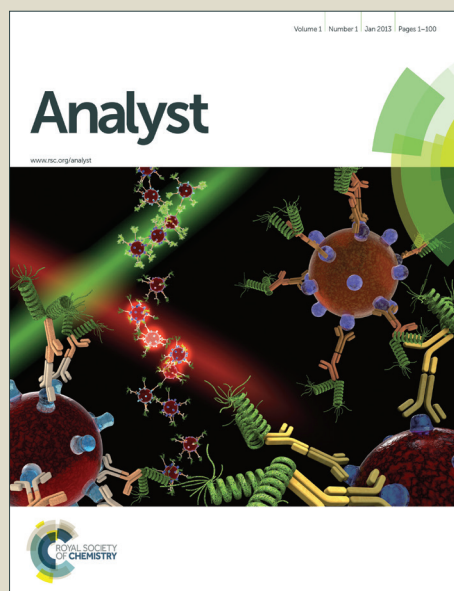


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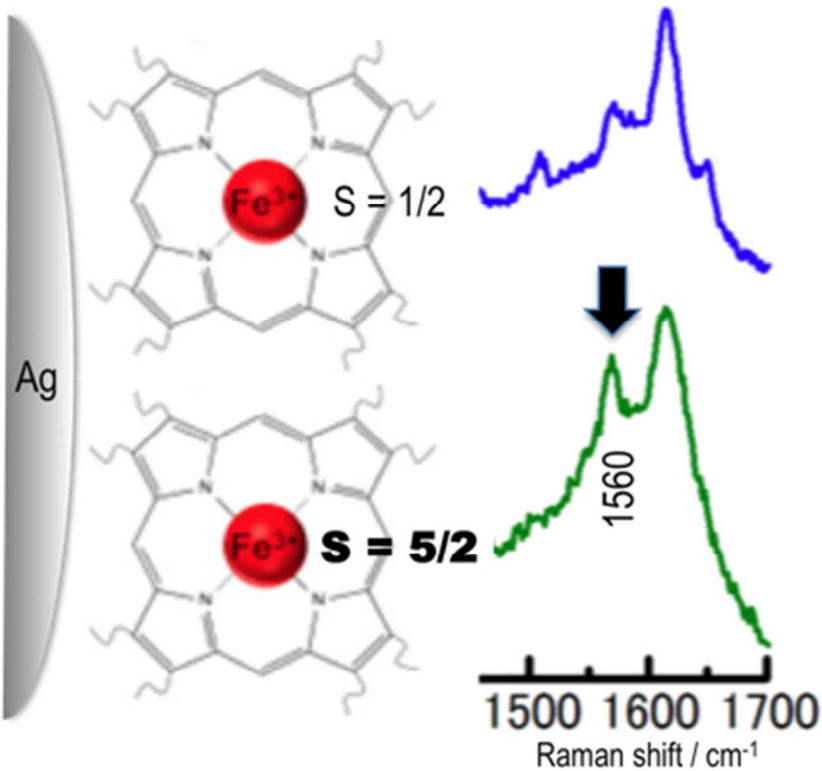


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The SERRS intensity ratio of the peak at 1560 cm^{-1} to that at 1620 cm^{-1} was applied to detect the spin states of heme in metmyoglobin sensitively.

70x69mm (150 x 150 DPI)

**Sensitive marker bands for the detection of spin states of
heme in surface-enhanced resonance Raman scattering
spectra of metmyoglobin**

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† Electronic Supplementary Information (ESI) available: SERRS and resonance
Raman spectra of myoglobin in 0—3700 cm⁻¹; Estimation of the SERRS peak
intensities. See DOI: 10.1039/c000000x/

Abstract

Surface-enhanced resonance Raman scattering (SERRS) spectra of myoglobin (Mb) with various ligands were measured. In the resonance Raman scattering (RRS) spectra, the peaks around at 1610 and 1640 cm^{-1} have been used to discriminate between the heme iron in high or low spin state so far. In the SERRS spectra, however, the spin state cannot be distinguished by the corresponding peaks. Alternatively, the intensity ratio of the SERRS peak at 1560 cm^{-1} to that at 1620 cm^{-1} was applied to detect the spin states sensitively (1.5×10^5 times compared with the RRS); namely, the high ratio was obtained from met-Mb in the high spin state at $\text{pH} \leq 7$ except for in a strong acid solution. The different marker bands between the SERRS and RRS may be due to the enhancement order by the surface selection rule.

Keywords: Surface-enhanced resonance Raman scattering, resonance Raman scattering, myoglobin, heme, spin state

Introduction

For analysis of biological and medical molecules, Raman spectroscopy is very useful, because it provides detailed information about the molecular structure through the sharp peaks of the vibrational modes of molecules in fingerprint region and is insensitive to the aqueous environment.¹⁻⁴ However, the Raman signal is often too weak to observe a small amount of a sample such as a protein. Thus, resonance Raman spectroscopy has been applied to study biological and medical molecules.³⁻¹⁰ By using resonance Raman effect, namely, excitation at an absorption band of a chromophore, only peaks from it are remarkably enhanced. A wide variety of applications of resonance Raman scattering (RRS) can be found for studies of carotenoids, retinal, bacteriorhodopsin, metal proteins, chlorophylls, and hemeproteins.³⁻¹²

Hemeproteins play a variety of important roles such as respiration, electron transports, and detoxification in a living body. Myoglobin (Mb), which is found in muscle working for oxygen storage, consists of a heme molecule and a polypeptide chain (17 kDa). A heme is composed of a protoporphyrin and an iron ion.¹²⁻¹⁴ The iron ion is coordinated by one and four nitrogen atoms of histidine group of the peptide and the protoporphyrin, respectively. O₂ and various ligands can be bound by the rest of the coordinate bond.¹²⁻¹⁴ The iron ion in heme of Mb is oxidized and then changed into Fe³⁺ (met-Mb). In a living body, however, the iron ion of Mb exists as Fe²⁺ in the presence and absence of O₂ (oxy-Mb and deoxy-Mb, respectively). Met-Mb is classified by five *d*-electrons of Fe³⁺ in high and low spin state, namely, $S = 5/2$ and $1/2$,

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5 respectively. For Fe^{2+} in Mb, six *d*-electrons form high and low spin state, namely, S
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8 $= 2$ and 0 , respectively. The spin state is related to O_2 and ligand-binding.¹⁵⁻¹⁷ In the
9
10 studies of hemeprotein through resonance Raman scattering,⁴⁻¹¹ strong peaks from heme
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12 can be measured by excitation at absorption bands of heme in ultraviolet and visible
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14 (UV-Vis) region. It is noted that marker bands for discrimination of the oxidation and
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16 spin states of the iron ion have been reported.⁴⁻⁸ However, the resonance Raman
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18 signal is still too weak to measure heme *in vivo* because of its low concentration, and
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20 the UV-Vis excitation light can induce fluorescence and photo-degradation.
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26 Recently, surface-enhanced Raman scattering (SERS) has been applied to
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28 highly sensitive detection.¹⁸⁻²⁹ On a noble metal nanoparticle, an electromagnetic
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30 (EM) field is enhanced by resonance of plasmon with excitation light due to dipolar
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32 oscillation of the conduction band electrons, which is called localized surface plasmon
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34 resonance. The EM field is enormously enhanced at a gap of the nanoparticles, and
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36 then causes SERS.¹⁹⁻²¹ In SERS with resonance Raman effect, namely,
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38 surface-enhanced resonance Raman scattering (SERRS), the fluorescence and the
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40 photo-degradation are prevented by energy transfer to the metal. Indeed, SERRS has
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42 been used for measurement of a single Mb molecule in aqueous solution and sensitive
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44 discrimination between hemoglobin and glycated hemoglobin.^{22,23} To the best of our
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46 knowledge, however, the discrimination of the oxidation and spin states of heme using
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48 SERRS has not been reported yet. The reason may be that SERRS spectra are strongly
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50 affected by adsorption and orientation of molecule on the metal surface.²⁴ Moreover,
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52 It has been found that the oxidation by Ag affects the SERRS spectra of Mb.^{25,26} In
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the present study, SERRS spectra of Mb with various ligands or at various pH which show the different oxidation and spin states of heme were measured, and then the marker band for highly sensitive discrimination of the spin state of heme was discovered by the SERRS measurements. Furthermore, we consider a reason for the different marker bands between the SERRS and RRS. Thus, the sensitive detection using SERRS can be applied to the *in vivo* discrimination at single molecule level and an evaluation of an infinitesimal amount of biomimetic molecules using heme for gas sensors and blood substitutes.^{11,30}

Experiments

We used Mb from equine skeletal muscle as purchased from Sigma-Aldrich Japan. An aqueous solution of Mb (0.3 mM) was used as a sample of met-Mb in the high spin state (Mb-H₂O). Another sample of met-Mb in the high spin state was obtained by addition of NaF (300 mM) to a stock of Mb-H₂O (Mb-F). On the other hand, samples of met-Mb in the low spin state were prepared by adding imidazole (15 mM) and NaN₃ (15 mM) to the stock (Mb-Im and Mb-N₃, respectively). We added NaBH₄ (15 mM) to the stock and then obtained a sample of deoxy-Mb in the high spin state. Moreover, pH of an aqueous solution of Mb was adjusted from 2 to 12 at intervals of one by adding HCl (0.1—1 M) and NaOH (0.05—1 M) aqueous solution to the sample. Absorption spectra of the Mb complexes were acquired by a UV-Vis spectrometer (Shimadzu, UV-3101PC). Their resonance Raman scattering (RRS) and SERRS spectra were measured by excitation using a 514 nm line of an Ar ion laser

(Spectra-Physics, Stabilite 2017-06S) whose intensity of 50 and 0.5 mW, respectively, through an objective lens (SLWD Plan (APO) 9×) of a reflection-mode Raman microscope (Photon design, Nanostar NFRSM800). The spectral resolution is about 2 cm^{-1} . Both measuring times were 540 s and the same. The SERRS were measured from a mix of the sample solution of the Mb (0.3 mM), a citrate-reduced Ag colloidal suspension,²⁷ and a NaCl aqueous solution (100 mM) at a volume ratio of 1:1000:500, respectively.

Results and Discussion

Figure 1 shows absorption spectra of the Mb samples. The prominent bands around at 400 nm are attributed to their Soret band. In the case of deoxy-Mb, two characteristic bands, α and β bands, appear in the 500—600 nm region. The spectral changes indicate that met-Mb was reduced to deoxy-Mb by the addition of NaBH_4 .^{31,32} Incidentally, the red-shifted Soret-band of Mb- N_3 has been already reported.^{33,34} The absorption bands originate from the heme moiety, and thus we used the excitation light at 514 nm for resonance Raman effect on heme. In a RRS spectrum obtained by the excitation near the Soret band, anomalously polarized peaks of heme, which are enhanced by excitation at the α and β bands, hardly appear.^{4,6}

It can be seen from Figure 2 that the RRS spectra are changed by the addition of the various kinds of ligands to Mb. The peak assignment in the RRS spectra is summarized in Table 1. In the case of deoxy-Mb, the peak appears at 1354 cm^{-1} , and in the other RRS spectra it is shifted to 1370 cm^{-1} . The peak, which is assigned to

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5 symmetrical stretch mode of pyrrole half-ring,¹⁰ is used for the discrimination between
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7 Fe^{2+} and Fe^{3+} in heme.^{4,6-8} Furthermore, a peak appears at 1614 cm^{-1} in the RRS
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9 spectra of Mb-H₂O, Mb-F, and deoxy-Mb, while it is shifted to 1640 cm^{-1} by the
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11 addition of imidazole (Mb-Im) and NaN₃ (Mb-N₃). These peaks, which are attributed
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13 to asymmetrical stretching mode of C_αC_m of heme,¹⁰ are a marker band for the detection
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15 of spin state of the iron ion through the core size of heme; namely, the peaks at lower
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17 and higher wavenumbers indicate the high and low spin states, respectively.⁴⁻⁸

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23 Figure 3 shows the SERRS spectra from the same sample as Figure 2 (the
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25 spectra in $0\text{--}3700\text{ cm}^{-1}$ are shown in Figure S1 in the ESI). Their signal to noise
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27 ratios are similar to those of the RRS spectra, while the excitation laser intensity and the
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29 concentration in the RSS measurement were 100 and 1500 times as strong and dense as
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31 the SERRS measurement, respectively. The broader SERRS peaks than the RRS
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33 peaks may be due to absence of motional narrowing, because the former and latter were
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35 observed from the molecules adsorbed on the metal and in an aqueous solution,
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37 respectively. It is noted that the similar SERRS spectra were measured by the addition
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39 of the various kinds of the ligands to Mb, although the RRS spectra were different. In
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41 the SERRS spectra of deoxy-Mb, its RSS peak at 1354 cm^{-1} is shifted to 1370 cm^{-1} .
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43 The former and latter peaks indicate Fe^{2+} and Fe^{3+} in heme, respectively.^{4,6-8} The
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45 upward shift of the SERRS peak may be due to the fact that deoxy-Mb is oxidized by
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47 citrate-reduced Ag nanoparticles. Indeed, it has been revealed that the oxidation
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49 occurred at the edge of a hemoglobin nanocrystal by a tip-enhanced Raman scattering
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51 (TERS) technique.²⁵ Moreover, the upward shift due to the oxidation has been
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prevented by using thiol-protected Ag nanoparticles and a TERS technique, in which deoxy-Mb avoids contact with Ag.²⁶

In the SERRS spectra of Figure 3, the peaks at 1640 cm^{-1} , due to asymmetrical stretching mode of $C_{\alpha}C_m$ of heme,¹⁰ is barely observed even from Mb-Im and Mb- N_3 in the low spin state. Thus, the peaks cannot be used as the marker band for detection of the spin state of heme. Alternatively, the peak at 1560 cm^{-1} , assigned to stretching mode of $C_{\beta}C_{\beta}$ in skeletal structure of heme,¹⁰ in the high spin state were stronger than those in the low spin state. The reason for the difference between the RRS and SERRS peaks could be orientation of Mb adsorbed on the Ag surface. SERRS mainly originates from the enhanced EM field at a gap of Ag nanoparticles.¹⁹⁻²¹ The peak whose vibrational mode parallel to the metal surface, namely, perpendicular to the enhanced EM field is enhanced insufficiently. However, both of the peaks at 1640 and 1560 cm^{-1} are caused by in-plane mode.¹⁰ On the other hand, it has been proposed that the relative enhancement should be in the order of $B_1 < A_1$ for flat and vertical orientation of the adsorbed molecules by the surface selection rule.^{18,19} Indeed, it has been already reported that SERS enhancement depends on symmetry type of normal Raman via the surface selection rule.^{28,29} The peaks at 1640 and 1560 cm^{-1} belong to B_{1g} and A_{1g} modes, respectively,¹⁰ and the former was barely observed in the present SERRS spectra.

We investigated pH dependence of the SERRS peak at 1560 cm^{-1} . In an aqueous solution at $\text{pH} \geq 8$, Mb from equine skeletal muscle, which is the same sample as the present study, exists as Mb-OH in the low spin state, while it is changed into

Mb-H₂O in the high spin state at pH ≤ 7 .⁵ In the case of Mb from sperm whale, it has been reported that the spin state changes at pH = 8.9 by using NMR.¹⁷ Figure 4a and 4b show the pH dependent RRS and SERRS spectra of Mb in aqueous solution, respectively (the spectra in 0—3700 cm⁻¹ are shown in Figure S2 in the ESI). The RRS peaks at 1640 cm⁻¹, which indicate the low spin state, appears at pH ≥ 8 in a similar way of the previous report.⁵ On the other hand, intensities of the SERRS peaks at 1560 cm⁻¹ are normalized by those at 1620 cm⁻¹, which correspond to two stretching modes of C_a=C_b in vinyl group of heme,¹⁰ and then plotted against the pH as shown in Figure 5. The peak intensity was defined as height from a datum line, which was estimated by linear interpolation between the points at the bases of the peak (for more details, see Figure S3 and the explanation in the ESI). At pH ≥ 8 , the normalized SERRS intensities at 1560 cm⁻¹ were smaller than those at pH ≤ 7 except for pH = 2 and 3. At pH = 2 and 3, Mb may be denatured by acid. Also in the RRS spectrum, different peaks are observed in the 1500—1700 cm⁻¹ region at pH = 2 (see the bottom in Figure 4a). Thus, the high and low ratio of SERRS intensities at 1560 cm⁻¹ to those at 1620 cm⁻¹ indicates met-Mb in the high and low spin state, respectively.

Conclusion

The SERRS spectra of heme complexes in Mb show similar signal to noise ratios to the RRS spectra, although the excitation laser intensity and the concentration in the SERRS were 1/100 (0.5 mW) and 1/1500 (0.2 μ M) compared with the RRS, respectively. In the SERRS spectra, the marker bands for detection of the oxidation

and spin states of the iron ion in heme, which appear in the 1350—1370 and 1610—1640 cm^{-1} regions in the RSS spectra, respectively,⁴⁻⁸ were always observed at similar wavenumbers despite changing the ligand. The latter marker band for the spin state (B_{1g} mode) may be barely enhanced due to the surface selection rule, which leads to the enhancement order of $B_1 < A_1$.^{18,19} Alternatively, we investigate the pH dependent SERRS spectra of Mb-H₂O and Mb-OH and then found that the intensity ratio of the SERRS peak at 1560 cm^{-1} to that at 1620 cm^{-1} , which are assigned to stretching modes of the skeletal structure (A_{1g} mode) and the vinyl group of heme, respectively,¹⁰ can be used as the sensitive marker band for detection of the spin state of heme; namely, the high and low ratio represents the Fe^{3+} of met-Mb in the high and low spin state ($S = 5/2$ and $1/2$), respectively. By the SERRS marker band, the spin state of heme will be detected *in vivo* sensitively at single molecule level, and an infinitesimal amount of biomimetic molecules using heme for blood substitutes and gas sensors can be evaluated.

Acknowledgements

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Table 1 **Wavenumbers and assignment of the resonance Raman peaks**
of heme in myoglobin

experiment	reference ¹⁰	assignment ¹⁰
1639	High spin : 1640—1644	$\nu(\text{C}_\alpha\text{C}_\text{m})_{\text{asym}}$
1620	1621	$\nu(\text{C}_\text{a}=\text{C}_\text{b})$
1611	Low spin : 1610—1614	$\nu(\text{C}_\alpha\text{C}_\text{m})_{\text{asym}}$
1581	1583	$\nu(\text{C}_\alpha\text{C}_\text{m})_{\text{asym}}$
1562	1563	$\nu(\text{C}_\beta\text{C}_\beta)$
1541	1544	$\nu(\text{C}_\beta\text{C}_\beta)$
1518	1521	$\nu(\text{C}_\beta\text{C}_\beta)$
1426	1426	$\nu(\text{C}_\alpha\text{C}_\text{m})_{\text{sym}}$
1399	1404	$\nu(\text{pyr quater-ring})_{\text{sym}}$
1370	Met-Mb : 1370—1375	$\nu(\text{pyr half-ring})_{\text{sym}}$
1354	Deoxy-Mb : 1355—1362	
1340	1341	$\nu(\text{pyr half-ring})_{\text{sym}}$
1303	1301	$\delta(\text{C}_\text{a}\text{H}=\text{)}_2$
1209	1209	$\nu(\text{C}_\text{m}\text{H})$
1170	1169	$\nu(\text{pyr half-ring})_{\text{asym}}$
1125	1121	$\nu(\text{C}_\beta\text{—methyl})$

Figure captions

Figure 1 Absorption spectra of the various ligand-binding myoglobin. The spectra are normalized. Inset is a chemical structure of heme in myoglobin (heme *b*).

Figure 2 Resonance Raman spectra of the various ligand-binding myoglobin. Red, blue, and green spectra were measured from the sample of met-Mb in the high spin state (Mb-H₂O and Mb-F), met-Mb in the low spin state (Mb-Im and Mb-N₃), and deoxy-Mb in the high spin state, respectively.

Figure 3 SERRS spectra of the various ligand-binding myoglobin. Red, blue, and green spectra were measured from the sample of met-Mb in the high spin state (Mb-H₂O and Mb-F), met-Mb in the low spin state (Mb-Im and Mb-N₃), and deoxy-Mb in the high spin state, respectively.

Figure 4 (a) Resonance Raman and (b) SERRS spectra of met-Mb in the low and high spin states (Mb-OH and Mb-H₂O, respectively) at pH from 12 (top) to 2 (bottom) at intervals of one.

Figure 5 Ratios of the SERRS intensities of met-Mb in the high and low spin states (Mb-H₂O and Mb-OH, respectively) at 1560 cm⁻¹ to those at 1620 cm⁻¹ at various pH.

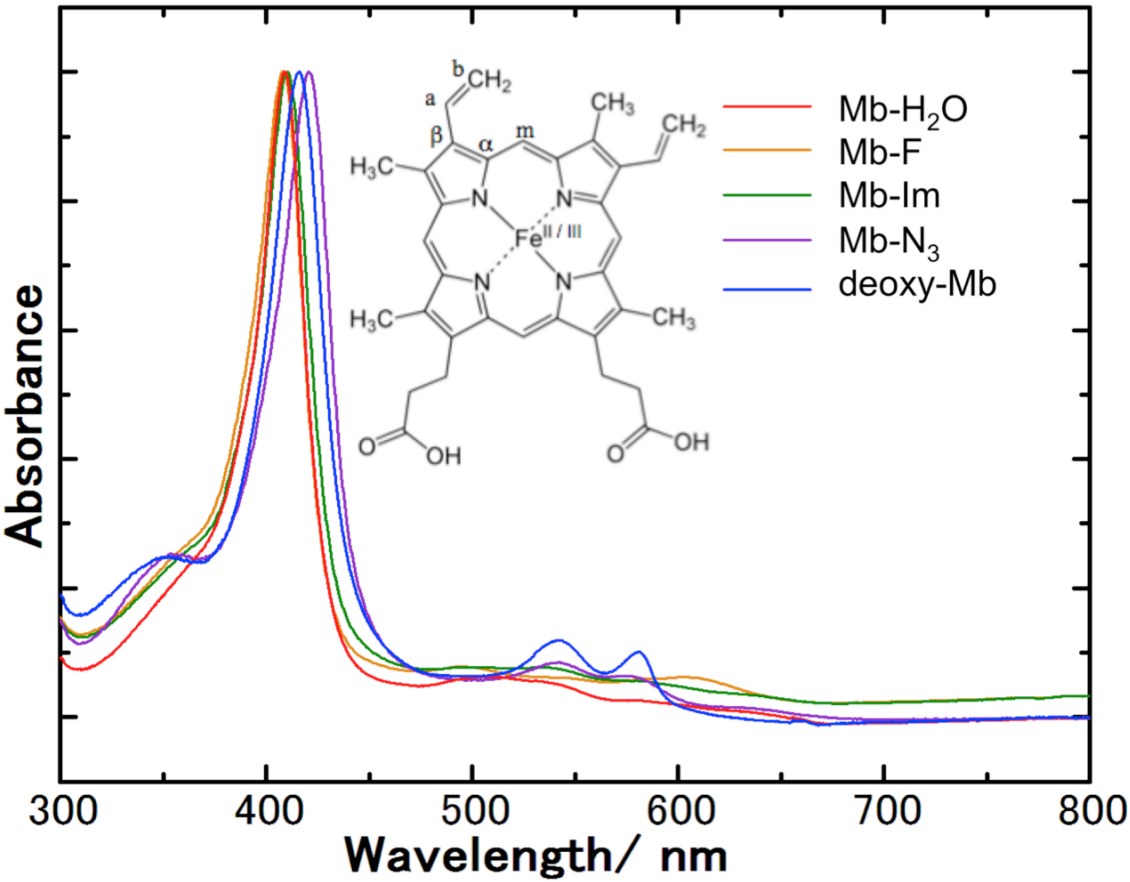


Fig. 1 Y. Kitahama et al.

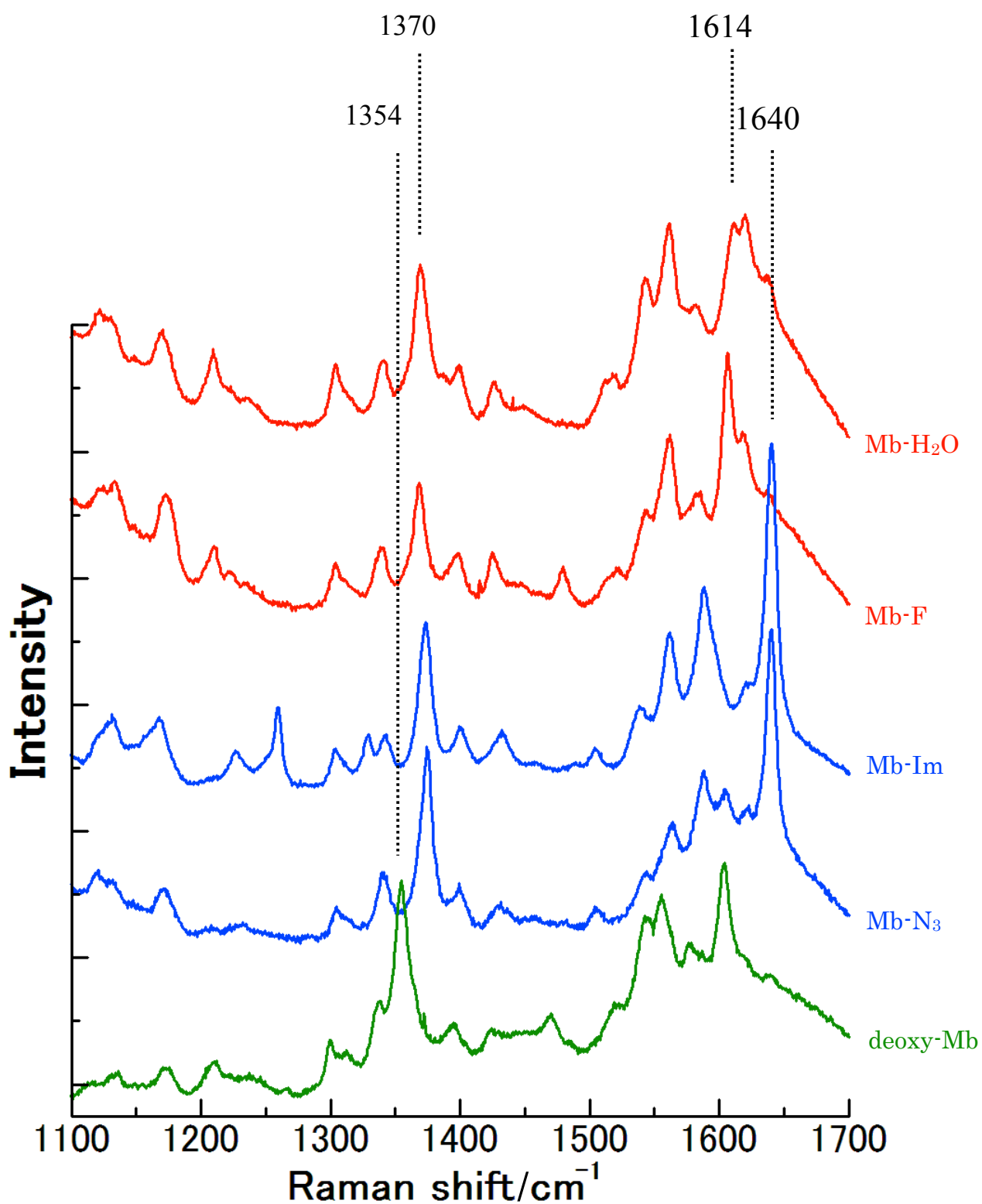


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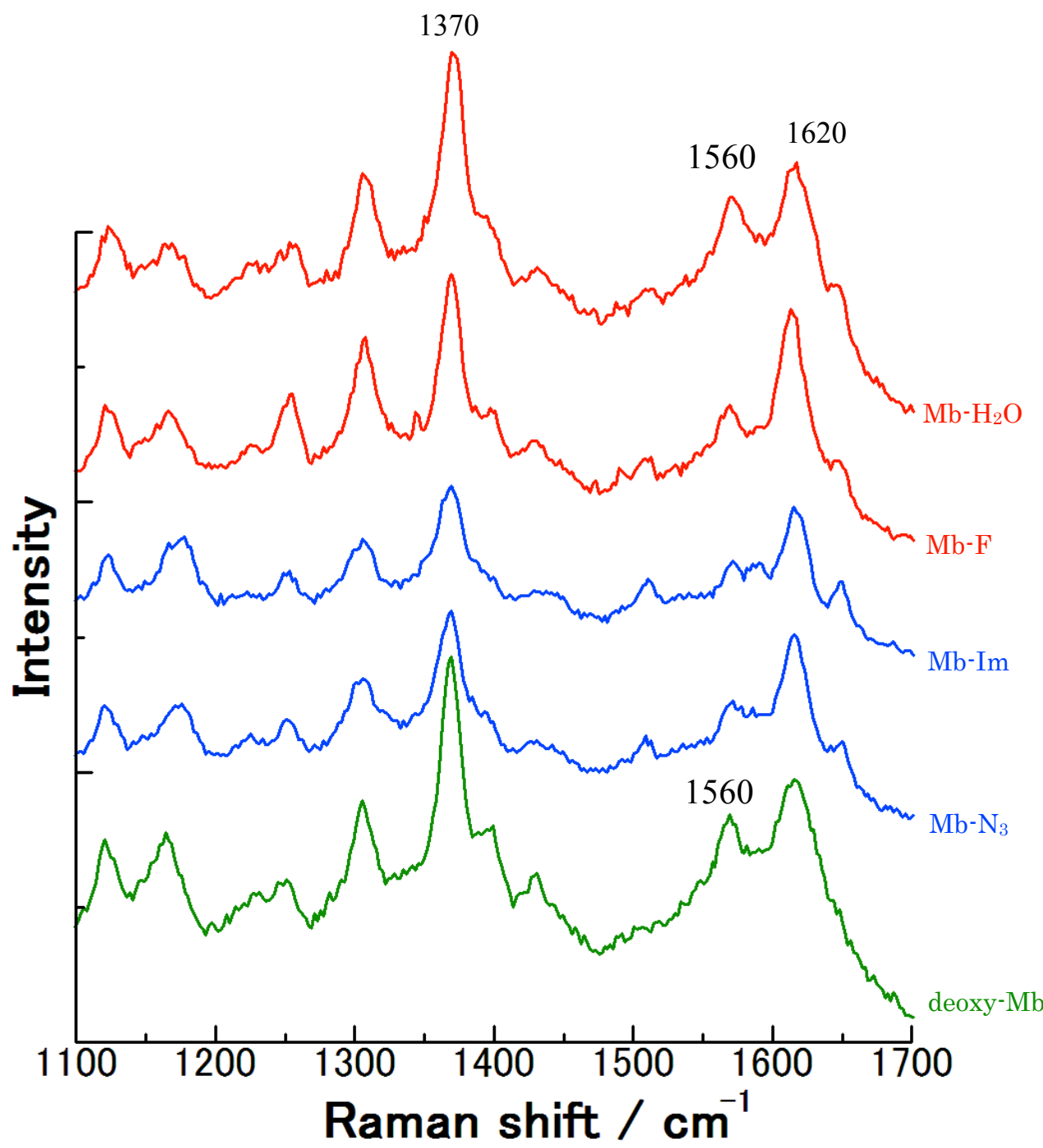


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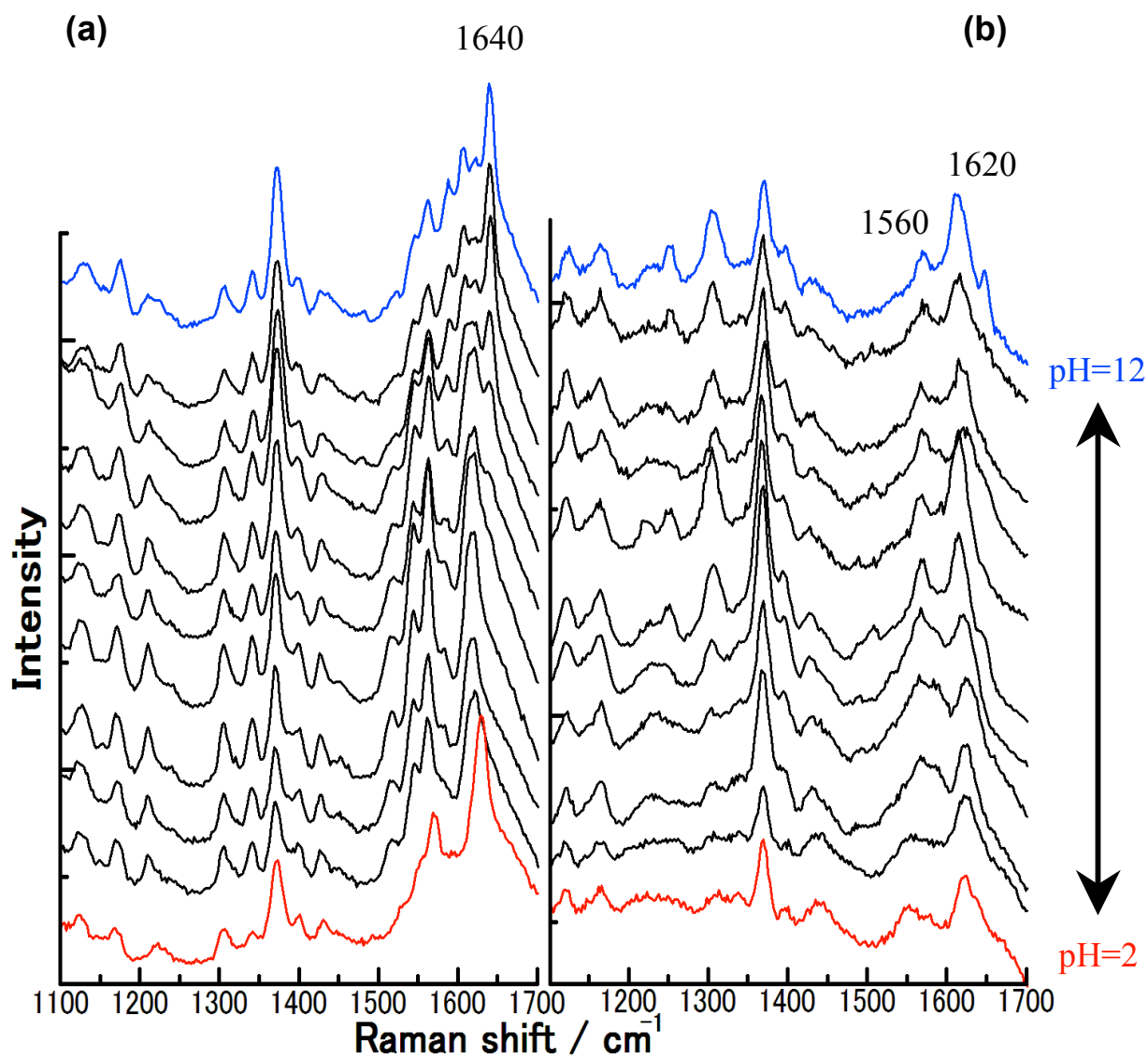


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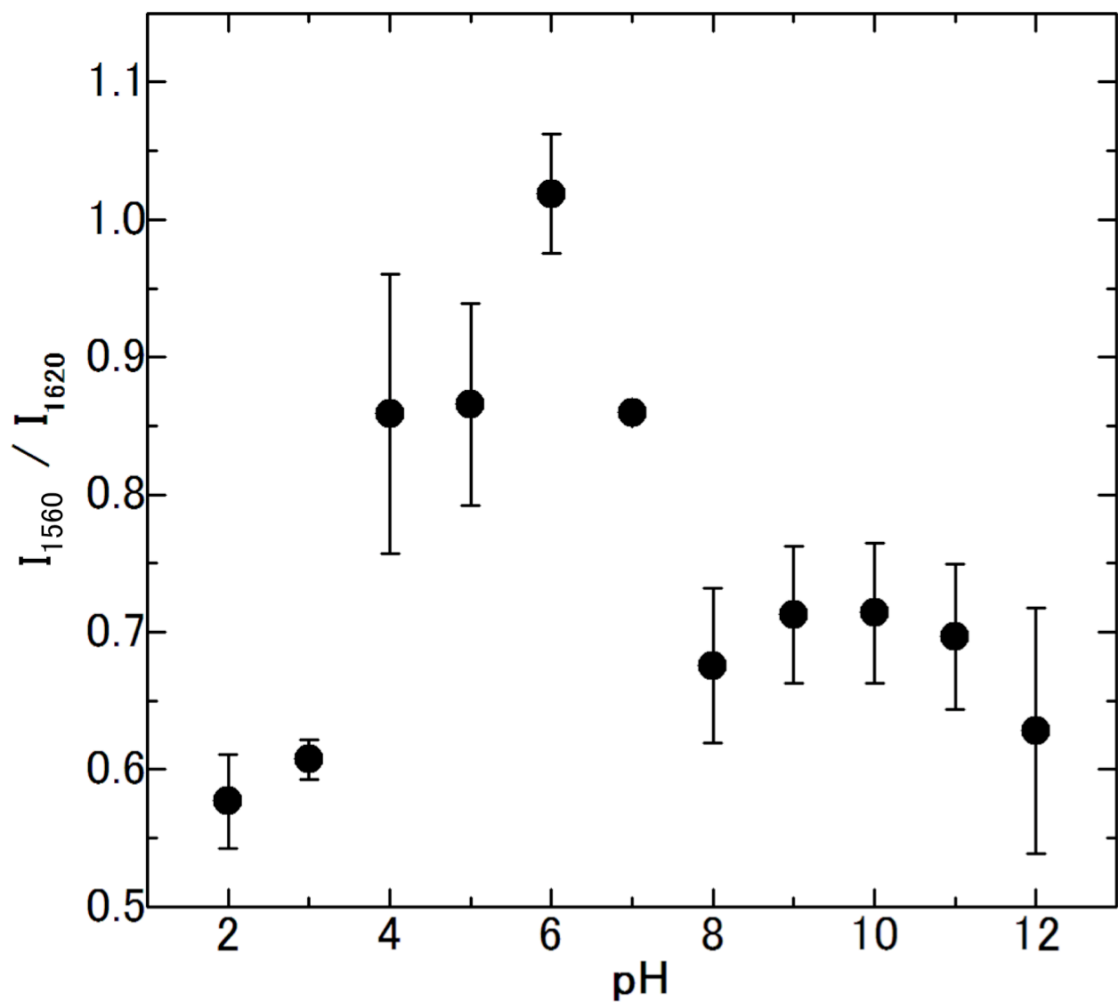


Fig. 5 Y. Kitahama et al.

TOC graphic

