Mechanochemistry of single red blood cells monitored using Raman tweezers

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Abstract: Two microparticles were biochemically attached to a red blood cell at diametrically opposite parts and held by optical traps allowing to impose deformations. The cell deformation was monitored from the microscopy images. Raman spectra of the cell under tunable deformations were studied. Vibrational spectra analysis at different stretching states was supported with two statistical methods. Principal Component Analysis distinguishes the most prominent changes in spectra while 2D correlation technique monitors the evolution of Raman bands during stretching. The measurements show significant changes in the cell chemical structure with stretching however the changes saturate above 20% of cell deformation. Mechanical deformation of the cell mainly affects the bands corresponding to hemoglobin but contributions from spectrin and membrane proteins can not be excluded. The saturation of bands at higher deformations suggests some structural relaxation that RBC has to undergo to bear extra load. The results confirm widely accepted belief that spectrin released from membrane proteins allows for significant shape changes of the cells. We therefore tentatively suggest that interaction between membrane and cytoskeleton during deformation can be efficiently probed by confocal Raman spectroscopy, in particular via the peak around 1035 cm⁻¹.

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1. Introduction

The primary role of red blood cells (RBCs) is redistribution of oxygen throughout the human body. This is possible due to characteristic structure, shape, and mechanical properties of the cells. Erythrocytes are built of lipid bilayer which contains many transmembrane proteins and underlying cytoskeleton. It forms flexible biconcave disks which, consist mainly of hemoglobin but lacks a cell nucleus and most organelles. Chemical content of RBCs provides information about human health conditions. Therefore blood is one of the most common samples submitted nowadays for medical diagnosis.

Since, in human body, erythrocytes adopt different squeezing/stretching states, mechanoochemical processes associated with deformation of RBCs have been of particular research interest. First studies on deformability of RBC were performed using pipette aspiration [1, 2] and permitted to observe a high degree of deformability. This extraordinary property allows RBC to squeeze through microvasculature and transport oxygen during blood circulation [3]. Micromanipulation techniques such as optical or magnetic tweezers have allowed for more sophisticated microrheological studies. Most of them were focused on measuring the static changes of cell length [4-7] or area expansion [8] in response to a deforming force. More recently two point microrheology measurements of single RBC [9] showed the frequency dependence of complex stiffness confirming strong nonlinear deformation of cells. Optical tweezers provide an excellent method for probing single cells at different stretching states, giving potentially powerful tool for diagnosis of diseases [10], since mechanical properties of RBCs can be affected by malaria, spherocytosis, elliptocytosis or sickle cell anemia [11].

Understanding of molecular origin of extraordinary mechanical flexibility or nonlinear response of the single cell requires more sophisticated experimental approach and extensive theoretical simulations. In this context combination of optical tweezers with single cell spectroscopy seems to be very promising. Linking spectroscopic techniques with optical tweezers opens unique possibility to directly extract biochemical information at single-cell level under controllable mechanical conditions. The simultaneous use of external force and spectroscopic detection can also provide direct insight into molecular changes caused by mechanical deformations. Here, Raman spectroscopy is of particular interest because of its high chemical resolution and relative simplicity. It yields rich, multi-peaks vibrational spectra containing structural information about RBC’s basic constituents [12-17]. As previously demonstrated, Raman spectroscopy combined with optical tweezers is extremely valuable tool for reliable estimation of cell’s vitality [18-21]. Sensitivity of this method is high enough for identification and monitoring of changes in intra-cellular components induced by cell stretching. In particular, direct evidences for deoxygenation of cells with stretching was shown [22].

Till now most of Raman studies on RBC were performed with cells being directly trapped with focused optical beam. This is not an ideal approach because of potential light induced cell damage within the trapping volume. Reducing the power of trapping beam leads to significant diminution of Raman signal-to-noise ratio, extensive averaging at the expense of single experiment acquisition time is required. In this case external forces applied to cells by optical tweezers are also strongly reduced. The problem can be avoided by attaching RBCs to chemically functionalized microbeads, which might hold the cell during Raman scattering experiments [23]. Although heating via beads (size dependent process) or effects of scattered light can not be still completely excluded, the risk of direct light-induced damage by trapping beams is significantly reduced. Therefore sensitive experiments at higher forces and lower noise for longer time periods become possible.

We studied changes in molecular structure of a single RBC when it is gradually stretched by optically trapped beads attached to the cell. We extended our previous Raman measurements [22] to many different RBC’s stretching states, which required longer time for experiments.
The applied force is intended to simulate step-by-step deformation experienced by cells in normal conditions and induced by blood flow as they squeeze through microvasculature. To improve further the sensitivity of the experiments and facilitate their interpretation, we used also statistical techniques (2D correlation and principal component analysis (PCA)), that permit us to observe previously inaccessible changes in Raman spectra. The purpose of this work is to unravel direct relationships between mechanical deformation of RBC and chemical changes occurring in the cell structure on molecular level.

2. Materials and methods

2.1. Experimental setup

The experimental setup is a combined dual-beam optical trapping system with confocal Raman spectrometer and has been described previously [22, 24, 25]. Briefly, a 1064 nm beam generated a dual trap. The expanded and collimated beam was directed through an interferometer. The back focal plane of the trapping objective was conjugated with movable mirror planes of an interferometer. The movements of the mirrors in either arm of the interferometer then result in movements of the traps in the focal plane of the objective without changes in its intensity and shape, thus keeping the trapping potential of the traps the same. The out-of-plane position of two trapping beams with respect to the Raman excitation beam was controlled by moving one of the lenses in front of the interferometer.

A 785 nm beam was used for excitation of Raman spectra with average power of 5 mW at the sample. Samples were injected into a custom-made fluid chamber. The chamber was placed on an inverse Olympus IX 51 microscope equipped with a 100 × 1.3 NA oil immersion objective and a micrometer controlled stage. The backscattered light was collected by the trapping objective, passed through a holographic notch filter and a confocal system with a 100-µm pinhole. The spectrometer had a 600 lines/mm grating and was fitted with a thermoelectrically controlled charge-coupled device (CCD), cooled to -60°C. A CCD camera attached to the microscope provided optical images used for calculations of cell deformation.

Raman spectra were recorded with a spectral resolution of 3 cm⁻¹ for 30 seconds at each acquisition. Measured Raman spectra were background subtracted, normalized using Multiplicative Scatter Correction (MSC), smoothed using smoothing spline technique [26] and median centered before performing 2D correlation and PCA. Analysis was done in Matlab platform.

2.2. Sample preparation

About 30 µl of blood was obtained from a healthy donor and washed two times. Final volume was diluted in 600 µl of Alsever’s solution and stored at 4°C. The sample was used within no longer than two days. 3µm COOH functionalized polystyrene beads were purchased from Spherotech (CPX-30-10). The beads were washed four times in 50 mM MES buffer (Sigma Aldrich M1317). Their functionalization was obtained with Lectin (Sigma Aldrich L9640) using chemicals and protocol provided with PolyLink Protein Coupling Kit from Bang Labs (PLO1N). After functionalization, beads were again washed 4 times, and finally resuspended and stored in MES buffer. For measurement, 20 µl of resuspended beads were washed in Alsever’s solution.

The beads were attached to a single RBC by proceeding as follows: two beads were optically trapped while RBC was held by the optical trap generated by the Raman excitation beam. The two trapped beads were carefully approached towards the diametrically opposite parts of RBC, and the connection of RBC with the beads was obtained in around 5-10 seconds. The position of beads were then adjusted with the help of movable mirrors to assure, that the Raman excitation beam is hitting the edge of RBC. Cells were stretched by moving one of the trap.
of Raman spectra were done on 15 different cells. Raman spectra of most of them behaved by application of mechanical deformation in a similar fashion.

2.3. Statistical processing of the Raman spectra

In order to get insight into spectral changes induced by cell deformation, in particular those which are inaccessible by direct visual inspection, we used multivariate analysis [27]. Multivariate methods have been developed to study complex data with many variables analyzed simultaneously. Specifically, Raman spectra from biological samples are a good example of such data, when extraction of important, characteristic variables from some parts of Raman spectra is quite complicated or even impossible without statistical methods.

Principal component analysis (PCA) reduces the dimensionality of the data by finding a set of orthogonal coordinates, principal components (PCs), which accounts for the maximum variance in the Raman spectral dataset, and describes the major trends in the data. In other words, with PCA it is possible to extract the principal contributions in spectral changes due to molecular deformations by RBC stretching. Loading plots represent the characteristic spectra in principal component coordinate system.

Other statistical technique used in this work is 2D correlation analysis [28]. Recently we demonstrated this processing by monitoring local pH in photodynamic therapy-treated live cancer cells with surface-enhanced Raman scattering probes [29]. The 2D correlation analysis identifies and extracts Raman bands which are changing the most with respect to the external perturbation and relative to each other. The output of the analysis consists of contour graphs, where the in-plane axes are the Raman shifts and the third axis is the level of correlation between Raman bands. The method produces synchronous and asynchronous graphs, which refer to the in-phase and out-of-phase relationship between bands, respectively, as a function of cell deformation. For the synchronous spectra, autopeaks occur along the diagonal of the plots, and are mathematically equivalent to the autocorrelation of a band, thus signifying bands that are susceptible to change under cell deformation. Cross peaks appear off diagonal and represent bands that increase or decrease together during the perturbation. The picture of the modes behavior is completed by the asynchronous spectrum where autopeaks and cross peaks represent bands that increase or decrease with perturbation but out of phase with one another.

3. Results

Figure 1 shows a typical set of Raman spectra of single RBC. Raman signals at fifteen different stretches were measured. Raman spectra of RBCs depend strongly on excitation wavelength [30, 31]. At 785 nm excitation the bands associated with proteins can be observed in addition to those associated with the porphyrin macrocycle [15]. Presented Raman spectra are characteristic of single RBC and for unstretched cells have been discussed in details in [15, 17, 31].

Deformation was conventionally measured using image analysis by calculating the distance between the beads attached to the diametrically opposite parts of the cell. It was calculated in the following way: \( \text{Deformation}(\%) = \left( \frac{\Delta L}{L_0} \right) \times 100 \) where, \( \Delta L = L - L_0 \) is the difference between diameters of stretched \((L)\) and relaxed \((L_0)\) cell. Even without statistical processing Fig. 1 demonstrates effects of the cell stretching on some Raman bands (see also color gradient of the plot), in particular the bands centered at about 991 cm\(^{-1}\), 1035 cm\(^{-1}\) and 1442 cm\(^{-1}\). 1035 cm\(^{-1}\) peak is associated with in-plane CH\(_2\) asymmetric mode of vinyl substituent in porphyrin [15, 17] and/or Phenylalanine (Phe) [14, 32]. The bands at 991 cm\(^{-1}\) and 1442 cm\(^{-1}\) are assigned to \( \nu_4 \) /Phe and CH\(_3\)/CH\(_3\) in plane modes of haem, respectively [17]. At low deformations the 1035 cm\(^{-1}\) and 1442 cm\(^{-1}\) peaks are unchanged. Their growth starts when the cell deformation is more than 10%, and the growth is saturated at higher cell deformations (above 20%).

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The most remarkable finding of our measurements - the extremely high sensitivity of 1035 cm$^{-1}$ peak to the cell extension - has not been reported yet. It permits us to suggest the monitoring of this peak intensity as a marker to characterize internal deformations in the cell in possible lab-on-a-chip applications. Other Raman bands also change with cell deformation, but their behavior could be revealed only using statistical techniques (see below).

4. Discussion

To obtain the dynamics of conformational changes we first performed the PCA analysis using data in the whole spectral range (Fig. 2). The score plot Fig. 2(b) shows that at small cell deformations (up to 10%), spectral changes are negligible (see also Fig. 1). Hence, the maximum intensity variation of spectra obtained at five initial values of deformation may be used to estimate a threshold to discard the peaks generated by the electronic and mechanical noise and errors by numerical calculations. Then we can consider only those Raman bands which are above this threshold.

To establish the numerical value of the threshold in the loadings plot Fig. 2(a) we performed PCA analysis of the Raman spectra obtained for the first five values of deformations. Multiplying each data point on the scores plot gives us the intensity of the corresponding spectra with respect to the median spectrum. The maximum intensity was found to be around 40 units (inset of Fig. 2(a)). To translate this threshold (in intensity) to the loadings plot of complete set of spectra we divided it by the highest value in the scores plot of Fig. 2(b). The threshold ($\pm$0.02 in loadings) was then used for further analysis.

We conclude that the loadings of the first principal component, which captures almost 80% of the total variance, are dominated by bands centered at 1083, 1196, 1484, and 1535 cm$^{-1}$ along with above mentioned bands at 991, 1035, and 1442 cm$^{-1}$. The former four bands can be assigned to $\delta(= C_b H_2)_{asym}$, $\nu_5 + \nu_{18}$, $\nu_3$ and $\nu_{38}$ modes respectively [15, 17, 33].

These selected peaks are also consistent with the most prominent features observed in the complementary technique, 2D correlation analysis, where strong correlation between these bands is observed (Fig. 2(c) and Fig. 2(d)). Positions of cross-correlation peaks in synchronous map is consistent with the PCA analysis. This confirms that this set of Raman bands is changing synchronously with the cell stretching.

In order to characterize the intensity behavior of correlated bands with deformation, we per-
formed the PCA analysis with reduced spectral windows to include the selected bands only. Resulting scores plots are intended to mimic the band intensity behavior of single band with stretching. In a way, PCA acts as a fitting that permits us to monitor the selected Raman bands even when the spectra are noisy and without distinguishable features. Results for bands at 991, 1035, 1083, and 1196 are shown separately in Fig. 3(b), 4(b), 5(b), and 6(b), respectively, where all the bands exhibit a similar tendency with the cell stretching. Raman peak at 1442 cm$^{-1}$ does not give new information and its behavior is very similar to Raman peak at 1196 cm$^{-1}$. If the cell is deformed less than 10%, these bands exhibit almost constant Raman intensity. Higher mechanical forces lead to almost linear increase (or decrease) dependence with stretching. This behavior is observed up to about 20% of cell deformation. Finally, the bands saturated for deformations exceeding 20%.

However, the bands at 991 and 1535 cm$^{-1}$ showed inverse behavior with increased deformation, i.e. their intensity decreased in the deformation range of 10-20% and saturated above 20% (see, for example, data shown for 991 cm$^{-1}$ band in Fig. 3.

To study the dynamics of band’s position and their shapes we took advantages of 2D corre-
Fig. 3. Statistical analysis for Raman band at 991 cm$^{-1}$. **Top:** PCA analysis (loading plot (a) and scores plot (b)). **Bottom:** Expanded view of 2D correlation maps from Fig. 2 (synchronous map (c) and asynchronous map (d)).

Fig. 4. Statistical analysis of Raman band at 1035 cm$^{-1}$. **Top:** PCA analysis (loading plot (a) and scores plot (b)). **Bottom:** Expanded view of 2D correlation maps from Fig. 2 (synchronous map (c) and asynchronous map (d)).

The simulation method. Figures 3, 4, 5, and 6 show 2D maps for the individual bands. Similar trends for some groups of peaks are clearly visible. In the asynchronous map we observe a characteristic pattern for band broadenings formed by four lobes in a cross-like shape. It can also be seen that this pattern is asymmetric. This is caused by the small shift of the Raman band maxima to higher wavenumbers. At 1035 cm$^{-1}$ we observe a similar pattern but with opposite signs in asynchronous maps indicating the band narrowing and shifting to smaller wavenumbers with stretching. Bands at 991 and 1535 cm$^{-1}$ have also the same cross-like shape but their intensity decrease (see, for example, data shown for 991 cm$^{-1}$ band, Fig. 3). Simulation of their behavior suggest that 991 cm$^{-1}$ band broadens and shifts to lower wavenumbers. 1535 cm$^{-1}$ band broadens and shifts to higher wavenumbers (data not shown). A different pattern was observed for 1083 cm$^{-1}$ band, which exhibits two lobes at both parts of the diagonal in the asynchronous
map (Fig. 5). This indicates that the width of the band remains the same but a shift to lower wavenumbers occurs. All observed peak frequency shifts are rather small and do not exceed 5 cm$^{-1}$, close to the spectrometer resolution.

Raman signals collected from RBC are average signals from the confocal volume consisting of large number of molecules that include haemoglobin, spectrin and other biomolecules. The stretching might move the cell, but obviously the basic constituents of cell under investigation do not change. In other words, net effect of stretching in Raman signals should not depend on the probed region of the cell. Moreover, the measurements were carried out at many stretching states and for various cells. These cells demonstrated unidirectional changes in their bands with stretching. That is why we can exclude that the cell movement within focal volume has any
noticeable effect on Raman spectra.

The main constituents of RBC are hemoglobin and cytoskeleton (including spectrin), all surrounded by thin membrane with many embedded proteins. RBC vibrational spectra, therefore, consist mainly of bands characteristic of porphyrin macrocycle and various proteins. The shape of cells is preserved by spectrin network and ankyrin proteins, which anchor cytoskeleton to membrane. About $10^6$ hemoglobins per cell are strongly bound to the inner wall of RBC membrane with possible intercalation [34, 35]. Stretching of the cell is expected to affect mostly membrane and cytoskeleton, which absorb most of the forces, and also hemoglobins, in particular those which are bounded. Raman excitation close to the edge of RBC allowed us to get signals from the above mentioned cell’s regions which undergo maximum deformation.

The statistical analysis, in particular the scores plots, shows the similar behavior of the Raman bands: their intensity remains almost unaffected at low cell deformations, and changes occur at intermediate forces following by band saturation at highest deformations achieved in the experiments. Most of these bands can be associated with different vibrational modes in hemoglobin. It indicates that RBC’s chemical structure is strongly perturbed by deformation. Such activation/deactivation of selected Raman transitions can be caused by significant deformation-induced changes in molecular symmetry. Although the role of hemoglobin in maintaining the shape of the cell is rather secondary we believe that via direct binding to membrane it acts as an important probe of membrane/cytoskeleton interaction.

The 785 nm Raman excitation beam is slightly absorbed by hemoglobin, and resonant enhancement of Raman bands may affect measurements. To the best of our knowledge, absorption measurements of single RBC at different stretching states have not been performed yet. Electronic structure of hemoglobin is expected to change with cell deformation, at least via deoxygenation [36, 22]. This leads to changes in RBC’s absorption spectrum, in particular, the absorption grows at 785 nm [36]. Raman spectra excited at 785 nm show strong enhancement of the $B_{1g}$ modes and the vinyl modes [15]. Therefore expected increase of absorption (at 785 nm) with stretching should further enhance all above mentioned modes almost proportionally. Our data show that intensities of some modes (in particular at 1035 cm$^{-1}$) grow much stronger with stretching than the others so this hypothesis appears to break down. Hemoglobin concentration in the cell may also affect proportionally all measured Raman intensities. Elongating the RBC decreases the internal volume of cell and leads to the corresponding increase in hemoglobin concentration [22]. This effect should not only promote Raman intensities at all wavenumber but also neighbor-neighbor interaction between hemoglobins. Such enhanced interaction can be partially responsible for observed broadening of the peak at 1196. Nevertheless hemoglobin concentration effect alone can not fully explain observed behavior of Raman bands. We have to consider significant structural changes caused by mechanical deformation. Exact nature of structural changes in RBC are not straight forward to determine mainly because Phenylalanine (Phe), which is an essential amino acid that can be found not only in hemoglobin but also in various membrane proteins e.g. ankyrin, band3 proteins and spectrin [37]. Although hemoglobin is most likely the main source of Raman signal perturbation, we can not completely exclude contributions from proteins embedded in membrane and cytoskeleton which bears most of the forces during deformation. Direct exposure of membrane to Raman excitation beam is supposed to enhance total scattering probability from it. Interestingly, in Raman studies of RBC ghost [14], strong peak at about 1035 cm$^{-1}$ was also observed which might suggest partial membrane contribution in our data. From many membrane proteins it is ankyrin which anchors cytoskeleton to membrane and that is why this protein together with spectrin presumably undergoes maximum deformation.

Taking into account all the above mentioned aspects, the behavior of Raman bands intensities as a function of applied deformation can be tentatively explained as follows. We suppose
that at low deformations, when bands intensities remain almost constant, spectrin bears most of the forces and rearranges itself without significant changes in its primary chemical structure. It is likely that in this range of deformation, structural changes might occur in its higher order structure. At intermediate deformation range (10-20%), the stress is high enough and can lead to significant structural perturbations of linker proteins, spectrin network as well as hemoglobin attached to membrane. Therefore significant changes in Raman bands intensities were observed. At higher deformations (when bands intensity growth saturates), we need to consider mechanical nonlinearity of RBCs. It was proposed that nonlinear response of the cells can originate from the release of spectrin filament from linker proteins (ankyrin) which then re-bond in a configuration of lower stress [9, 23]. We believe that observed saturation of the peaks corresponds to filament release from the linkers. This process is followed by creation of new bonds but in a configuration of similar or even lower stress. Behavior of all the bands discussed here are consistent in a way that they remain constant up to 10% cell deformation, increase (or decrease) in intermediate deformation range, then saturates for a small region and finally decrease (or increase) slightly at higher deformation (above ~25%).

5. Conclusion

We have presented Raman spectra of RBC at relaxed and various stretched states and discussed the spectral changes induced in RBC by mechanical deformation. Statistical techniques, such as principal component analysis and 2D correlation spectroscopy were applied to facilitate detailed analysis of spectral changes. Mechanical deformation of the cell mainly affects the bands corresponding to hemoglobin but the contribution from spectrin and membrane proteins cannot be excluded. While Raman intensity changes with deformation were provided by both - 2D contour maps as well as PCA, other spectral details such as bands broadening and their shift were extracted from the 2D correlation analysis. We found that most of the affected bands show similar intensity behavior with stretching. The saturation of bands at higher deformations suggests some structural relaxation that RBC has to undergo to bear extra load. The results confirm widely accepted belief that spectrin release from membrane proteins allows for significant shape changes of red blood cells. We therefore tentatively suggest that interaction between membrane and cytoskeleton during deformation can be efficiently probed by confocal Raman spectroscopy, in particular via the peak around 1035 cm⁻¹.

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