Confocal Raman Microscopy on Single Living Young and Old Erythrocytes

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ABSTRACT:

Raman confocal microscopy, including the techniques of point Raman spectra, line mapping, 2D mapping, and time-dependent spectrum monitoring performed with 514.5 nm excitation light, was used in a comparative study on the distribution and oxidation states of hemoglobin (Hb) in young and old mature erythrocytes. It is demonstrated that in contrast to the homogeneous distribution of the Hb in young cells, there are more Hb distribution around the cell membrane in old erythrocyte. The proteins exhibit some extent of aggregation and conformational change, present less ability of oxidation, and lower oxygenation speed than the Hb in young erythrocytes. Our results also provide the first direct evidence of some intermediate oxygenated states of Hb between the two fully oxygenated (R) and deoxygenated (T) states in living erythrocyte, and give detail information about the conformational change of the intracellular Hb with time during the reoxygenation process. © 2008 Wiley Periodicals, Inc. Biopolymers 89: 951–959, 2008.

Keywords: Raman microscopy; living erythrocyte; hemoglobin; distribution and oxidation

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INTRODUCTION

Confocal Raman scattering scanning microscopy or Raman microspectroscopy has advantages over other forms of spectroscopy. It can provide detail information on the constitution of specific components in single living cells with microscopic resolution, but without requiring any sample preparation and obvious disturbance on them. It allows specific spatial analysis of regions within a living cell, and even offers 3D scanning and mapping of the molecules in it. Therefore, it becomes a powerful tool in biological applications.[1–7]

Erythrocyte, as an ideal model for single cell investigation, is one of the most attracting subjects for Confocal Raman scattering study. It is relatively a simple cellular system without intracellular membrane and organelles, and about 90% of its intracellular molecules are hemoglobin. Hemoglobin is very sensitive to Raman scattering and the high symmetry and chromophoric structure of its heme result in strong Raman scattering and a rich spectrum for different laser excitation wavelengths. The hemoglobin's Raman characteristics in fully oxygenated (R) and deoxygenated (T) states, and in the circumstances of heme aggregation and denaturation, as well as the Raman imaging of functional erythrocytes[8–13] have been studied. Some interesting results were observed and the validity of Raman microspectroscopy on the cell was also proved.

Mature erythrocytes can be fractionated into young and old erythrocytes using gradient centrifugation. Old erythrocytes are known to be more fragile, denser, and more spherocytic than the young ones, while their surface area, volume, and diameter are smaller than those of the young cells. The total contents of sialic acid of old erythrocytes are less than

those of young erythrocytes.[14–18] However, basically no one knows if there are also differences in the structure and function as well as the distribution of the intracellular hemoglobin in the two kinds of erythrocytes.

Accordingly, in this article we use the technique of confocal Raman microscopy and Raman imaging to study living young and old mature erythrocytes. Our object is to obtain information about the structure and function of the in situ hemoglobin and their distribution in the two kinds of cells at different oxygenation states by taking point spectra, line and 2D mapping measurements. We also measure the time-dependent conformation change of the intracellular Hb in the old and young cells respectively, thus comparing their transition speeds from T to R state. We believe that not only the results of such a study are important for the comprehension of erythrocyte's cell aging, but also the methods developed for obtaining these kinds of information will be very helpful for the in vivo studies of cell biology at molecular level.

MATERIALS AND METHODS

Erythrocytes Preparation

Blood (2–3 ml) was obtained by venipuncture from healthy volunteers and placed in glass tubes containing EDTA as an anticoagulant. The blood was washed (2000 rpm for 20 min) with isotonic phosphate buffered saline (PBS: 90 m*M* NaCl, 50 m*M* sodium phosphate, 5 m*M* KCl, 6 m*M* glucose, pH 7.4, 290 mOsmol) three times.

Then the erythrocytes were fractionated according to their cell age by density gradient centrifugation.[19] The linear gradient solution of five layers was formed in a conical-glass centrifuge tube consisting different content Percoll (Pharmacia) with densities from 1.065 to 1.080 g/ml. Then the linear gradient solution was loaded with the erythrocytes previously diluted with PBS (1:20) and centrifuged at 2700*g* for 20 min at 4°C. The young and old erythrocytes were later separated and put in a CO_2 incubator at 37°C for about 30 min. After that, the samples were placed in a refrigerator at 4°C preparing for use.

Glass slides were coated with a little amount of 0.01% poly-L-lysine hydrobromide (Sigma, molecular weight between 70,000 and 150,000 kDa) solution and then dried. The cells were transferred to the slides and allowed to settle about 10 min before spectra recording.

This research protocol was approved in advance by the Institution Ethics Committee of Ji Nan University and conformed to the Chinese Public Health Service Policy on Human Care and Use of Laboratory Animals.

Raman Spectra of Erythrocyte

For single erythrocyte, the Raman spectrum is mainly originated from Hb. The Raman spectrum of Hb is very sensitive to its conformation, so it can be used to identify the conformation change of the protein upon condition variation. For example, the Raman spectra of the T and R states of Hb show distinct difference (see Table I) and usually taken as a means of monitoring the molecular dynamics of Hb's transition from T to R state. In our experiment, the Raman spectrum of erythrocytes were recorded and analyzed not only for the information of the protein's distribution in the cell, but also for the transition of the protein from T to R state.

Raman spectra of living young and old erythrocytes were recorded by a Horiba JY RAM INV system using 514.5-nm excitation line from an Ar^+ ion laser through an inverted Olympus optical microscope with a 60× objective. The acquisition band was 600–1800 cm^{-1} with a spectrum resolution of 1 cm^{-1}. The 520.7 cm^{-1} band of silicon wafer was used to calibrate the instrument on daily basis.

To avoid damage on the cells under the exposure of the 514-nm laser light, the laser power was adjusted to about 1 mW and the laser spot size was ~2 μm in diameter. The exposure time was 1 s and the accumulation number was 1. The cell morphology was found to maintain totally intact and exhibit no sign of laser scarring after the exposure, the spectra were also consistent over the time scale.

Cosmic rays were removed during the postprocessing of the spectra.

Table I The Raman Band, Assignment, and Local Coordinates for the T and R States of Hb (for 514-nm Excitation Line)

	Band			
Band region (cm^{-1})	*T* (cm^{-1})	*R* (cm^{-1})	Assignment	Local Coordinate[a]
1650–1500		1637–1641	ν_{10}	$\nu(C_\alpha C_m)_{asym}$
	1603–1609		ν_{19}	$\nu(C_\alpha C_m)_{asym}$
	1580–1585	1586–1588	ν_{37}	$\nu(C_\alpha C_m)_{asym}$
		1567–1568	ν_2	$\nu(C_\beta C_\beta)$
	1540–1548		ν_{11}	$\nu(C_\beta C_\beta)$
1450–1300	1421	1421	ν_{28}	$\nu\ (C_\alpha C_m)_{sym}$
	1397	1397	ν_{20}	ν(pyrrole quarter-ring)
	1356–1359	1370–1374	ν_4	ν(pyrrole half-ring)$_{sym}$

[a] Refer to heme labeling scheme adopted in Ref. 20.

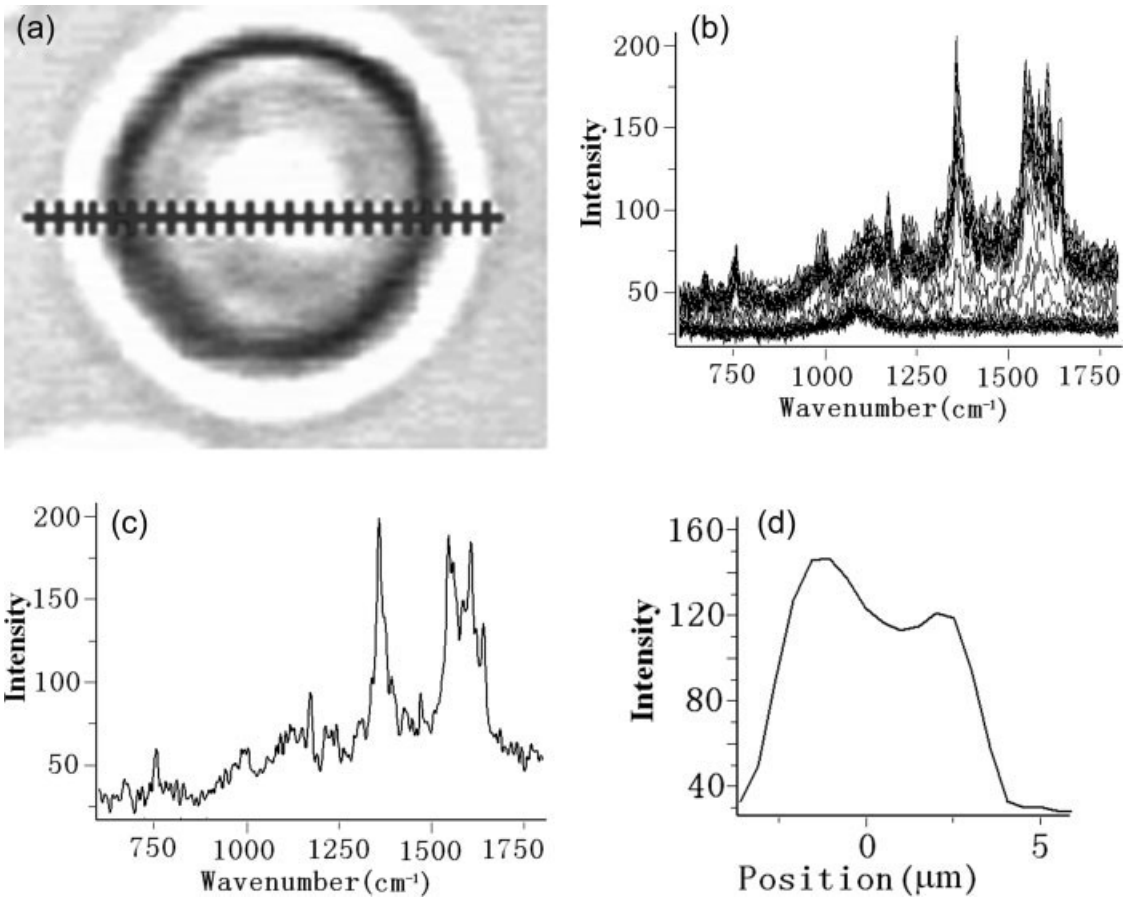

FIGURE 1 The process of line mapping. (a) Setup a scan line with scan step on an erythrocyte; (b) The Raman spectra of all the scanned points; (c) The band of interest selected for mapping; (d) A typical line mapping curve.

Raman Line Mapping of Erythrocyte

Line mapping technique was used to study the distribution of Hb in single erythrocyte. The line mapping was performed using the 1358 cm^{-1} ($\nu 4$) band for Hb under the 514.5-nm excitation light, for its signal is strong and almost without variation under the measuring condition,[10] from the $\nu 4$ band one can also obtain the information about the electron population in the π^* orbital.[1,8,21]

In line mapping, the scan step was set to ~0.5 μm, the laser power at sample was 0.6 mW, and the other scan parameters were the same as mentioned above. Both the young and old erythrocytes were placed in a refrigerator (4°C) for half an hour before measurement to ensure that their Hb molecules were at T state, which, is characterized by the disappearance of the marker band for O_2 concentration at 1640 cm^{-1} (ν_{10}).

When the line mapping was applied, the line was across the cell (shown in Figure 1a). The Raman spectra of all the points on the line were obtained (see Figure 1b) and the band of interest for line mapping was selected (Figure 1c). The line mapping curve was achieved from the intensity of the interest band at each point (Figure 1d).

The signal-to-noise ratio (data not shown) in the line mapping measurement shows that we can detect the protein intensity variations with an accuracy of about 2.8% in line mapping.

Raman 2D Imaging

To obtain the two-dimensional (2D) distribution of Hb in living erythrocytes, the technique of band mapping was employed. Similar to line mapping, the band mapping needs to select the band of interest. The 1358 cm^{-1} band was chosen as the imaging band in the experiment, not only for it is a strong resonance Raman band, but also for it is a sensitive oxidation-state marker that can reflect the electron population in the porphyrin π^* orbital. The band mapping was also performed under the 1 mW 514.5-nm light excitation, the exposure time was set for 0.5 s, and the scan step was 1 μm. The result of the 2D mapping is shown as an image, in which the brightness of each point indicates the intensity of the selected band.

The Transition Speed of the Hemoglobin from T to R State

To detect the transition speed of the hemoglobin from T to R state in a living erythrocyte, a simple method, which is somehow different from that proposed by Wood and McNaughton[9] was developed. The erythrocytes were prepared in a centrifugal tube under 4°C for half an hour, so they were mainly in a deoxygenated state that is characterized by the disappearance of the band at 1638 cm^{-1}. Then, they were suspended in a PBS at room temperature for 10 min to

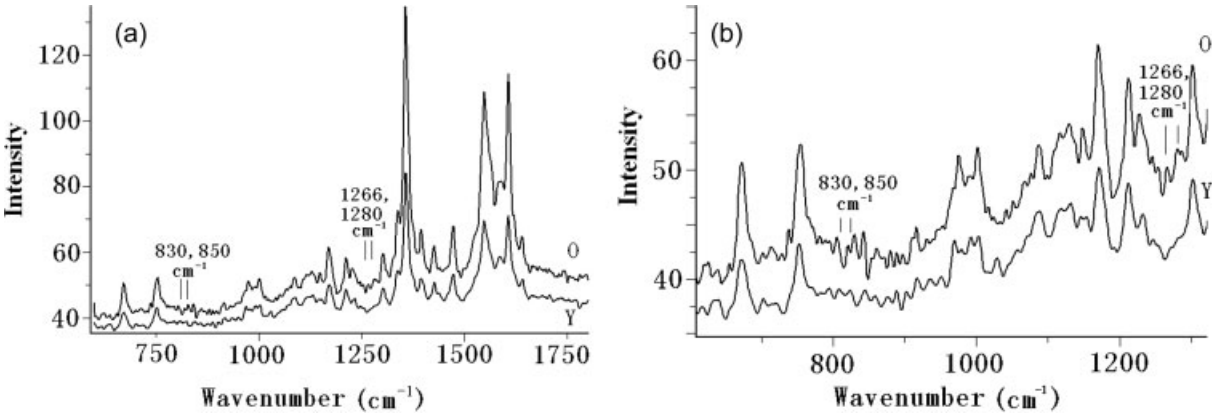

FIGURE 2 The Raman spectra of young (Y) and old (O) erythrocytes in T state. (a) The band width is from 600 to 1800 cm^{-1}; (b) The detail spectra in the range of 600–1000 cm^{-1}.

gradually equilibrate with atmospheric oxygen. At each 5-min interval, 100 μl of the cell suspension were taken to spread on a poly-L-lysine coated glass slide for Raman microscopy. The Raman spectra were recorded for at least 18 erythrocytes each time. The variation of the molecular conformation with time was therefore deduced from the time course of the Raman spectra.

RESULTS

Raman Spectra of Young and Old Erythrocytes

Figure 2 shows the Raman spectra of young and old erythrocytes recorded at room temperature with 1-s exposure time and one accumulation. We can see that the most characteristic Raman bands of young and old erythrocytes are similar, but the old one has more intense bands at 640, 830–850, 1266, and 1280 cm^{-1}.

Corresponding to the different oxygenation states of the hemoglobin in erythrocytes, there are different types of Raman spectra. We can see in Figure 3a that, for an erythrocyte in fully deoxygenated (T) state, the intensities of the bands at 1358 and 1606 cm^{-1} are very strong, but the band at 1640 cm^{-1} (ν_{10}) disappears. When the erythrocyte becomes fully oxygenated (R), the intensities of the bands at about 1640 and 1371 cm^{-1} are very strong, while the band at 1606 cm^{-1} is less intensive and the band at 1358 cm^{-1} even disappears. We also found that there are some other types of spectra, which are different from those of T and R states. The corresponding Raman spectra are denoted as M1, M2, M3, and M4, respectively (see Figure 3a). For the M1 spectrum, almost all the bands are similar to the spectrum of T state but a band at 1640 cm^{-1} appears. The difference between the spectra of M1 and M2 is the appearance of the band at about 1378 cm^{-1} (ν_4) and a slight increase in the intensity at the band of 1640 cm^{-1}. For the spectrum labeled M3, the band intensities at 1640 and 1375 cm^{-1} further increase, especially the ν_4 band (1375 cm^{-1}), can increase to about a half of the band intensity at 1358 cm^{-1}. For M4 spectrum, the intensity of the band at 1640 cm^{-1} increases to a value close or even exceeding to the band at 1606 cm^{-1}. Similar situation can also be found for the ratio between the bands at about 1373 cm^{-1} (ν_4) to that at 1358 cm^{-1}. To see if these spectra are the mixture of the T and R state, we constructed a "Mix" curve by mixing the Raman spectra of Hb in T and R states using the average-weighted method. The "Mix" curve is also illustrated in Figure 3 as a reference to indicate the difference of it from the spectra of M1, M2, M3, and M4. Figure 3b shows the detail Raman spectra of Figure 3a in the region of 1300–1400 cm^{-1}, so that the detail variation of the ν_4 position in the T to R transition process can be seen more clearly.

Line Mapping

The line mapping curves of young and old erythrocytes are presented in Figure 4. The baseline calibrated mean value and SD for the intensities of young and old erythrocytes at the mapping band (1358 cm^{-1}) are 34.05 $\pm$ 2.81 and 49.03 $\pm$ 11.03, respectively. We can see that, in contrast to the homogeneous distribution of the Hb in young cells, the protein intensity is much higher at the edge of the old cell than in its center, indicating that there are more Hbs distributing around the cell membrane.

2D-Mapping

Figures 5a and 5b show the band mapping images of young and old erythrocytes, respectively. Similar to the result of line mapping, the signal of Hb in young erythrocytes (Figure 5a)

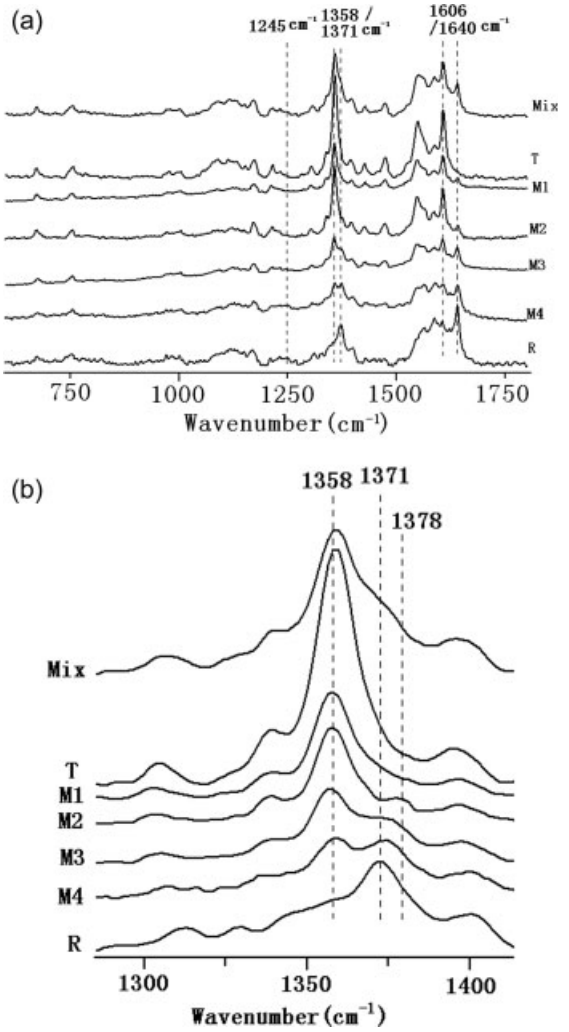

FIGURE 3 The diversity of human Hb's Raman spectra. (a) From top to bottom they are the Raman spectra of "Mix" (mixture of the spectra of T and R, weighted average), T state, M1, M2, M3, M4, and R state, respectively. (b) The detail Raman spectra in the region of 1300–1400 cm^{-1}.

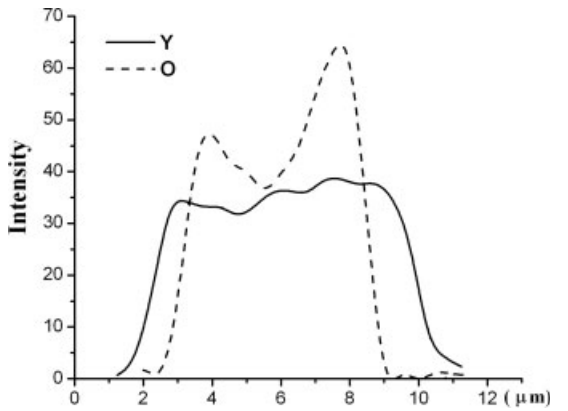

FIGURE 4 The line mapping of young (Y—solid line) and old (O—dashed line) erythrocytes at about 1358 cm^{-1} band.

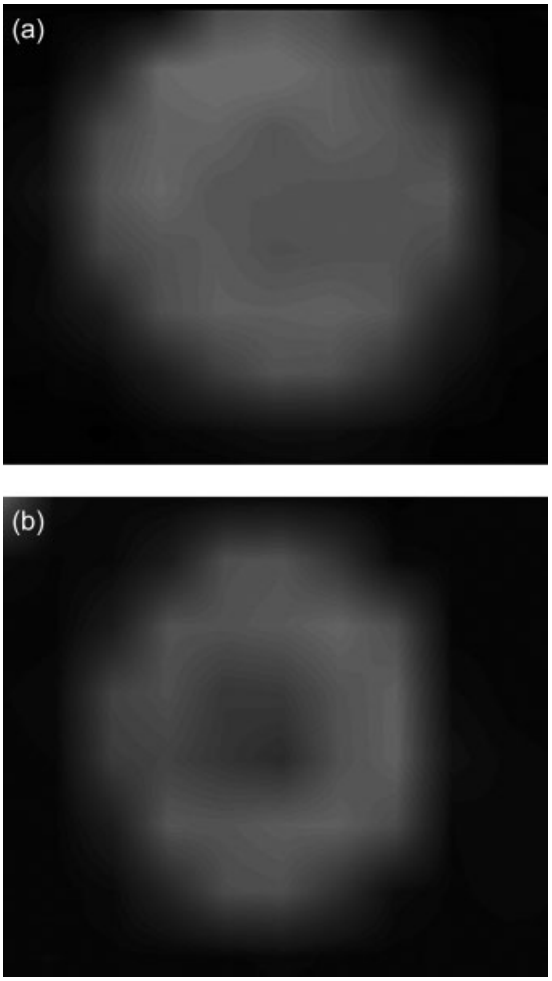

FIGURE 5 The 2D Raman images of young and old erythrocytes. The spectra range was taken from 600 to 1800 cm^{-1}, the exposure time was 1 s, the scan step was 1.0 μm (about 6-min scan time). (a) and (b) are the Raman images with band mapping (centering at 1358 cm^{-1}) of young and old erythrocytes, respectively.

is more homogeneous than that of old erythrocytes (Figure 5b), indicating that the Hbs in young cell distribute more homogeneously than in old erythrocytes.

The Transition Speed of the Hb from T to R State

Figure 6 shows the transition of the hemoglobin in young and old cells from T to R state as a function of time, in which the spectra of the T state, those labeled M1-M4 and the R state are converted to the artificial scores of 0, 1, 2, 3, 4, and 5 according to their oxygenation states, respectively. The error bar of each point indicates the SD calculated from the scores of more than 18 erythrocytes at the time. We can see that the Hbs in both kinds of the cells transits from T (Score 0) to M2 (Score 2) in about 15 min, then the Hbs in young erythrocytes become oxygenated in about 45 min for they

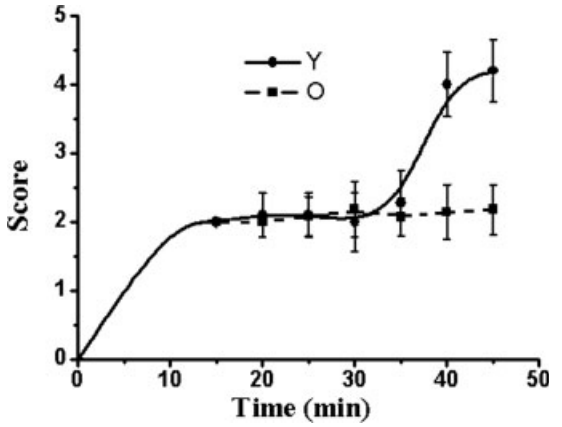

FIGURE 6 The transition curves of the Hb in young (Y) and old (O) erythrocytes from deoxygenated state to more oxygenated states.

have transited to M4 (Score 4), but the Hb in old ones keep at M2 unchanged up to 55 min. This suggests that the Hb of young erythrocytes has a faster transition speed from T to R than that of old ones.

DISCUSSION

The Diversity of Hb's Raman Spectra

The Raman spectra of T and R states of Hb have been studied by several authors,[8–13,22–26] in their reports, the oxidation of the protein was mainly analyzed based on the two extreme states. However, our results indicate that there are some intermediate states between them as shown in Figure 3.

From Figure 3b we can see that, during the T to R transition, the band position of ν_4 in M1-M4 spectra changes from 1378 to 1371 cm^{-1}, this suggests that there should be some intermediate states other than T and R states. Otherwise, the position of ν_4 should only appear either at about 1371 cm^{-1} (the peak in R state) or 1358 cm^{-1} (the peak in T state), but not at 1373–1378 cm^{-1} (refer to the Results section). This can be further proved by the "Mix" curve. As mentioned above that the "Mix" curve was achieved by mixing the spectra of T and R states using the average-weighted method. Though the weighted factor can be changed to obtain different mixing curves in different shapes, the ν_4 band keeps at the position from 1358 to 1371 cm^{-1}. Therefore, with appearance of the ν_4 band at the region from 1373 to 1378 cm^{-1}, and distinct bands that are obviously not from T or R state, the time-evolved spectra M1, M2, M3, and M4 indicate that at least one intermediate state is present.

The diversity of the Raman spectra is consistent with the variety of Hb's conformation during the process of oxygenation, and gives the detail information about the conformational change step by step. The binding of oxygen to the sixth coordination positions of a Fe atom can trigger the transition from T to R state. When a Fe atom moves into the porphyrin plane, it will pull the proximal F8 histidine coordinate to the Fe in the fifth position, thus shifting the F helix. The resulting conformational change is then transmitted to the subunit interfaces; the salt bridges between the terminal amino acid groups on the subunits would be broken, leading to a T to R state transition.[27] During the process, the Hb may undergo successive conformational changes, thus presenting different types of Raman spectra. According to previous researches, the ν_4 position of the Hb appears at about 1378 cm^{-1} when it begins binding oxygen. As more O_2 molecules bind to it, the ν_4 band will shift to low wave number until 1371 cm^{-1} with all the subunits of Hb are in R state.[24] Therefore, the appearance of the band in different positions between 1378 and 1371 cm^{-1} suggests that there are intermediate states existing in the allosteric process of Hb.

Actually, some previous researches also proposed the existence of some stable intermediate states[28–34] other than R and T states. Using the method of crystallization, Silva et al. proved a third quaternary structure (named as R2 state) of human HbA.[35] The R2 differs from the R state by rotating His-97β2 away from threonines 38al and 41a1, breaking contact with these residues and allowing water access to the center of the a182 interface. With a steric barrier that is less than that for the R–T transition, the R2-state is believed to function as a stable intermediate along an R-R2-T pathway, it is also an energetically accessible structure as the T and R states. Spiro and coworkers by taking time-resolved absorption measurement and UV resonance Raman spectra, also reported a successive stepwise formation of a T quaternary in allosteric pathway of Hb.[36,37]

Besides experimental evidence, theoretically it can be demonstrated that there is an intrinsic tendency of Hb to undergo T → R2 transition. By using a simple mechanical model based on Gaussian fluctuations of residues, Xu et al. showed that the passage from T into R2 is favored by the global mode of motion, the transition can be induced by purely elastic forces of entropic origin that are uniquely defined for the particular contact topology of the T form.[38] The KNF model (sequential model) proposed for explaining the allosteric effect of Hb also supports multiconformations of Hb during the T to R transition.[39]

Though the coexistence of T-, R-, and R2-states in solution was proved by crystallography,[35] more direct evidence is needed. The UV Raman spectra indeed prove the existence of the intermediate states of Hb, but they were obtained from isolated Hb and based on the information of the residues of

globin. Therefore, the Raman spectra of erythrocytes we obtained using 514.5-nm light excitation are significant, for it not only provides the first direct evidence of the intermediate states of Hb in living erythrocyte, but also reveals the allosteric effect of Hb and provides more direct and distinct information about heme, the key part for oxygenation in the protein.

The Raman Spectra Difference Between Young and Old Erythrocytes

From the Raman spectra of young and old erythrocytes, we can see that there is no obvious difference between them in the main characteristic Raman bands associated with heme (see Figure 2a). They are mainly different in signal intensity and smoothness, and in some bands associated with the peptide chain of globin. For most old erythrocytes, their Raman spectra have better smoothness and the spectrum intensities are stronger than those of young ones. The most likely explanation for it is the increase in the concentration of the Hb in an old erythrocyte owing to its volume reduction.[14] Another possible reason is that the Hb in an old erythrocyte may aggregate to some extent, thus resulting in a decrease of the intermolecular distance between the heme groups. As the electron population in π^* orbital increases, the vibration mode becomes stronger.[11]

For heme, it has no characteristic bands at about 850 cm^{-1}.[8,11,20] Siamwiza et al. had shown that the doublet of 830 and 850 cm^{-1} arises from a Fermi resonance between a ring-breathing vibration and the overtone of an out-of-plane ring-bending vibration of Tyr.[40] Therefore, the band at 850 cm^{-1} is usually assigned to the Tyr of globin,[1,40] while the band at 830 cm^{-1} is assigned to the γ(Cm-H) of heme,[8,20] and it also has the contribution from the Tyr of globin.

From Figure 2 we can see that the band at 850 cm^{-1} for old cells is more intensive than that of young ones thus can be recognized obviously. According to Narayanan,[41] when the tyrosine residue is buried, the band at 850 cm^{-1} is less intensive than the band at 830 cm^{-1}. However, if the tyrosine residue is exposed, the intensity of the band at 850 cm^{-1} would increase and even stronger than that at 830 cm^{-1}.[1,40–42] Therefore, the intensity change of the 850 cm^{-1} band can be used to evaluate the state of Tyr. The ratio of I_{850}/I_{830}, on the other hand, is used as a sensitive marker to estimate the buried or exposed states of Tyr in protein conformation. As we can see in Figure 2b that, old erythrocyte has stronger band intensity at 850 cm^{-1} than at 830 cm^{-1}. Therefore, it is reasonable to believe that the Tyr of the old cell is exposed. The function of Tyr in Hb is to stabilize the structure of Hb through the effect of H bond to H helix and F helix, so the allosteric effect in old erythrocytes would be gradually degenerated.

The spectra of young and old erythrocytes also show differences at the bands associated with the peptide chain of globin, the old one has more intense bands not only at 830–850, but also at 640, 1266, and 1280 cm^{-1}. The bands at 1266 and 1280 cm^{-1} are respectively assigned to the unordered coil and the α-Helix protein conformations of amide III.[1] Therefore, the increase of the band intensity at 1266 cm^{-1} relative to 1280 cm^{-1} in the old erythrocytes suggests that the peptide chain of their globin may have more unordered coils and some extent of degradation.

For a functional Hb, its hydrophilic amino acids should be more exposed and its hydrophobic amino acids are mainly buried, so that the Hb can be dissolved in water without deposition and the water molecule can not enter into the inner part of the heme pocket, thus preventing the Fe^{2+} from being oxidized to Fe^{3+} (MHb). By contrast, with exposed Tyr and more unordered coils, the Hb in old erythrocytes should be easily oxidized to MHb and deposited.

The Distribution of Hb in Young and Old Erythrocytes

As can be seen from Figures 4 and 5, Hbs distribute quite homogeneously in young cells; while in old erythrocyte, the protein intensity is much higher at the edge of the cell. It indicates that there are more Hbs distribution around the cell's edge area. This is in consistent with the results obtained previously by Demein, Hiroshi, Rauenbuehler, and Salhany using the methods of centrifugation, stopped-flow fluorescence, and optical spectrum, for demonstrating the possible binding of Hb to the red cell membrane.[43–46] The binding of Hb to the cell membrane can not only lead to a reduction of the membrane flexibility,[46] but also influence the oxidation ability of Hb. The reactive oxygen species generated during autoxidation are not efficiently neutralized by the cellular antioxidant enzymes when Hb is bound to the membrane.[47,48]

Comparing the two kinds of mapping techniques, 2D mapping provides more plentiful information than line mapping, but needs much longer time for the mapping. Under the radiation of 514.5-nm laser, the photodissociation effect in living erythrocyte can be induced easily. When using the mapping technique, especially 2D mapping, the laser power, exposure time, and accumulation number should be selected very carefully to avoid burning the cells.[49] Therefore, line mapping may be considered first as it can provide almost all the major character of Hb's distribution in an erythrocyte but has less risk of burning the cell. Another choice is to use

longer wavelength light for the excitation, such as 633-nm laser light reported by Wood et al.,[11] but 514.5-nm exciting light has the advantage in obtaining stronger signal at the characteristic oxidation marker $\nu 4$ band (1358 cm^{-1}) and more detail information about the structure of the band. Our results show for the first time, the applications of 514-nm radiation to the line mapping and 2D mapping on living erythrocytes, the methodology is expected to be also suitable for other kinds of cells.

T to R State Transition

By taking the time course of the Raman spectra of the Hb in living erythrocytes and using the six oxygenated states classification, we obtain the detail information about the variation of the protein's conformation with time, thus deducing the Hb's transition speeds from deoxygenated to oxygenated state in young and old erythrocytes, respectively. As could be seen from the curves in Figure 6, young erythrocytes can transit from T state to M4 with less time than old erythrocytes. This can be explained by the evidences provided in both line mapping and 2D mapping measurements. As the Hb in old cells may be in aggregation to some extents, their ability of carrying oxygen should be weakened, thus the old cell has slower speed in the T to R state transition.

CONCLUSIONS

In summary, Raman confocal microscopy was used to perform comparative study on the distribution and oxidation state of hemoglobin in living young and old mature erythrocytes. It is demonstrated that in contrast to the homogeneous distribution of the Hbs in young cells, there are more Hbs distribution around the cell membrane or bound to the cell membrane in old erythrocyte, the proteins exhibit some extent of aggregation and conformational change, thus has less ability of oxygenation than the Hbs in young erythrocytes. Our results also suggest for the first time that there are some intermediate oxygenated states for the Hbs in a living erythrocyte between T and R states. By using the six oxygenated states classification, one can obtain more detail information about the conformational change of the intracellular Hb with time during the reoxygenation process, and have evidence in explaining the allosteric effect of Hb at the same time. These findings are believed to be not only helpful in understanding the mechanism of the cell's aging process, but also useful in diagnosing normal, diseased, and dysfunctional erythrocytes, and finding out the ways to prolong the life of erythrocyte. Moreover, the methodologies developed in this study can be applied to the in vivo investigation of other interesting phenomena, such as the effect of oxidative stress in erythrocytes[8]; the variation of the cell's structure and function at both cellular and molecular levels under different conditions like pH, osmotic gradient, and radiations[50]; and the effects of drugs and treatments on diseased and dysfunctional erythrocytes, or other kinds of biological cells.

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