Detection and identification of a single DNA base molecule using surface-enhanced Raman scattering (SERS)

Katrin Kneipp,* Harald Kneipp, V. Bhaskaran Kartha, Ramasamy Manoharan, Geurt Deinum, Irving Itzkan, Ramachandra R. Dasari, and Michael S. Feld

G. R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

(Received 4 November 1997; revised manuscript received 30 January 1998)

Nonresonant Raman cross sections of $\sim 10^{-16}$ cm^2 per molecule are shown to be related to surface-enhanced Raman scattering (SERS) on colloidal silver clusters at near-infrared (NIR) excitation. The enhancement is found to be independent of cluster sizes between 100 nm and 20 μm. These experimental findings demonstrate that NIR SERS on colloidal silver clusters is an excellent technique for single molecule detection that is applicable for a broad range of molecules including ''colorless'' biomolecules, for example nucleotides in DNA sequencing. As an example, we present the detection of a single adenine molecule without any labeling based on its intrinsic surface-enhanced Raman scattering. [S1063-651X(98)51106-3]

PACS number(s): 87.64.Ni, 82.65.Pa, 42.62.Be, 78.30.−j

Extremely large surface-enhanced Raman-scattering (SERS) cross sections of $\sim 10^{-16}$ cm^2/molecule or, in other words, total SERS enhancement factors on the order of 10^{14} have been obtained at near-infrared (NIR) nonresonant excitation for molecules attached to colloidal silver clusters [1,2]. Similar enhancement factors have also been reported for rhodamine 6*G* on spatially isolated silver nanoparticles at resonant visible excitation [3]. Such effective cross sections are sufficient for Raman detection of single molecules [2,3].

In this Rapid Communication, we show that extremely large SERS cross sections at NIR excitation are related to colloidal clusters as SERS-active substrates. Very strong electromagnetic field enhancement that is independent of cluster size and is particularly effective in the NIR has been theoretically predicted for such structures [4–7]. SERS experimental results reported here explain why NIR SERS in ''heterogeneous'' colloidal solutions works well as a tool for single molecule detection and why it should be applicable for a broad range of molecules. As an example, we present the detection of single adenine molecules attached to colloidal silver clusters in water.

To study the role of colloidal clusters, we compare SERS enhancement factors for crystal violet (CV) adsorbed on spatially isolated 10–25–nm spherical colloidal silver particles and on colloidal aggregates (clusters) of various sizes between 100 nm and 20 μm. The insets in Fig. 1 show typical electron micrographs of isolated spheres and small clusters [8]. Raman experiments are performed at 407-nm excitation (single-particle plasmon resonance) and at 830-nm NIR excitation. From the absorption spectrum of CV [9], it can be concluded that at these wavelengths practically no molecular resonance Raman effect contributes to the observed total enhancement.

Figure 1 compares SERS at 407-nm excitation for crystal violet on isolated small spheres [Fig. 1(a)] and on small colloidal clusters [Fig. 1(b)]. SERS enhancement is estimated to be on the order of 10^6 for spatially isolated small colloids and 10^7–10^8 for colloidal clusters by comparing the signal strength of the 1174-cm^{-1} CV SERS band and the 1030-cm^{-1} methanol Raman band, and by taking into account the different concentrations of both molecules [10]. Since ablating silver in distilled and deionized water made the isolated small colloids, no special ''chemical activation'' (except silver ions) should exist. The enhancement on the order of 10^6 is in agreement with electrostatic estimates of enhancement factors for isolated spherical silver particles [11].

At 830 nm excitation, no SERS signal is measured for molecules on small isolated spheres due to the absence of single-plasmon resonance at this wavelength [12]. However, for colloidal clusters, a tremendous enhancement appears at NIR excitation, which can be estimated from the obtained pumping of molecules to the first excited vibrational state due to the strong Raman process [1]. Vibrational pumping is reflected in deviations of anti-Stokes–to–stokes SERS signal ratios from the expected Boltzmann population. In agree-

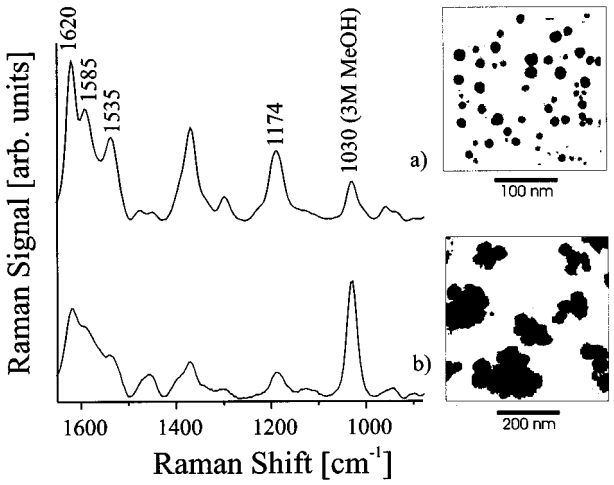

FIG. 1. SERS spectra of (a) $10^{-6}M$ CV on isolated small silver spheres and (b) $10^{-8}M$ CV on small colloidal silver clusters. Excitation wavelength 407 nm. The insets show electron micrographs of the SERS-active structures.

*Present address: Physics Department, Technical University Berlin, D-10623 Berlin, Germany.

TABLE I. Anti-Stokes and Stokes SERS; signals measured at 830 nm excitation with a power of $\sim 10^6$ W/cm^2 from CV attached to various colloidal clusters (see circles in Fig. 2). The anti-Stokes–to–Stokes ratio to toluene Raman scattering establishes the Boltzmann population.

Crystal Violet Stokes and anti-Stokes signals at 1174 cm^{-1}			
Anti-Stokes	Stokes	Ratio	Location
[cps]	[cps]		(see Fig. 2)
37	780	4.8×10^{-2}	a
53	1100	4.8×10^{-2}	b
28	545	5.1×10^{-2}	c
132	2550	5.2×10^{-2}	d
50	1000	5.0×10^{-2}	e
51	1055	4.8×10^{-2}	f
Toluene Stokes and anti-Stokes signal at 1211 cm^{-1}			
10.4	1920	5.4×10^{-3}	

of $\sim 10^{-16}$ cm^2/molecule or enhancement factors of $\sim 10^{14}$ can be inferred from measured anti-Stokes–to–Stokes signal ratios [13]. The increase of about 6 to 7 orders of magnitude for SERS enhancement on colloidal silver clusters when the excitation wavelength is shifted from 407 to 830 nm is in relatively good agreement with estimates applying electromagnetic theory from Ref. [6], explaining a mainly electromagnetic origin of the extremely large SERS enhancement. Therefore, effective SERS cross sections that are sufficient for single molecule detection should be available for a wide range of molecules, including ''colorless,'' biologically interesting molecules, for example, amino acids and nucleotide bases.

Table I gives some typical anti-Stokes–to–Stokes ratios measured from CV on clusters of different sizes shown in Fig. 2 [14]. The ratios are constant within the 10% accuracy of our measurement, implying uniform effective cross sections independent of cluster size [15]. The uniform enhancement factor is the prerequisite for exploiting SERS in solutions of ''heterogeneous'' colloidal clusters as a tool for single molecule detection, and explains the relatively well-

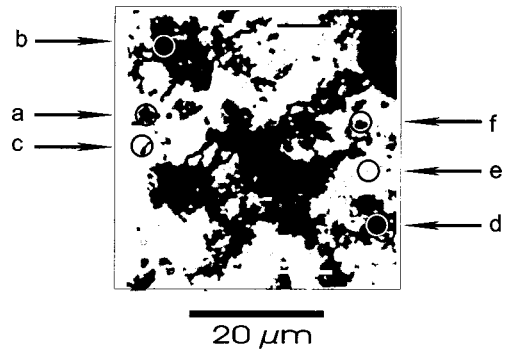

FIG. 2. Microscopic view of μm clusters [14]. The areas between them are covered with 100–500-nm (submicroscopic) silver clusters [shown in Fig. 1(b)]. The circle indicates the laser excitation site (spot size approximately 3 μm).

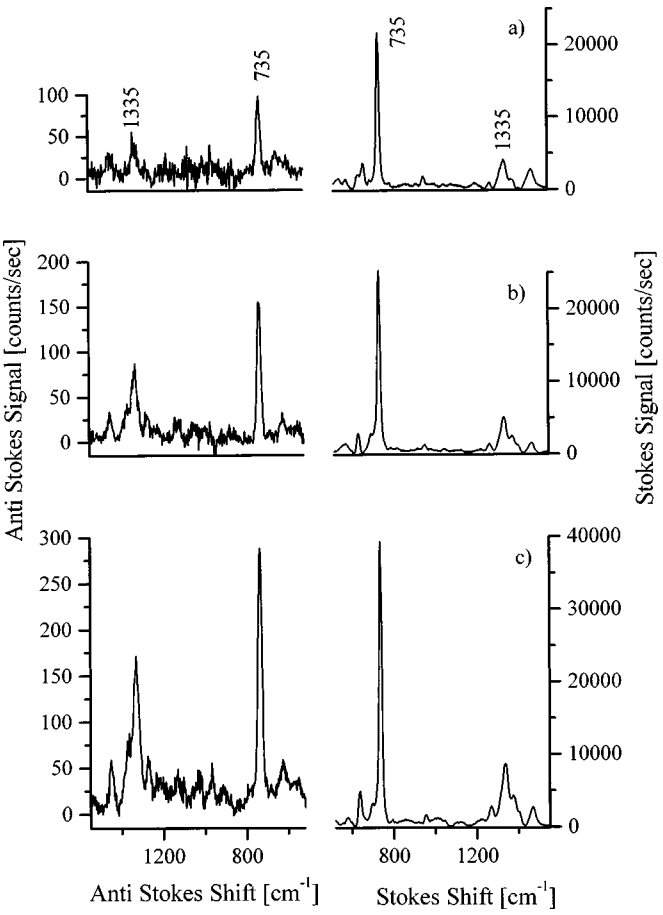

FIG. 3. (a) NIR-SERS Stokes and anti-Stokes spectra of AMP and adenine measured (b) from 100 to 150-nm sized silver clusters and (c) from a cluster about 8 μm in size. On the order of hundreds of molecules contributed to the spectra, with slightly varying numbers in spectra *a*, *b*, and *c*. All Stokes and anti-Stokes spectra were collected in 1 sec.

quantized SERS signals for one, two, and three molecules, which we found in Ref. [2] despite the nonuniform sizes and shapes of the SERS-active particles.

Due to very similar cluster formation of colloidal silver and gold [16,17] and very similar dielectric constants in the NIR [18], gold should be a useful material for NIR SERS-active clusters as well, and might provide some advantages due to its chemical inactivity.

NIR excitation has a basic advantage in single molecule detection and spectroscopy because background (fluorescence and Raman signal of the surrounding medium or solvent) is strongly decreased. Additionally, NIR excitation is nonresonant for most molecules, which should allow for use of high excitation intensities up to saturation without photobleaching. Due to shorter vibrational relaxation times compared to electronic relaxation times (in the case of fluorescence-based detection), the number of Raman photons per unit time that can be emitted by a molecule under saturation conditions can be higher than the number of fluorescence photons by a factor of 10^2–10^3 [2]. This should allow shorter integration times for detecting a molecule, or higher rates for counting single molecules, by Raman scattering rather than by fluorescence.

One of the most exciting challenges for single molecule

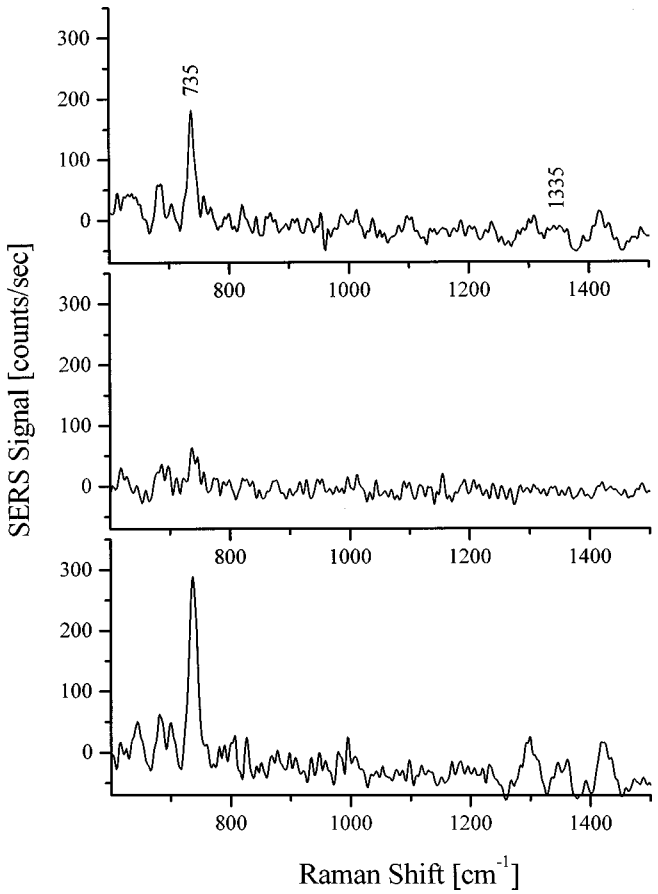

FIG. 4. Typical SERS Stokes spectra representing approximately ''1'' (top), ''0'' (middle), or ''2'' (bottom) adenine molecules in the probed volume (collection time: 1 sec; 80-mW NIR excitation).

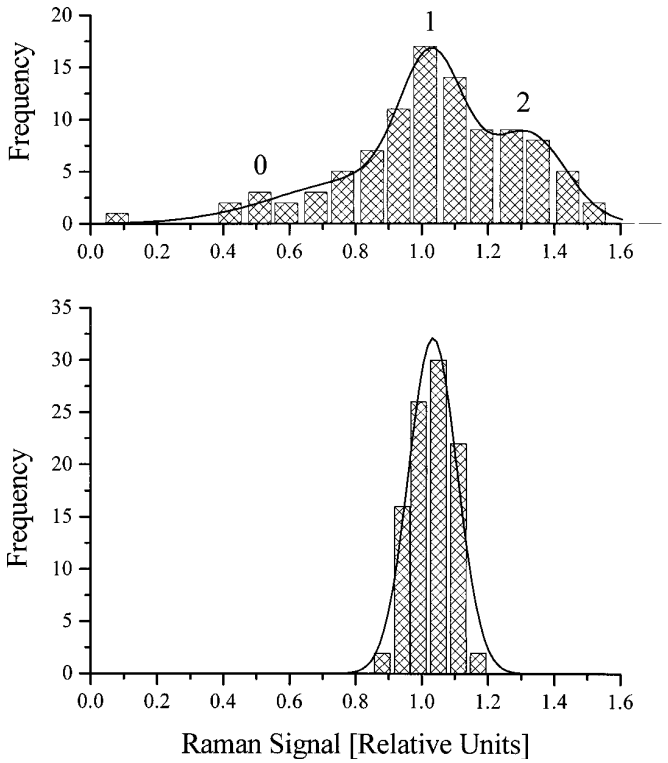

FIG. 5. Statistical analysis of 100 SERS measurements on an average of 1.8 adenine molecules (top) and 18 adenine molecules (bottom) in the probed volume. The *x* axes are divided into bins whose widths are 5% of the maximum of the observed signal. The *y* axes display the frequency of the appearance of the appropriate signal levels in the bin. The experimental data of the 1.8-molecule sample were fit by the sum of three Gaussian curves (solid line), whose areas are roughly consistent with a Poisson distribution for an average number of 1.3 molecules. As expected, the data of the 18-molecule sample could be fit by one Gaussian curve.

detection appears in rapid DNA sequencing where one approach is based upon spectroscopic detection and identification of single nucleotides. To detect and identify single DNA bases by fluorescence, they must be labeled by fluorescent dye molecules [19,20]. NIR-SERS provides a method for detecting and identifying a single DNA base, which does not require any labeling because it is based on the intrinsic surface-enhanced Raman scattering of the base.

Figure 3 shows surface-enhanced Stokes and anti-Stokes Raman spectra of adenosine monophosphate (AMP) and adenine. The spectra display the strong Raman line of the adenine ring breathing mode at 735 cm^{-1} and lines in the 1330-cm^{-1} region [21–23]. SERS spectra of adenine and AMP are identical, showing that sugar and phosphate do not prevent the strong SERS effect of adenine.

Effective Raman cross sections of the order of 10^{-16} cm^2/molecule can be inferred from the observed anti-Stokes–to–Stokes signal ratios as described above [24]. A comparison between anti-Stokes and Stokes adenine spectra, measured from clusters of various sizes between about 100 nm and 10 μm [for example, compare Figs. 3(b) and 3(c)], confirms SERS enhancement factors independent of cluster size.

Samples for single molecule detection are prepared and checked as described in Ref. [2]. The concentrations of small colloidal clusters and adenine are 2×10^{-10} M and 3×10^{-11} M, respectively, resulting in clusters containing zero or one adenine molecule [2,3]. Brownian motion of single-adenine-molecule-loaded silver clusters into and out of the probed volume results in strong statistical changes in SERS signals measured from such a sample in time sequence. Figure 4 represents selected typical spectra collected in 1 sec from samples that contain an average of 1.8 adenine molecules in a probed 100-fl volume. The drastic changes disappear for ten times higher adenine concentration when the number of molecules in the probed volume remains statistically constant. Figure 5 gives the statistical analysis [2] of adenine SERS signals (100 measurements) from an average of 1.8 molecules in the probed volume (top of Fig. 5) and from 18 molecules (bottom of Fig. 5). The change in the statistical distribution of the Raman signal from Gaussian (bottom) to Poisson (top) reflects the probability of finding 0, 1, 2 (or 3) molecules in the scattering volume during the actual measurement and is evidence that single molecule detection of adenine by SERS is achieved [25]. Comparing the 1.3 molecule fit with the 1.8 molecule concentration-volume estimate, we conclude that 70–75% of the adenine molecules were detected by SERS.

Due to the electromagnetic origin of the enhancement, it should be possible to achieve SERS cross sections at the same order of magnitude as for adenine for other bases when

they are attached to colloidal silver or gold clusters. The nucleotide bases show well-distinguished surface-enhanced Raman spectra [22,23]. Thus, after cleaving single native nucleotides from a DNA or RNA strand into a medium containing colloidal silver clusters, for instance into a flowing stream of colloidal solution [26], or onto a moving surface with silver or gold cluster structure, direct detection and identification of single native nucleotides should be possible due to unique SERS line(s) of their bases.

We are grateful to Mike Frongilio from the MIT Center for Material Science Engineering for his support in electron microscopy. This work was carried out at the NIH-supported MIT Laser Biomedical Research Center and the NSF-supported MIT Laser Research Facility.

[1] K. Kneipp, Y. Wang, H. Kneipp, I. Itzkan, R. R. Dasari, and M. S. Feld, Phys. Rev. Lett. **78**, 2444 (1996).

[2] K. Kneipp, Y. Wang, H. Kneipp, L. T. Perelman, I. Itzkan, R. R. Dasari, and M. S. Feld, Phys. Rev. Lett. **78**, 1667 (1997).

[3] S. Nie and S. R. Emory, Science **275**, 1102 (1997).

[4] V. M. Shalaev and M. I. Stockman, Zh. Eksp. Teor. Fiz. **92**, 509 (1987) [Sov. Phys. JETP **65**, 287 (1987)].

[5] V. M. Shalaev and M. I. Stockman, Z. Phys. D **10**, 71 (1987).

[6] M. Stockman, V. M. Shalev, M. Moskovits, R. Botet, and T. F. George, Phys. Rev. B **46**, 2821 (1992).

[7] V. M. Shalaev, R. Botet, J. Mercer, and E. B. Stechel, Phys. Rev. B **54**, 8235 (1996).

[8] Solutions of isolated small silver spheres were made by laser ablation of silver foil in water as described in Refs. [27,28]. We used 355 nm laser light for ablation with a pulse energy of about 50 mJ and a 10-Hz repetition rate. Solutions from small colloidal clusters (100–500 nm) were prepared as we described in Ref. [2] based on a standard citrate reduction procedure [29].

[9] I. Persaud and W. E. L. Grossman, J. Raman Spectrosc. **24**, 107 (1993).

[10] Methanol does not show enhancement of the Raman signals in colloidal silver solution and can be used as an internal intensity standard [30].

[11] D. S. Wang and M. Kerker, Phys. Rev. B **25**, 1777 (1981).

[12] Addition of NaCl to the solution of isolated spheres (NaCl concentration $<10^{-2}$, M), as it was applied for small colloidal clusters to achieve better SERS conditions [2], did not result in the appearance of a NIR–SERS signal of molecules adsorbed on small spheres excluding a strong ''chemical'' effect due to NaCl activation.

[13] SERS cross sections are inferred from anti-Stokes–to–Stokes SERS ratios $P_{aS}^{\mathrm{SERS}}/P_{S}^{\mathrm{SERS}}$ normalized to the ratio in a normal Raman experiment $P_{aS}^{\mathrm{NRS}}/P_{S}^{\mathrm{NRS}}s$ (Boltzmann population) according to Ref. [1].

$$\frac{P_{aS}^{\mathrm{SERS}}/P_{S}^{\mathrm{SERS}}}{P_{aS}^{\mathrm{NRS}}/P_{S}^{\mathrm{NRS}}}=\sigma^{\mathrm{SERS}}(\nu_m)\tau_1(\nu_m)e^{(h\nu_m)/kT}n_L+1,$$

where σ^{SERS} is the effective SERS cross section, ν_m is the vibrational frequency, τ_1 is the lifetime of the first excited vibrational state (assumed to be on the order of 10 ps), T is the sample temperature (300 K), and n_L is the photon flux density of the excitation laser beam (for typical values, see Table I).

[14] When small droplets of sample solution are dried on a microscope cover slide, dye-loaded silver clusters of various sizes are fixed on the glass slide [31] and the excitation laser can be focused onto desired μm clusters or onto areas between them that are covered with 100–500-nm (submicroscopic) silver clusters.

[15] Comparing vibrational pumping effects for various SERS active structures allows a direct conclusion on effective SERS cross sections without knowing the number of the molecules involved in the SERS process.

[16] D. A. Weitz and M. Oliveria, Phys. Rev. Lett. **52**, 1433 (1984).

[17] K. Güldner, R. Liedtke, K. Kneipp, and H. J. Eichler, Europhys. Conf. Abstracts **21**C, 128 (1997).

[18] D. W. Lunch and W. R. Hunter, in *Handbook of Optical Constants of Solids*, edited by E. D. Palik (Academic Press, New York, 1985), p. 275.

[19] R. Keller *et al.*, Los Alamos Annual Report, 1990 (unpublished).

[20] R. Keller, W. P. Ambrose, P. M. Goodwin, J. H. Jett, H. C. Martin, and M. Wu, Appl. Spectrosc. **50**, 12A (1996).

[21] J. R. Perno, C. A. Grygon, and T. G. Spiro, J. Phys. Chem. **93**, 5672 (1989).

[22] R. Sheng, F. Nii, and T. M. Cotton, Anal. Chem. **63**, 437 (1991).

[23] J. Thornton and R. K. Force, Appl. Spectrosc. **45**, 1522 (1991).

[24] The ratios of anti-Stokes–to–Stokes signals of the 1330- and 735-cm^{-1} lines normalized to appropriate ''normal'' Raman-scattering ratios were found to be 12 and 1.5, respectively, at $\sim 10^6$ $\mathrm{W/cm}^2$ excitation intensity in an approximate agreement with the values obtained for CV.

[25] Compared to our previous measurements [2], we are working at a higher concentration of the targeted molecule and colloidal clusters, and with smaller scattering volume (the focus is 2.5–3 μm, the depth of docus in $\sim$15 μm) for single molecule detection. Therefore, the average dwell time of a molecule in the probed region becomes comparable with our measurement time. This results in a broadening of the Poisson distribution.

[26] P. M. Goodwin, W. P. Ambrose, and R. Keller, Acc. Chem. Res. **29**, 607 (1996).

[27] A. Fojtik and A. Henglein, Ber. Bunsenges. Phys. Chem. **97**, 252 (1993).

[28] J. Nedderson, G. Chumanov, and T. M. Cotton, Appl. Spectrosc. **47**, 1959 (1993).

[29] P. C. Lee and D. J. Meisel, J. Phys. Chem. **86**, 3391 (1982).

[30] K. Kneipp, R. R. Dasari, and Y. Wang, Appl. Spectrosc. **48**, 951 (1994).

[31] B. Vlckova, X. J. Gu, and M. Moskovits, J. Phys. Chem. B **101**, 1588 (1997).