates that the SERS signature is very robust and can be distinguished independent of the presence of ICG aggregation, making it applicable in different physiological environments (e.g., different cellular compartments).

Figure 3 illustrates the detection limits of ICG−HSA in gold colloidal solution. The strong Raman line at 945 cm^{-1} in spectrum D is still detectable at an ICG concentration of 10^{-10} M. This corresponds to an average number of 3 dye molecules in the probed 50-fL volume. Such single-molecule experiments represent very high SERS enhancement factors without averaging effect.[17,18]

We also collected spectra from silver nanoparticles and compared them to those obtained on gold. Figure 4 shows SERS spectra of 10^{-7} M indocyanine green in silver and gold colloidal solutions obtained using 786-nm excitation. Both substrates yield the same strong and characteristic ICG signatures (compare traces A and B in Figure 4). Comparing the spectra in Figures 2−4, which were obtained with excitation wavelengths of 680, 830, and 785 nm, respectively, we observe that they exhibit the same Raman frequencies. The differences in relative Raman signals in Figures 2−4 are a consequence of altered resonance conditions for ICG due to the different excitation wavelengths, which is sensible if one also compares the absorption spectrum of ICG (Figure 1b). The strong and distinct SERS spectrum of ICG attached to silver and gold nanoparticles makes it suitable as a reporter molecule in a SERS optical probe. We were not able to detect a Raman spectrum of ICG without the silver and gold colloids (even at saturation concentration of ~10^{-3} M or from higher concentrated dry samples). In those control experiments, we observed a fluorescence signal in agreement with the use of the dye as approved fluorescence label for biomedical applications.[1,19]

In our studies, silver and gold nanoparticles gave rise to the same strong SERS enhancement. However, comparing the universal biocompatibility of both metals, gold is clearly favored for biological applications.

Measurements in Living Cells Using ICG−Gold SERS Probes. Cultured cells were incubated with 60-nm gold nanoparticles loaded with ICG molecules as described in the Experimental Section. We used HSA-bound ICG for the construction of our probe to prevent ICG aggregation[12] and to stabilize the gold nanoparticles.[13−15]

The size of the gold (and silver) particles determines probe delivery/uptake into/by the cells, but also SERS enhancement, confinement of the local fields, and spatial confinement of the collected information. Our experiments show that 60-nm gold spheres do not influence the viability of the cells and also provide a strong SERS enhancement factor. Microscope inspection of the cell culture provided evidence that the cells were dividing after incubation with culture medium containing the ICG nanogold probes. While incubated with colloidal gold, the cells were visibly growing, and no evidence of cell detachment from the growth surface as a sign of cell death was found. A slightly lower density of cells after 20-h incubation was observed when compared with control cells growing in unaltered culture medium. This was probably a result of nutrient depletion, since the culture medium was diluted when the particle suspension was added. Controls in diluted medium with ICG/NaCl and NaCl-diluted culture medium showed growth rates similar to those incubated with the gold−ICG label.

Uptake of gold particles into the cells was verified by the appearance of surface-enhanced Raman signals collected from the cell and confirmed by light and electron microscopy as shown previously.[20] Figure 5 shows examples of SERS spectra measured in single living cells incubated with the ICG−gold hybrid probe at 830-nm excitation. Trace A in Figure 5a displays an ICG SERS spectrum measured from a dye−gold hybrid probe. The probe can be localized inside the cells by image reconstruction based on its specific spectral information. The images are reconstructed from Raman data collected by raster scanning over single living

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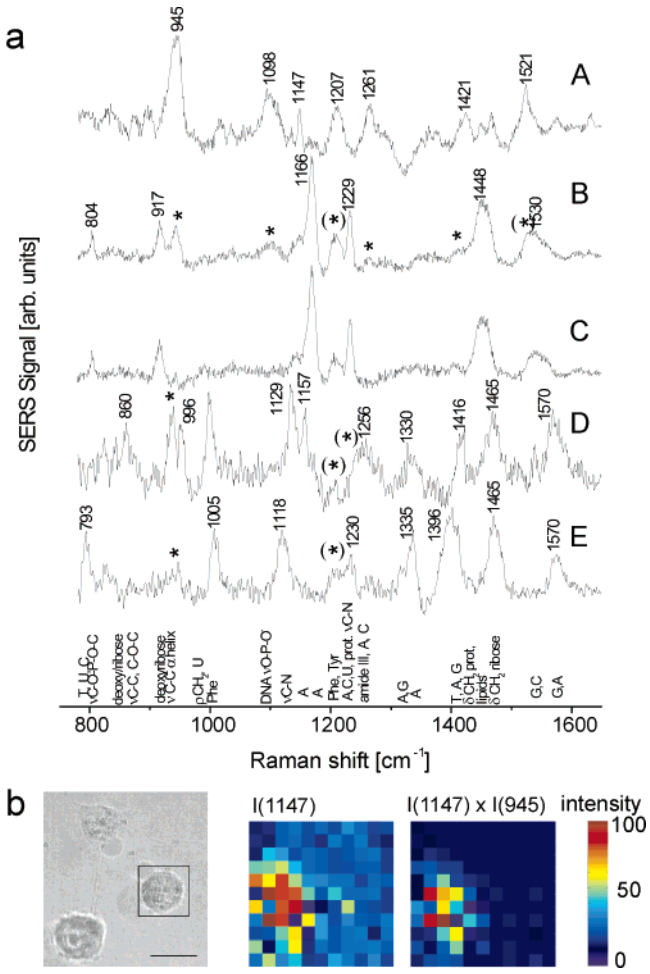

Figure 5. (a) Examples of SERS spectra measured in single living cells incubated with the ICG−gold nanoprobe at 830-nm excitation. Trace A represents the ICG signature. Assignments of major bands in spectra B−E are given below spectrum E (based on refs 21−24). ICG bands are marked with an asterisk, while an asterisk in parentheses indicates contribution of both ICG and cell. Trace C shows the difference between spectra B and A and displays only Raman lines of the cell. Abbreviations: A, adenine; G, guanine; C, cytosine; T, thymine; U, uracil; Phe, phenylalanine; Tyr, tyrosine; prot, proteins; ν, stretching; δ, deformation; ρ, rocking. (b) Spectral map of the ICG−gold nanoprobe in a cell based on 1147-cm^{-1} ICG line and on the product of two ICG lines at 1147 and 945 cm^{-1}. Intensities are scaled to the highest value in each area. A photomicrograph of the cell, indicating the studied area, is shown for comparison. Scale bar 20 μm.

cells in 2-μm steps using 830-nm excitation. As our data indicate, the SERS spectrum of ICG consists of more than 10 characteristic bands distributed over a broad frequency range. For imaging of the label, this offers the advantage that spectral correlation methods can be used to enhance the contrast between the label and the cellular background. Figure 5b shows a spectral map of the probe in a cell based on the 1147-cm^{-1} ICG SERS line and on the product of two ICG SERS lines at 1147 and 945 cm^{-1}. As illustrated by the example of Figure 5b, using two bands already increases the contrast and enables better localization of the probe. A photomicrograph of the cell, indicating the studied area, is shown for comparison.

In addition to its own detection by the characteristic SERS spectrum of the reporter dye ICG, the probe also delivers vibrational information from molecules and molecular groups in its vicinity, since they experience SERS enhancement. As our data show, SERS spectra of cell components can be measured along with SERS signatures of the reporter (traces B, D, and E in Figure 5a). The relative contributions of ICG and cell components depend on the coadsorption of both kinds of molecules. The capability to extract qualitative vibrational information on the biological matrix is illustrated in trace C (Figure 5a), which displays a spectrum of native cell components, obtained after subtraction of the ICG spectrum (spectrum A) from spectrum B.

Although the Raman shifts, relative peak intensities, and line widths with SERS may slightly differ from those in normal Raman spectra due to the interaction of the molecule with the metal and due to large field gradients, many bands show similarities with spectra collected from cell constituents in normal Raman measurements.[21−24] However, compared to normal nonresonant Raman measurements on single cells, the large effective scattering cross section in SERS allows application of very low laser powers and very short data acquisition times of 1 s and less. We were not able to measure Raman spectra of the cells without colloidal gold particles using the applied relatively low excitation powers (<4 mW) and collection times up to 100 s. Higher excitation intensities were not applied as they resulted in cell death.

The spectra in Figure 5a provide clear evidence of various vibrations originating from the DNA backbone and of C−N and ring stretching modes from DNA and RNA bases (see annotations in Figure 5a). Spectrum D contains a signature of CH_2 groups (rocking vibration at 996 cm^{-1} and deformation at 1465 cm^{-1}, the frequency of the latter suggesting that they are contained in ribose, which is also in accordance with the presence of the 860-cm^{-1} C−C stretching band associated with ribose ring breathing). Spectrum D also shows amide III contributions and C−C stretching vibrations of protein α-helices. The band at 1005 cm^{-1} in spectra D and E originates from the phenylalanine ring breathing mode. The variety of combinations of bands from different molecules in the cell can be assessed best by multivariate methods and will be discussed elsewhere in detail. Nevertheless, the few examples shown here illustrate that, in addition to the detection of the ICG signature itself, chemical probing of the cellular environment is a characteristic of the ICG−gold hybrid SERS probe.

The high molecular specificity of SERS[5] suggests the potential use of other chemically similar cyanine dyes [19,25] to construct gold nanoparticle probes with different spectroscopic signatures to enable multilabel probing, particularly when correlation techniques as suggested in Figure 5b are used. In further developments, the proposed SERS probes will be used for simultaneous targeted vibrational probing of specific cellular compartments or other biological substructures after controlled transfer of probes with different SERS reporters to these structures. In such a multilabel approach, the spectral signature of the reporter that appears along

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with the spectrum of the cell components then indicates which part of the cell or biosample the spectrum is from. Efficient methods for targeting gold particles, e.g., by using protein localization sequences, are already applied in other studies.[15]

CONCLUSION

We are proposing a robust and sensitive optical probe based on the SERS spectroscopic signature of indocyanine green on gold nanoparticles. In addition to its own detection by the characteristic SERS spectrum of the reporter dye ICG, the in vivo compatible probe also delivers local molecular structural information from its biological environment. The SERS probe can be used to develop new tools for targeted molecular probing of cells and tissues and provides a new means to improve our understanding of cellular processes on the molecular level.

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