Surface enhanced Raman optical activity as an ultra sensitive tool for ligand binding analysis

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Surface enhanced Raman optical activity as an ultra sensitive tool for ligand binding analysis

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Abstract. The Surface Enhanced Resonance Raman Scattering (SERRS) and Surface Enhanced Resonance Raman Optical Activity (SERROA) spectra of myoglobin and the myoglobin–azide complex were measured on very dilute samples (100 nM protein) in order to analyze the sensitivity of SERROA spectroscopy when inducing small structural changes. While the SERRS spectra of the two compounds were virtually identical, comparison of the SERROA spectra revealed several differences, including frequency shifts and changes in signal intensity, consistent with structural change in the porphyrin prosthetic group of the protein upon azide complexation. Application of this method allows for rapid analysis of ligand binding in metalloproteins in dilute aqueous solution and could in the future, when combined with theoretical studies, increase the obtainable structural resolution of proteins beyond that of X-ray analysis.

Keywords: Spectroscopic analysis, colloid, chiral, SERRS, SEROA, metalloprotein

1. Introduction

A major concern in analysis of biological samples in spectroscopic chemical research, as well as in clinical or industrial applications, is sample concentration. While biological assays often are optimized to measure very dilute samples [1], or in complex mixtures such as a living cell [2], spectroscopic analysis frequently requires pure samples of concentrations close to saturation [3]. Additionally, in order to obtain a high level of detail, e.g. studying structure and function through spectroscopic techniques, long acquisition times are usually required. The issues of time and sample concentration represent major limitations in the application of spectroscopic techniques in screening processes.

Utilizing laser induced surface plasmon resonance or Surface Enhanced Raman Spectroscopy (SERS), enhancement of Raman intensity can be achieved when molecules are found in close proximity of colloidal nano particles. This allows for immediate acquisition of Raman spectra from very diluted samples [4,5], and therefore provides a solution to the concentration issue, down to the single molecule level [6].

One drawback of both Raman and SERS spectroscopy is the limited amount of structural data obtained by these methods, especially when analyzing biomacromolecules such as proteins [7]. Recently, we were able to experimentally combine the surface plasmon enhancement effect with Raman Optical Activity (ROA) into Surface Enhanced Raman Optical Activity (SEROA) and apply this method to

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the structural analysis of dilute samples of biomolecules [8,9]. Measuring on colloidal silver suspensions the enhancement observed in SERS can be transferred to ROA, providing information about the chirality of the sample by differential scattering of circularly polarized light, hence allowing for more stringent structural analysis of sample compounds. While ROA is a well established method in structural analysis of proteins, this method is very much limited by its requirements for higher concentration and long acquisition time [7]. By combining SERS and ROA into SEROA, we were able to reduce sample concentration and acquisition time many folds, while still obtaining considerable structural information, verified by standard ROA measurements [8,9].

Here, we further develop the application of SEROA analysis by a case study of ligand binding in myoglobin at very low concentrations (100 nM), while at the same time exploiting the Resonance Raman (RR) effect in chiral analysis. We show that, while the Surface Enhanced Resonance Raman Scattering (SERRS) spectrum of myoglobin in aqueous solution is nearly identical to the SERRS spectrum of the myoglobin-azide complex, the corresponding Surface Enhanced Resonance Raman Optical Activity (SERROA) spectra can clearly be distinguished, proving that sensitivity to structural perturbations inherent in ROA spectroscopy are transmitted to SERROA, hence allowing for rapid analysis of complex biological macromolecules in dilute samples. The possibility of combining SEROA measurements with theoretical calculations, hence gaining structural information at a detail level comparable, or exceeding X-ray analysis, is discussed.

2. Materials and methods

2.1. Preparation of colloids and samples

Silver colloids were prepared according to the modified citrate reduction method developed by Lee and Meisel [10].

Freeze dried horse myoglobin (purity 95%) was purchased from MP Biomedicals and used without further purification. The sample was dissolved in Milli Q water to a stock solution of 100 μM and kept at 4°C until use. For the myoglobin-azide complex samples, freeze dried myoglobin (final concentration 100 μM) was dissolved in a solution of 5 mM NaN_3 in order to secure surplus of azide ions and left to equilibrate at least over night at 4°C. Prior to analysis, the stock solutions were filtered through a 22 μm Millipore filter, followed by dilution with Milli Q water and mixed with Ag colloids and NaCl to a final protein concentration of 100 nM. The concentration of Ag colloids in the final mixture used in the SERROA measurements was determined by the optimization procedure described previously [9, 11], consisting of 70% (v/v) Ag colloids to 30% (v/v) cytochrome c and 10 μl 1 M NaCl to a final concentration of 0.05 M salt in 200 μl of the sample solution. After mixing the components for the SERROA measurements, the samples were left for 15 minutes before measuring.

2.2. Spectroscopic analysis

All measurements were carried out on a ChiralRAMAN scattered circular polarization spectrometer (BioTools Inc., USA). This instrument simultaneously provides Raman and ROA spectra and is equipped with a 532 nm laser source (Excel, Laser Quantum, UK) and a back thinned CCD detector, optimized for recording in the spectral range 100–2400 cm^{-1}. In order to keep the sample at ambient temperature during acquisition, and in order to avoid bleaching of the sample, the illumination is controlled by a shutter (incident shutter) mounted between the laser and the sample. Another shutter (scattered shutter)

mounted between the sample and the CCD is also used in order to prevent saturation of the CCD. The ROA spectra are presented as circular polarization intensity differences ($I_R - I_L$), where I_R and I_L denote the intensities of the scattered light's right- and left-circularly polarized components, respectively. The parent Raman spectra are presented as corresponding circular polarization intensity sums ($I_R + I_L$).

All spectra were obtained with a laser power of 70 mW at the sample and 20 minutes acquisition.

3. Results and discussion

As myoglobin is one of the most extensively studied proteins, the RR spectrum of the compound has previously been thoroughly analyzed and assigned [12–14], likewise the SERRS spectrum of the compound [15]. All observed signals in the RR/SERRS spectra can be assigned to resonance enhanced skeletal porphyrin vibrations [13,16,17]. The focus of the following analysis will therefore be on the SERROA spectra of myoglobin and the myoglobin-azide complex. The SERRS spectrum, and corresponding SERROA spectrum, of myoglobin in colloidal suspension are presented in Fig. 1, and the spectra of myoglobin with excess sodium azide added are shown in Fig. 2. The spectral range analyzed here has been restricted to 1500–1100 cm^{-1}, as our previous study on myoglobin [9] has identified this region as containing the most profound SERROA signals.

Comparing the SERRS spectra of the protein and the protein-azide complex, seen in Fig. 1a and Fig. 2a, respectively, the spectra appear extremely similar, in spite of slightly better signal-to-noise ratio

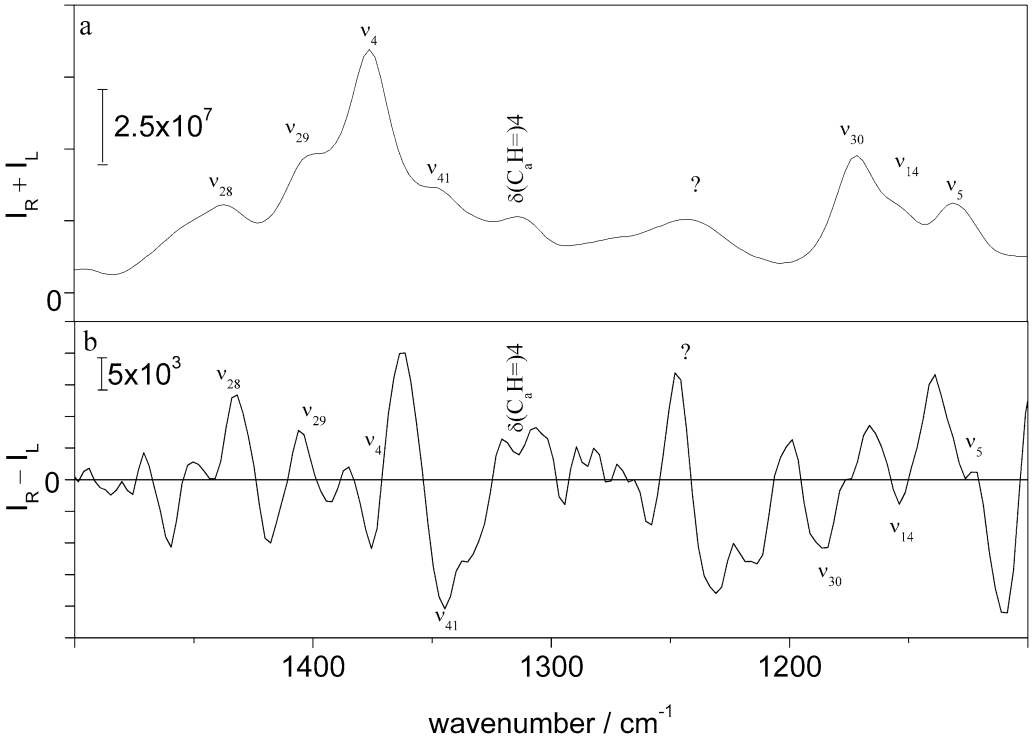

Fig. 1. Surface enhanced resonance Raman scattering (a) and surface enhanced resonance Raman optical activity (b) spectra of myoglobin in aqueous/colloidal solution (100 nM protein). Both spectra were obtained simultaneously as described in the text. For details regarding the assignment of signals in the SERRS spectrum, see [16].

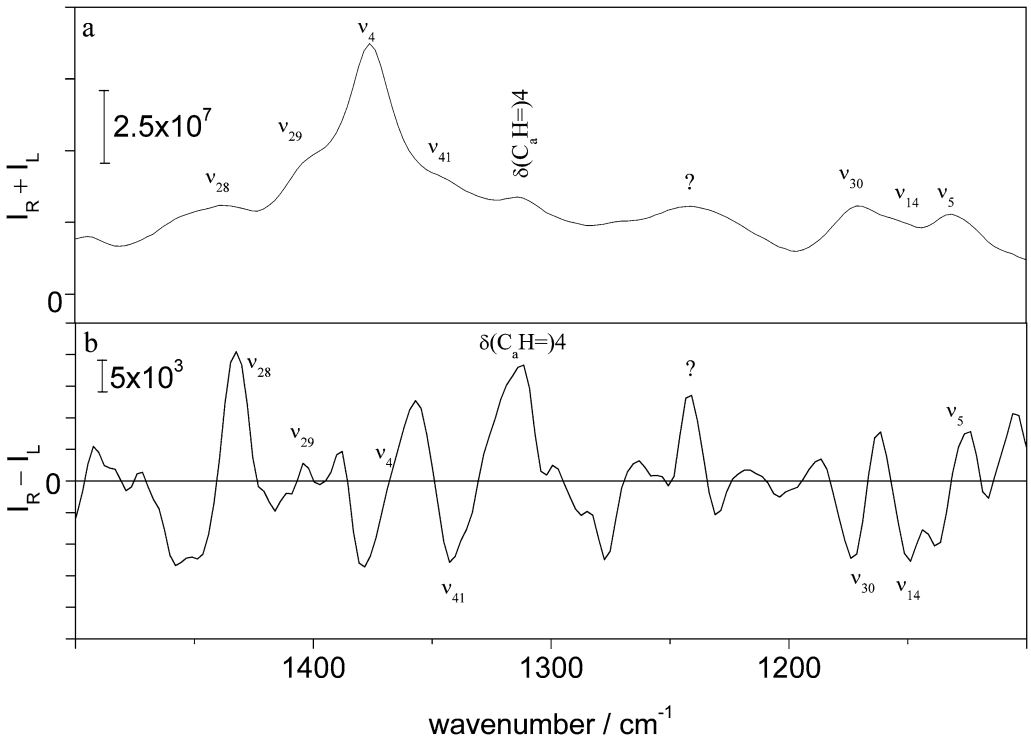

Fig. 2. Surface enhanced resonance Raman scattering (a) and surface enhanced resonance Raman optical activity (b) spectra of the myoglobin-azide complex in aqueous/colloidal solution (100 nM protein, azide ions added in excess). Both spectra were obtained simultaneously as described in the text. For details regarding the assignment of signals in the SERRS spectrum, see [16].

in the myoglobin SERRS spectrum, which is likely to be due to better colloidal aggregation in the presented spectrum when comparing with the corresponding myoglobin-azide SERRS spectrum. The peaks observed in Figs 1a and 2a are all readily assignable (see [13]), except a broad signal around 1250 cm^{-1}. Restricting the analysis to SERRS spectroscopy, it would be reasonable to assign this signal to a spectral contribution originating from the aggregating colloids, but as the SERROA spectra of the proteins and protein-azide complex indicate, the signal is due to an optically active component.

The SERROA spectra of myoglobin and the myoglobin-azide complex are presented in Figs 1b and 2b, respectively. Here, in contrast to the SERRS spectra, spectral differences are obvious throughout the presented wave number range. In the region above 1400 cm^{-1}, the signals assigned to ν_{28} and ν_{29} are wave number conserved, when comparing the myoglobin and myoglobin-azide spectra. The positive-negative couplet corresponding to ν_{28} is well balanced in the SERROA spectrum of myoglobin (see Fig. 1b), while positively biased in the myoglobin-azide spectrum (see Fig. 2b), and the positive signal, followed by a weak negative contribution, assigned to ν_{29}, appears somewhat weaker in the myoglobin-azide spectrum (Fig. 2b). Even though changes in couplet structure could indicate a structural change in the porphyrin, the ν_{28} couplet is not wave number shifted upon addition of the azide-ion, and we therefore believe that the normal mode responsible for ν_{28}, a C_α–C_m stretching mode, is not strongly influenced by the ligand binding. The observed positive bias in the myoglobin-azide spectrum could very well be due to a polarization effect often observed in ROA spectra [18], while the difference in intensity of the ν_{29} signal between the two spectra can be explained by better signal-to-noise ratio in

the myoglobin sample rather than an effect of the complexation of azide. The contributions from ν_{29} is followed by a peak assigned to the ν_4 symmetric half-ring stretching vibration, strongly resonance and surface plasmon enhanced in the SERRS spectra (see Figs 1a and 2a). The corresponding SERROA signals, together with signals due to ν_{41} and the vinyl in-plane bending mode at 1305 cm^{-1}, make up the main porphyrin signature in the myoglobin spectra. In the SERROA spectrum, the ν_4 transition can be assigned to a negative–positive couplet, as the peak intensity of the SERRS signal is found at the inflection point of this feature (see Figs 1b and 2b). Assigning ν_4 as a couplet further allows us to assign the negative contribution following this feature to ν_{41}, also originating from a symmetric half-ring stretching vibration. The final feature in this region, a broad positive signal, noisy in the myoglobin spectrum (see Fig. 1b), can then be assigned to the vinyl in-plane bending mode.

In the region 1400–1200 cm^{-1}, we do observe wave number shifts, in addition to changes in intensity ratios, indicating that structural changes in the porphyrin caused by the ligand binding can be measured. Upon binding of azide to the porphyrin, the couplet assigned to ν_4 broadens approx. 8 cm^{-1}, blue shifting the negative contribution while red shifting the positive contribution. As a result, the SERROA signals assigned to ν_{41} and the vinyl in-plane bending mode appear to be slightly red shifted, taking into account that the noise observed in the myoglobin spectrum does not allow for unambiguous peak intensity assignment. An obvious explanation for this broadening of the SERROA porphyrin signature could be structural changes in the porphyrin caused by small displacements of the iron ion upon complexation of the azide ion.

As mentioned initially in this section, the broad SERRS signal observed around 1250 cm^{-1} in both spectra (see Figs 1a and 2a) cannot be assigned to a porphyrin transition. Interestingly, both SERROA spectra include a spectral contribution in this region, a positive-negative couplet, red shifted approx. 8 cm^{-1} in the myoglobin-azide spectrum. As no protein signals are found in this region, the most feasible explanation for this signal is that citrate ions, covering the surface of the nano particles in the areas that are not occupied by sample molecules, are locked in a predominant conformation and becoming optically active.

Below 1200 cm^{-1}, three signals observed in the SERRS spectra can be assigned to porphyrin vibrations originating from transitions ν_{30}, ν_{14} and ν_5 (see Figs 1a and 2a). The SERRS signal corresponding to the ν_{30} transition appears to be more highly enhanced in the myoglobin spectrum (see Fig. 1a), when comparing the over all signal to noise ratio of the two SERRS spectra. On the other hand, the SERROA spectrum of myoglobin presents no real signal at 1171 cm^{-1}, the peak intensity of the SERRS signal assigned to ν_{30} (see Fig. 1b), while the SERROA spectrum of myoglobin-azide shows a clear negative signal at this wave number (see Fig. 2b).

When inspecting the entire spectral range below 1200 cm^{-1}, it is reasonable to assign ν_{30} in the SERROA spectrum of myoglobin to the negative signal situated at 1181 cm^{-1} which then must be red shifted approx. 10 cm^{-1} by azide complexation. As the ν_{30} transition originates from an asymmetric half-ring stretching vibration, this red shift is in accordance with the general broadening of the spectrum observed upon addition of azide. Assigning the next negative contribution in the SERROA spectra to ν_{41} is again consistent with the general shifting trend of the wave number of the peak assigned to collective porphyrin stretching mode as this signal is red shifted approx. 5 cm^{-1} in the myoglobin-azide spectrum (see Fig. 2b). The last signal observed in the presented SERRS spectra is assigned to ν_5, a pyrrole stretching vibration with considerable contribution from the methyl groups attached to the porphyrin. The SERROA spectrum of myoglobin displays no signals immediately corresponding to the SERRS signal, whereas the myoglobin-azide SERROA has a positive contribution at 1131 cm^{-1}. Assigning the ν_5 transition to the nearest positive signal in the SERROA spectrum of myoglobin would put a

considerable distance between the chiral and absolute signal, and as the presented SERROA spectra in general are rather inconsistent below 1150 cm^{-1}, we must leave the SERROA assignment of this transition as inconclusive in this study.

Viewing the assignment of the SERROA spectra presented above as a whole, one could argue that the amount of information obtained from such analysis is limited, when compared with the structural details available, e.g., from X-ray diffraction analysis. Also, in the RR spectra of myoglobin, spectral changes are observed above 1500 cm^{-1} upon complexation of azide, why the range presented here could be argued to be of less importance. In fact, our studies have shown that in the range above 1500 cm^{-1} little SERROA information is available from myoglobin, indicating that the localized porphyrin transitions found in this region is less susceptible to chiral analysis. Also, when comparing SERROA to more conventional methods, one must bear in mind that the spectra presented in this analysis were collected on very dilute samples over a short period of time, when comparing with complete NMR or X-ray analyses. In addition, the structural changes induced by complexation of azide or other small ligands in porphyrin containing metalloproteins are very small (less than 0.5 Å), and cannot be determined directly by X-ray diffraction, as the structural resolution of X-ray analysis is still above this limit.

Even so, the SEROA method, with or without the additional benefits of the resonance effect, is still in the developmental phase. The signal intensity of SEROA spectra is very low, why one could speculate on the authenticity of the spectra with regard to noise. Unfortunately, it appears that the conventional definition of shot noise imposed on CCD operated Raman spectrometers may not apply directly in the case of SERS/SEROA. As the peaks observed SERS spectra often are of very high intensity and very narrow, the corresponding calculated shot noise level will be unreasonably high. In our experiments, we were able to reproduce SEROA spectra of both samples to a reasonable level, indicating that the spectra are more consistent than would be the case if they were dominated by noise.

We believe that ligand binding analysis by SERROA could be developed into an extremely powerful method in the future, as a more solid understanding of the signals associated with this surface plasmon enhanced differential spectroscopy is achieved. Understanding and recognizing prosthetic group signatures could in the future be combined with *ab initio* calculational studies restricted to the prosthetic group, possible on high performance computer systems today, as the porphyrin signals observed in this and a previous study of cytochrome c [19], appear to be only slightly dependent on the surrounding protein structure, and calculations restricted to the active site should produce reliable results. Recently, theoretical formalism including surface plasmon enhancement of Raman and ROA has been developed and may be implemented in future theoretical studies [20], when included in more general calculational software such as Gaussian.

4. Conclusion

The Surface Enhanced Resonance Raman Scattering (SERRS) and Surface Enhanced Resonance Raman Optical Activity (SERROA) spectra of myoglobin and the myoglobin–azide complex at highly diluted (100 nM) conditions were obtained. Assignment of spectral contributions to the SERRS spectra were transferred to the corresponding SERROA spectra, revealing an increased sensitivity to minute structural changes, achieved when combining surface plasmon enhancement and Raman Optical Activity, allowing for fast, detailed analysis of ligand binding in dilute aqueous solutions.

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