Spectroscopy of Single Hemoglobin Molecules by Surface Enhanced Raman Scattering

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We demonstrate the detection of molecular vibrations in single hemoglobin (Hb) protein molecules attached to isolated and immobilized silver nanoparticles by surface enhanced Raman scattering (SERS). A comparison between calculation and experiment indicates that electromagnetic field effects dominate the surface enhancement, and that single molecule Hb SERS is possible only for molecules situated between Ag particles. The vibrational spectra exhibit temporal fluctuations of unknown origin which appear to be characteristic of the single molecule detection limit.

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Single molecule spectroscopy (SMS) has the unique potential to probe how an individual molecule reacts to varying outer stimuli, and thus may provide detailed information inaccessible in ensemble averaged measurements. Until recently, optical SMS was essentially synonymous with single molecule fluorescence spectroscopy [1] and advances in this field over the past decade have indeed revealed a rich field of new physical phenomena [2]. However, compared to fluorescence, inelastic light scattering (Raman scattering), which can occur from vibrational excitations in all molecules, is a more general process which usually carries a higher information content. Although the ordinary Raman effect is extremely weak—typical cross sections are of the order $10^{-30}$ cm$^2$ per molecule—a dramatic signal enhancement may occur if the analyte molecule is adsorbed to metal particles of subwavelength dimensions. This technique, known as surface enhanced Raman scattering (SERS) [3], was recently employed by Nie and Emory [4] and Kneipp and co-workers [5,6], who demonstrated that the effective Raman cross section of small aromatic molecules could be increased by a staggering 14–15 orders of magnitude—a surface enhancement which proved enough for successful SMS.

In this Letter we present evidence of single molecule SERS for one important biological macromolecule: the oxygen transport protein hemoglobin (Hb). We find that the minimal aggregation number for effective SMS is the dimer, i.e., a pair of Ag nanoparticles bridged by a Hb molecule. We estimate the effective enhancement for such a “hot” site and compare the result with calculations of the local electric-field strength.

The SERS active silver colloid was prepared by citrate reduction of AgNO$_3$ according to Ref. [7], with NaCl (0.25 mM final concentration) added as an activation agent. The final Ag particle concentration in the solution was estimated to $\approx 3.5 \times 10^{-11} M$. Adult human Hb obtained from Sigma Chemical Co. was incubated with the Ag hydrosol for $\approx 3$ h in order to allow for efficient adsorption. In order to immobilize the SERS particles a droplet of the Hb/Ag solution was deposited on a polymer coated Si wafer [8] which was then rinsed in Milli-Q Plus purified water. For a deposition time of a few seconds, a surface with well-separated Ag particles suitable for micro-Raman spectroscopy was obtained.

Figure 1 shows extinction spectra of the Ag colloid at various Hb concentrations. The broad and asymmetric double peak is typical of the combined absorption and Mie scattering spectrum from Ag particles of a size about $D = 100$ nm [9]. Whereas the pure hydrosol was found to be stable over a period of more than six months, the addition of even minute amounts of Hb resulted in aggregation. As can be seen from Fig. 1 the dominant effect is a reduction in optical density (OD) caused by a decrease in the number of scattering centers, but already at [Hb] = $10^{-9} M$ an increase in OD at longer wavelengths signals large diameter colloidal aggregates. For even higher concentrations ([Hb] > $10^{-7} M$) the aggregation resulted in a precipitation of the colloid and the extinction spectrum became dominated by supernatant oxy/deoxy Hb absorption.

The Raman spectra were collected through a confocal optical microscope coupled to a single grating
The incident laser power was •• with the same collection time marker modes discussed in the text. All spectra were measured molecule detection limit. The vertical lines indicate the Hb intensities of the Hb and from a dense colloid SERS measurements, obtained from a Hb crystal facet 1100 \( \text{cm}^{-1} \). Excitation at 4358 nm results in a resonantly enhanced Raman spectrum dominated by in-plane porphyrin ring modes of the heme groups covering the frequency range 1100–1700 cm\(^{-1} \) [10].

In Fig. 2 we show a collection of Raman spectra from hemoglobin. Spectra A and B are “ordinary” Raman and SERS measurements, obtained from a Hb crystal facet and from a dense colloid film incubated with a surplus of hemoglobin, respectively. The positions and relative intensities of the Hb “marker” modes in A (at about 1375, 1586, and 1640 cm\(^{-1} \)) agree well with published data on met-Hb [10], whereas the positions in B indicate a transition to oxy/deoxy Hb [10], induced by the reduction agent present in the Ag hydrosol.

In order to reach the SMS limit it is necessary to use as low a concentration of analyte molecules as possible. Here we used [Hb] = 1 \( \times 10^{-11} \text{M} \), yielding a Hb-molecule-to-Ag-particle ratio of Hb:Ag \( \sim 1:3 \). This should ensure that only a very small number of Hb molecules adsorb to each investigated Ag particle. At this very low Hb concentration we find that only a few hot sites give rise to any detectable SERS intensity, in agreement with the study of Nie and Emory on rhodamine 6G [4].

Spectra C and D in Fig. 2 are examples of a time series of successive SERS spectra obtained from two such hot sites. The majority of the spectra (e.g., C2, C4 and D1, D3) are in general agreement with the high-concentration SERS (B) and crystal Hb spectra (A), although there are clear differences in the precise peak positions and relative intensities. However, the most striking difference concerns the variation with time. Whereas spectra A and B are stable in time, the two time series obtained at extremely low analyte concentrations exhibit striking spectral fluctuations. In C2–C4, for example, a set of new intense peaks suddenly appears and disappears. A similar effect occurs in D1–D3, where a new peak at 1150 cm\(^{-1} \) seems to correlate with a broadening of the Hb marker modes. At present we are not able to present a detailed explanation of these temporal fluctuations. What is clear, however, is that their mere existence signals a departure from the normal time-invariant ensemble average. Together with the extremely small number of Hb molecules expected for each Ag particle complex, this strongly indicates that we have reached the single molecule detection limit.

Let us now consider the identification of the hot sites. After the Raman measurements of a particular sample were completed, the sample was brought to the scanning electron microscope (SEM) or atomic-force microscope (AFM) for inspection. Each individual Ag particle complex could easily be found and imaged by using markers on the Si wafer. The results of these investigations can be summarized as follows: (1) the Ag sol incubated with a 1 \( \times 10^{-11} \text{M} \) Hb solution for 3 hours contained approximately 80% single particles, 15% dimers, and 5% triplets or larger aggregates, (2) of the more than 100 hot sites examined none consisted of a single Ag particle, and (3) the majority of the dimers/triplets found on SMS surfaces by SEM/AFM were hot in Raman. Moreover, among the hot dimers, those with their dimer axis oriented parallel to the incident electric field consistently produced the highest SERS intensity. In Fig. 3 we show some examples of Ag aggregates found in these investigations.

Although these findings might seem surprising at first glance, they are qualitatively anticipated from the

![FIG. 2. Confocal Raman spectra of crystalline met-Hb (A), dense layer of Hb/Ag aggregates (B), and two time series (C1–C6 and D1–D6) of “hot sites” obtained at the single molecule detection limit. The vertical lines indicate the Hb marker modes discussed in the text. All spectra were measured with the same collection time (30 s) and collection efficiency. The incident laser power was 1 mW in A and 1 \( \mu \text{W} \) in B–D.](image-url)
the vibrational frequency are denoted by $\omega_v$. The potential $\phi$ entering the integral equation

$$\int G(\vec{r}, \vec{r}')\nu(\omega, \vec{r}')\vec{E}^L(\vec{r}', \omega) d\vec{r}'$$

where $G(\vec{r}, \vec{r}')$ is the tensor Green function relating the electric fields at points $\vec{r}$ and $\vec{r}'$. The potential $\nu(\omega, \vec{r}')$ is set to $(\omega/c)^2(\epsilon_0 - \epsilon_A(\omega))$ inside the Ag particle and zero outside. With the dielectric constant of silver taken from Ref. [13] and $\epsilon_0 = 1$ we have calculated $M$ for the midpoint between two spheres of diameter $D$ separated by a distance $d = 5.5$ nm, i.e., the diameter of a Hb molecule. The calculations were performed according to the scheme developed by Inoue and Ohtaka [11], and we refer the interested reader to this article for mathematical details.

Figure 4 shows the results for $D = 60, 90,$ and $120$ nm and $\vec{E}^l$ either parallel or perpendicular to the dimer axis. For simplicity we have assumed a negligible Raman shift, i.e., $\omega_v = 0$. We also show the enhancement at a point situated $d/2$ nm outside an isolated sphere as a comparison. While the single particle enhancement is strongly peaked near the surface plasmon dipole resonance at about 400 nm, the dimer exhibits a second long-wavelength peak for the parallel configuration. As outlined by, e.g., Gersten and Nitzan [12], this second peak can be seen as the multipole broadened “bonding orbital” resulting from the mutual repulsion between the two single sphere dipole resonances. As can be seen from Fig. 4, the enhancement for the dimer system is approximately 4 orders of magnitude larger than for the single Ag sphere, if both are excited in the most favorable configuration. In contrast, excitation of the dimer in the perpendicular configuration results in an exclusion of the field between the spheres and consequently an enhancement factor below unity. In the inset of Fig. 4 we show the variation in enhancement with $D$ for
the dimer in the parallel configuration. Here we have used 
\( \omega_1 = 514.5 \text{ nm} \) and \( \omega_v = 1500 \text{ cm}^{-1} \) in order to sim-
ulate the experimental situation. We find that the maximum 
enhancement occurs for spheres with a diameter of about 
90 nm, in excellent agreement with the hot particle dimen-
sions we observe experimentally. The question of inter-
est is now whether the theoretically estimated maximum 
enhancement, \( M = 6 \times 10^6 \) from Fig. 4, is in agreement 
with an experimental estimate. From Fig. 2 we see that the 
intensity of the Hb marker modes are of similar magnitude 
in spectrum A and D1. As the collection efficiency is the 
same in both cases and the incident laser power is known, 
\( M \) is simply determined by the number of Hb molecules 
contributing to the total scattered intensity. For an illumi-
nation area of 1 \( \mu \text{m}^2 \) and a collection/illumination depth 
of 2 \( \mu \text{m} \), these numbers are of the order \( N_A^1 = 1 \times 10^7 \) 
and \( N_{D1}^1 = 1 \), where we have assumed a close packed ar-
rangement of Hb molecules in A and the SMS limit for 
spectrum D1. By taking into account the factor \( 10^3 \) dif-
ference in illumination intensity between A and D1, we 
\( \text{obtain } M_{\text{SMS}} = 10^{10} \), i.e., more than 3 orders of mag-
nitude higher than our calculated value for the ideal case. 
However, the field enhancement also depends critically on 
the distance \( d \) between the Ag particles. Changing \( d \) from 
5.5 to 1 nm (with constants \( \omega_1, \omega_v, \text{ and } D \)) results in an 
increase in \( M \) from \( \approx 6 \times 10^6 \) to \( \approx 2.5 \times 10^{10} \). It is thus 
clear that the experimentally estimated enhancement fac-
tor is well within the reach of the electromagnetic model, 
even though we can not exclude that additional “chemi-
cal” charge transfer effects [14] contribute. One should 
also note that deviations from perfect sphericity may well 
increase the local field above that calculated above [3,12]. 
Calculations of these more complicated effects are under-
way and will be presented elsewhere.

Let us now briefly return to the temporal fluctuations 
described above. We note that spectral fluctuations of 
unknown origin were also observed in the single mol-
cule SERS study of rhodamine 6G by Nie and Emory [4] 
and that similar effects are well known from SMS fluores-
cence studies (see, e.g., Ref. [2]). In the present case, it is 
unclear whether the effects are signs of spontaneous var-
iations in the protein conformation and adsorption state, 
or whether photoinduced processes are involved. In the 
case of photochemical effects, which may be expected 
for illumination near the \( \pi-\pi^* \) Hb resonances, it is likely 
that both the electric-field enhancement and chemisorption 
processes could affect the photochemical yield [12]. It is 
also possible that photonic forces induced by the electric-
field gradients near the Ag surfaces are contributing to 
the fluctuations. It is clear that these effects could lead 
to conformational changes and, possibly, to the eventual 
disintegration of the protein, and therefore require consid-
erable attention in future SMS studies by SERS.

In summary, we have obtained vibrational Raman 
spectra from single hemoglobin molecules attached to 
100-nm-sized immobilized Ag particles. Our results 
indicate that Ag particle aggregation is a necessary 
condition for this effect, and that the observed protein 
molecules actually bind the Ag particle clusters together. 
We estimate the effective surface enhancement factor to 
\( M_{\text{SMS}} = 10^{10} \), and argue that the dominant part of this 
enhancement is due to an increase of the local electric 
field between the Ag particles. The spectra exhibit 
temporal fluctuations which appear to be characteristic for 
the single molecule detection limit.

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[2] For a review of the SMS field, see Single-Molecule 
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T. Basché et al. (VCH, Weinheim, 1996).
[8] We used a self-assembled monolayer of APTMS, as 
described by R. G. Freeman et al. [Science 267, 1629 (1995)].
[9] For a review, see U. Kreibig and M. Vollmer, Optical 
[10] For a review, see T. G. Spiro, in Iron Porphyrins, Part II, 
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