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Novel Chiroptical Analysis of Hemoglobin by Surface Enhanced Resonance Raman Optical Activity Spectroscopy

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ABSTRACT The metalloprotein hemoglobin (Hb) was studied using surface enhanced resonance Raman spectroscopy (SERRS) and surface enhanced resonance Raman optical activity (SERROA). The SERROA results are analyzed and compared with the SERRS, and the later to the resonance Raman (RRS) performed on Hb. The SERRS measurements careful optimization, with respect to the concentration and volume ratio of the analyte to colloids, enables for the first time SERROA of this molecule. We observed that the most intense SERROA signals were attributed the ν_4, ν_{20}, and ν_{21} vibrations, which are sensitive to the redox state of the heme's iron ion, and to the presence of its sixth site, bound to exogenous ligand; O_2, NO or CO. However, in this study, the SERROA signals corresponding to these vibrations appear more sensitive to the Hb oxygen-binding properties than they appear in the SERRS or RRS. Moreover, the SERROA signal of Hb has successfully been monitored as a function of time, and was observed to be stable for 4–5 min. To our knowledge, the SERROA results of Hb, and its comparison to SERRS and RRS, are here reported for the first time. *Chirality 21:S307–S312, 2009.* © 2010 Wiley-Liss, Inc.

KEY WORDS: SEROA/SERROA; chirality; hemoglobin; resonance raman spectroscopy; SERS/SERRS

INTRODUCTION

Raman optical activity (ROA) is a vibrational spectroscopical tool, which mainly determines the chirality of molecules by measuring the tiny difference in the Raman scattered signal by right-circular and left-circular polarized light. This tool has shown to be more sensitive to the backbone of a molecule than the side chains, whereas conventional Raman on the other hand is very sensitive for. Therefore, ROA and Raman scattering are considered complementary in full studies of molecules, especially in biology.[1,2] However, in spite of its wealth of information, provided for the folding, secondary and tertialy structures, ROA remains less widely spread than Raman, because the ROA signal is three to four orders of magnitude weaker than the Raman signal. This is because of the measured intensity difference, which is called the circular intensity difference, CID, usually referred to as the dimensionless Δ.

The surface enhanced Raman scattering (SERS) on the other hand provides much stronger signal, when a molecule is found in the vicinity of a nanoparticle. SERS was observed by Fleischmann and co-workers.[3] on pyridine adsorbed on a roughened silver surface, and it was first identified independently by Jeanmaire and Van Duyne,[4] who attributed it the charge transfer taking place between the molecule and the nanoparticle, and Albrecht and Creighton[5], who described it because of the electromagnetic effect. SERS has during the last couple of decades been applied for all kinds of samples, making use of its capability of measuring and detecting structural changes in concentrations below the micromolar level.[6–10] In the following study, we succeeded to combine the SERS method, using colloid solution of silver nanoparticles (Ag NPs), with ROA. The combination of these two methods, into surface enhance Raman optical activity (SEROA), was first treated theoretically by Efrima[11,12] who has shown that such combination could be applicable, and enhancement could be achieved in the chiral signal. In spite of these studies and the following theoretical developments of the method,[13–16] no reliable experimental study has verified the method, although two reports[17,18] have addressed SEROA, but the results were not convinc-

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ing because of the signal-to-noise ratio. The proceedings of abstracts of the[19] International Conference on Raman spectroscopy ICORS 2004, Australia, reported the first reliable experimental observation of SEROA [Abdali, ICORS IXX Proceedings]. However, it was not until 2006, when measured SEROA on the pentapeptide enkephalin was published.[19] This was followed by a series of reports, where the first one,[20] studied myoglobin, showed the sensitivity of SEROA due to the circular polarized light, which makes the measurement more sensitive than the case for SERS. This has required optimizing of the SERS effect with respect to the measured matrix, composed of the sample three components; the analyte, the colloids and the aggregating agent. The optimization showed that there was a strong interplay correlated to the concentration of each component in the measured sample, and by first obtaining the best SERS signal, SEROA was achieved. Additionally, the optimization process was later repeated on cytochrome c (cyt-c),[21] and the optimized conditions of SERS successfully provided another SEROA spectrum, adding to that of enkephalin and myoglobin.

The SEROA applied on myoglobin has also shown high sensitivity compared with other powerful methods, e.g., X-rays, when a SEROA spectrum of this molecule was compared to a spectrum of a mixture of myoglobin and azide.[22] In this study, the authors proved that SEROA could be an excellent method for studying the functionality, as the effect of the ligand binding was clearly detected in the SEROA spectrum.

Hemoglobin is one of the most important heme-containing proteins. Its significant role in the organism and the challenging quaternary structure make Hb an attractive subject in many studies. As it is known, Hb consists of four subunits (two α and two β) and each of the subunits have a protein (globin) part and a heme–the porphyrin ring with iron (Fe) ion.[23] The porphyrin is composed of four heterocyclic pyrrol rings connected to one another by methine bridges ($=C_mH-$). The iron atom is coordinated by six sites, composed of four nitrogen atoms of the porphyrin, the imidazole nitrogen atom of a histidine and the sixth site for binding of exogenous ligand; [oxygen (O_2), nitric oxide II (NO) or carbon monoxide (CO)]. Raman spectroscopy was shown to be a very sensitive technique to study the conformations of globin and porphyrin, the presence of the ligand and the binding of the modulator molecule (2,3-diphosphoglycerate and its synthetic analogues, CO_2 or H^+).[24–27] Studying the Hb properties inside a living erythrocyte under normal pathological conditions has also been shown possible by using the resonance RS (RRS).[28–30] Isolated Hb has also been studied by SERS,[8,31] and in both reports the Ag colloids were prepared by reduction of Na-citrate,[32] and they employed 514 nm excitation laser. This excitation lies in the shorter wavelength of the Hb absorption, yielding preresonance Raman, while longer wavelength, e.g. 532 nm would provide better resonance conditions of this molecule. We have in the following demonstrated that more informative SERRS spectrum of the Hb could be achieved by using higher excitation wavelength than that observed before, i.e. 514 nm.

Obtaining SERROA signal of Hb is a very challenging task, but such spectrum provides additional information about changes in the Hb conformations, the iron ion state, and the effect of modulator molecules. We report here on the employment of an optimization method, by which it was for the first time possible to obtain SERROA (and SERRS) spectra of Hb, thus, it could be considered as another novel SERROA observation of a metalloprotein, besides the earlier SERROA studies of Mb[20] and cyt-c.[21]

MATERIALS AND METHODS

Ag NP Colloids

The Ag NPs were prepared by the reduction of $AgNO_3$ with hydroxylamine hydrochloride (HHCl) in NaOH solution, following primarily the method of Leopold and Lendl.[33] However, modification of this method was found necessary to achieve stable colloids, as reported earlier.[21] These colloids showed reproducibility and stability up to three months, a period of time that allowed consistency for the measurements.

Hb Preparation

In all the following experiments, Hb was freshly isolated from the cytoplasm of rat erythrocytes (male Wistar rat line). First, to obtain erythrocyte suspension, rat blood was centrifuged for 3 min at 14,000 rot/min, equivalent to 4500 g, and the blood plasma was then removed. 100 μL of erythrocyte suspension was then diluted in 1 mL phosphate buffer saline (PBS) of composition; mM: 4 Na_2HPO_4, 1 NaH_2PO_4; pH 7.2. All chemical were purchased from Sigma in the purest grade available. The PBS with specified salt composition represents hyposmolar conditions for erythrocytes, which cause water influx into the erythrocyte cytoplasm, erythrocyte swelling and then disruption of the plasma membrane and erythrocyte lysis. As a result, PBS contains Hb molecules leaks from the erythrocytes, containing remains of the erythrocytes. Further centrifugation for 10 min was carried out in order to separate the supernatant with isolated cytosolic Hb from the remains of the erythrocytes containing the plasma membrane and submembrane Hb. The isolated cytosolic Hb was then used for the RRS, SERRS, and SERROA measurements. For RRS measurements, we used supernatant that was diluted 4 times, and for SERRS and SERROA measurements, the supernatants were diluted 200 times. The approximate concentration of Hb in the samples used for RRS was estimated to lie between 10^{-5}–10^{-6} M, and for the SERRS and SERROA measurements was estimated to lie between 10^{-7}–10^{-8} M.

RRS, SERRS, and SERROA Measurements

All the spectra were recorded using ChiralRaman instrument (BioTools), which utilizes a 532 nm laser source. Considering the absorption by Hb at this wavelength, the resonance conditions were fulfilled and RRS, SERRS, SERROA spectra were achieved. The instrument contains two shutters: the incident shutter that is mounted in the incident beam path to protect the sample, especially the biological samples, from any induced heat by the laser, and the scattering shutter that is mounted in the scattered path of the beam to minimize the noise at the CCD. Full

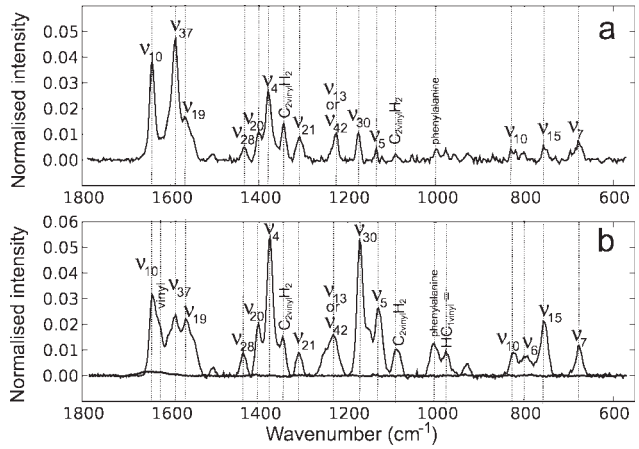

Fig. 1. (**a**) RRS spectrum of Hb, in which the main vibrational modes are shown. (**b**) SERRS spectrum of Hb, in which the same vibrational modes can be seen, with different intensity, but mainly at the same positions as in RRS (a). The flat spectrum (in grey) in the bottom of SERRS spectrum is the background/reference spectrum, representing SERRS of a mixture of water and Ag NPs solution, equivalent to the amount in SERRS for Hb (black line). The intensity in RRS and SERRS spectra are normalized to the sum of intensities in the 1618–1503 cm^{-1} region in each spectrum. The spectrum of the mixture of water with Ag NPs (gray line) was also normalized to the sum of intensities in the 1618–1503 cm^{-1} region of the SERRS spectrum of Hb. The Peak assignments were done according to earlier experimental and theoretical studies.[21,35–37]

description of the instrument can be found elsewhere.[34] Samples were measured in 1.5 mL glass-cells (Hounisen, DK), illuminated directly by a beam of 15–20 mW power, focused by a lens (1–2 mm), as this instrument does not employ any microscope.

Previously, we had found that the Ag NP volume content in the sample had a great influence on SERRS spectra of myoglobin.[20] In this study, we carried out a similar iterative optimization, i.e. varying the volume ratio of Ag NP solution to Hb in the solution. The volume ratio [Ag colloid]:[Hb solution] = 2:3 was found to provide pronounced and clear SERRS spectra of Hb. However, slightly different ratio (±5% v/v) shown to provide a slight enhancement of the SERRS signal, but possible damaging effect of higher Ag NP concentration on Hb could take place. Therefore, the ratio of 2:3 was chosen.

SERRS and SERROA spectra were recorded directly after the mixing of Hb solution with Ag NP colloid. Each of the RRS and SERRS spectra was the result of 10 accumulations recorded for 10 s.

RESULTS AND DISCUSSION

To demonstrate that Ag NPs do not cause any artificial shift of the peaks of the SERRS spectrum, a comparison of RRS and SERRS spectra of Hb is presented in Figure 1a and b, respectively. As absolute intensities of Raman and SERS signals depend on the concentration of the studied molecule, it seems helpful to perform normalization of the intensity of the reference peak (or group of peaks) or over a certain spectrum region. Therefore, we have normalized each spectrum to the sum of the intensities in the region 1618–1503 cm^{-1}. This region was chosen because it originates from certain vibrations of the heme bonds in the Hb molecules; hence, it is proportional to the total amount of Hb. Here, it is worth mentioning that the "Hb marker" peak at 1375 cm^{-1} (ν_4) in SERRS and RRS spectra before normalization had approximately the same absolute intensity. Assignments of the peaks in the RRS spectrum of Hb and their attributions to vibration of certain heme bonds and the sensitivity to ligands were done according to earlier studies.[21,35–37] Analysis of RRS and SERRS spectra of the Hb shows that most of the peaks have the same positions, which indicates the absence of artifact peak shifts caused by Ag NPs. The reference spectrum of a mixture of Ag NP colloids and water only can also be seen in Figure 1b (grey), which shows clearly that the SERRS peaks are attributed to the Hb in the sample. However, RRS and SERRS spectra display significant difference in the overall shape. For instance, there are more details in the SERRS spectrum and they appear with higher intensities, e.g. in the frequency region between 1200–600 cm^{-1}, than in the RRS spectrum. The dominant peaks in SERRS spectrum are those at 1375 cm^{-1} (symmetric pyrrol half-ring vibrations, ν_4) and at 1172 cm^{-1} (asymmetric pyrrol half-ring vibrations, ν_{30}), whereas in the RRS spectrum the dominant peaks appear at 1640 cm^{-1} (C_aC_m, C_aC_mH, C_aC_b bond vibrations, ν_{10}) and at 1588 cm^{-1} (C_aC_m, C_aC_mH bond vibrations, ν_{37}) appear with the highest intensities. The SERRS peak at 1640 cm^{-1} (ν_{10}) has a shoulder at 1623 cm^{-1} (vibration of vinyl residue of heme), which is absent in the RRS spectrum. A similar difference was observed by Han et al. in their SERRS study of myoglobin.[38] They showed that the intense 1640 cm^{-1} peak in the RRS spectrum of Mb disappeared in SERRS spectrum, but a new peak at 1623 cm^{-1} appeared. Moreover, in SERRS spectrum of Hb we observed a peak with a maximum at 974 cm^{-1} (vibration of = HC_{1vinyl} bonds in the vinyl radicals of the heme), which can hardly be seen in the RRS spectrum.

High dilution of the Hb sample causes its oxygenation by O_2 in PBS, and therefore all peaks in RRS and SERRS spectra are attributed to the vibrations of heme bonds in oxyhemoglobin (HbO_2). The observed difference between RRS and SERRS spectra of Hb is not surprising, and attributed the plasmon enhancement, in SERRS, of vibrational transitions that are weak or even unpronounced in the RRS.[39,40]

The SERRS spectrum, discussed above will be in the following compared with the SERROA spectrum of the Hb, and both spectra can be seen in Figure 2a and 2b, respectively. Several pronounced peaks are seen, with maxima at 1662 cm^{-1}, 1640 cm^{-1}, 1401 cm^{-1}, 1375 cm^{-1}, 1305 cm^{-1}, 974 cm^{-1}, and 774 cm^{-1}. Most of these peaks appear in SERROA at the same wavenumber as the corresponding SERRS peaks and, therefore, it is natural to conclude that the SERROA peaks are originated from the heme bonds. It seems that both bonds of pyrrol rings and methine bridges (C_mH) can give SERROA signals, thus, the SERROA peak at 1375 cm^{-1}, with the highest absolute intensity, and the peak at 1401 cm^{-1} can be attributed to the symmetric pyrrol half-ring vibration (ν_4) and the pyrrol quarter-ring vibration (ν_{20}), respectively. Interestingly, the

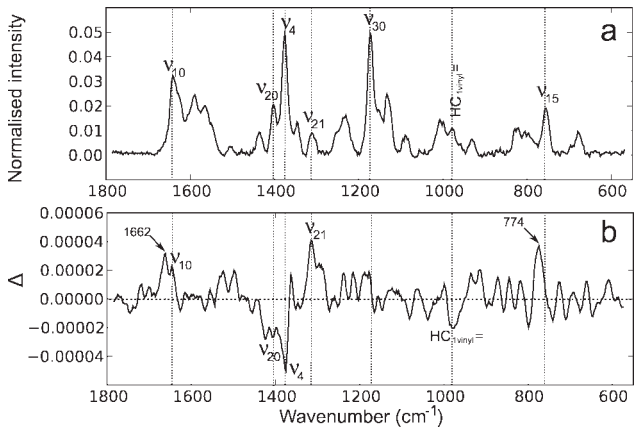

Fig. 2. (**a**) SERRS spectrum of Hb (from Fig. 1b), in which the main vibrational modes are shown.(**b**) SERROA spectrum of Hb, with acquisition time of 1 min. The intensity Δ is the dimensionlesscircular intensity difference (CID), measuring the difference in the scattered signal by right- and left-polarized light. The vertical dotted lines, go through both spectra, are drawn to show the main peaks position in both spectra, presenting better comparison.

asymmetric pyrrol half-ring vibration, producing the intensive SERRS peak at 1172 cm^{-1} (ν_{30}), seen in Figures 1b and 2a, does not give any SERROA signal (Fig. 2b). Peaks at 1640 cm^{-1} and, possibly, at 1662 cm^{-1} can be attributed the methine bridge (C_aC_m, C_aC_mH bonds) and to the C_aC_b bond vibrations. The SERROA peaks at 1305 cm^{-1} and 974 cm^{-1} may correspond to the vibrations of CmH and pyrrol bonds (ν_{21}) and to the vibration of = HC_{1vinyl} bond in the vinyl radicals, respectively. The pronounced SERROA peak at 774 cm^{-1} can result from the pyrrol breathing (in SERRS spectra, the ν_{15} peak, the pyrrol breathing vibration, appears at 754 cm^{-1}). It is well known that vibrations of pyrrol bonds, especially pyrrol half-ring vibrations, are very sensitive to the redox status of the heme Fe ion, and to the presence of the sixth ligand (O_2, NO, CO).[24,29,30,35] Hence, it is possible that any change in Hb saturation with O_2 will be manifested in changes in the intensity or the position of the ν_4 peak. However, specific designed experiments are needed to verify the sensitivity of SERROA signal (over SERRS) to specific changes in Hb conformation and oxygenation.

Comparing the SERROA spectrum of the Hb, seen in Figure 2b, to earlier reported SERROA spectra of Mb and cyt-c metalloproteins,[20,21] it is noticeable that these proteins share similar features. The peak features around the ν_4 peak in the region 1390 cm^{-1} to 1360 cm^{-1} are similar in the SERROA spectra for the three metalloproteins. The same can also be seen for the peak features around 774 cm^{-1} and around the ν_{15} peak, resulting from the pyrrol breathing. These features, and those typically of the heme, mentioned earlier in this section, show strongly that the SERROA signal of Hb is attributed to the molecules intrinsic property.

The intensity and stability of the SERROA signal was also studied as a function of time, and monitoring of the spectrum for 4 min can be seen in Figure 3. This is the first time such study is experimentally reported, and here we noticed that the SERROA signal suffered changes much faster than the SERRS of the same molecule, as no changes were seen in the SERRS spectrum, shown in Figure 2a, for more than 40 min. However, the acquisition time of the SERROA is adequate for most analyses and applications, and more stability is usually not needed when surface enhanced effect is performed. Nonetheless, the degradation of the signal in the SERROA is natural, because of the sensitivity of this method to the geometry of the molecule-surface, as it has earlier been seen experimentally,[20,21] and recently it has theoretically been shown valid.[41] The SERROA signals are more stable for these metalloproteins because of the orientation of the heme, which may have been insulated by the surrounding protein from other geometries, which otherwise could have yield faster degradation of the signal.[41] Such degradation can clearly be seen in the ν_4 peak at 1375 cm^{-1} and in the ν_{10} peak at 1640 cm^{-1}. On the other hand, all other peaks seem stable due this period of time, especially the peaks at 774 cm^{-1} corresponding to the pyrrol breathing, and the peak at 1662 cm^{-1} corresponding to the methine bridge (C_aC_m, C_aC_mH bonds) and to the C_aC_b bond vibrations, as aforementioned. The results shown in Figure 3 are of great importance for further study of the SERROA effect, which is lately noticed to acquire more interest.[41,42]

Further studies of SERROA spectroscopy of, e.g. absolute configuration and properties of proteins at low concentrations, with an immense reduction of acquisition time, e.g., to few minutes compared with hours of acquisition normally used in ROA, can find different important applications, and can be used as a new analytical/diagnostic tool of biological macromolecules. Another possible advantage of SEROA/SERROA is that they could be more sensitive than SERS/SERRS or conventional Raman spectroscopy to the conformational changes of proteins. Therefore, the method may be used for early diagnostics of diseases and cell pathologies induced by change of protein conformation.

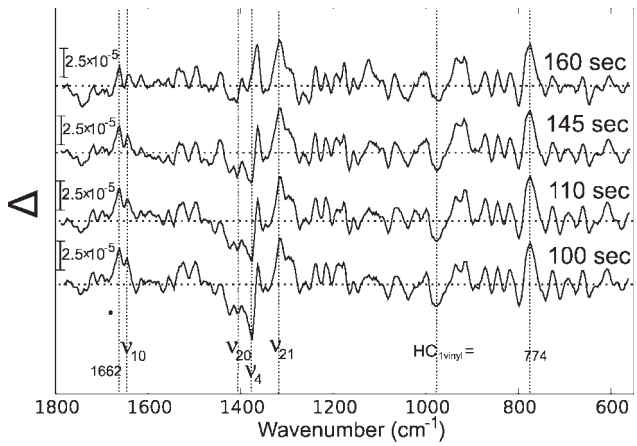

Fig. 3. SERROA spectra of Hb sample as a function of time. Above each spectrum the time elapsed from the beginning of the measurement is indicated, and for comparison, the spectra are shifted, however, Δ, the circular intensity difference (CID) is shown on the scale bare to the left of the spectra.

CONCLUSIONS

The metalloprotein hemoglobin was studied by using resonance Raman spectroscopy, surface enhanced resonance Raman spectroscopy and surface enhanced resonance Raman optical activity, RRS, SERRS, and SERROA, respectively. The later combines SERRS with Raman optical activity spectroscopy (ROA). Up to this time there is a limited number of the SERS/SERRS studies of proteins and, to our knowledge there is no SEROA/SERROA study of large proteins with the advanced quaternary conformation such as Hb. Optimization of the SERRS conditions, with respect to volume fraction of the Ag colloids and the molecule, was found necessary in order to achieve pronounced and stable SERROA signal. Comparison of RRS and SERRS spectra of Hb has revealed that all peaks in SERRS spectra of Hb were, due to resonance, originated from the vibrations of the heme bonds. Moreover, the SERROA spectrum demonstrates several pronounced peaks, appear at the same positions as in Hb SERRS and RRS spectra. This verifies that SERROA spectrum is genuine and not influenced by any artifact. Finally, the stability of the SERROA was also studied, and for the first time is reported as a function of time, and the signals were observed stable for more than 4 min. Our data verify that the SERROA signal achieved here is an intrinsic Hb property, and therefore, SERROA may open for significant applications in biomolecular absolute configuration and can be successfully used both in fundamental and applied sciences. The method is also proposed for analytical and biomedical studies of cell pathologies, especially those that are induced by changes of the proteins conformation such as channelopathies, Alzheimer, and prion diseases.

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