Biophysical Journal, Volume 97

Supporting Material

Close-up on erythrocytes with SERS

Nadezda A. Brazhe, Salim Abdali, Alexey R. Brazhe, Oksana G. Luneva, Nadezda Y. Bryzgalova, Evgenia Y. Parshina, Olga V. Sosnovtseva, and Georgy V. Maksimov

Supporting material to the manuscript "New insight into erythrocyte with *in vivo* surface enhanced Raman spectroscopy"

N.A. Brazhe[1*], S. Abdali[2], A.R. Brazhe[1], O.G. Luneva[1], N.Y. Bryzgalova[1], E.Y. Parshina[1], O. V.Sosnovtseva[3], G. V. Maksimov[1]

[1] *Biophysics Department, Biological Faculty, Moscow State University, Leninskie gory 1/12, 119991 Moscow, Russia*

[2] *Danish Cancer Society, Strandboulevarden 49, 2100 Copenhagen, Denmark and Department of Medical Engineering, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark*

[3] *Department of Biomedical Sciences, Copenhagen University, Blegdamsvej 3B, 2200 Copenhagen, Denmark*

[*] *Corresponding author: una@biophys.msu.ru*

Physiological salines for erythrocytes

Normal Tris-based physiological saline with normal osmolarity used for the first blood dilution in SERS and Raman experiments has the following composition, mM: 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 5.4 Tris-HCl. For the preparation of hyperosmolar Tris-based saline (used for the second dilution of blood used in the SERS experiments) we increased concentrations of all salts as many times as was needed to achieve normal osmolarity in a mixture of sample 2 with Ag NPs colloids. For example, in experiments with a volume ratio of [Ag NP solution]:[experimental erythrocyte sample]=2:3 we used hyperosmolar Tris-based saline with the salt concentration increased by a factor of 5/3 in comparison to normal Tris-based saline, mM: 241.67 NaCl, 8.33 KCl, 1.67 $CaCl_2$, 1.67 $MgSO_4$, 9 Tris-HCl.

Normal Alen saline with normal osmolarity has the following composition, mM: 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 4 Na_2HPO_4, 1 $NaH_2PO_4 \times 2H_2O$. Hyperosmolar Alen saline was prepared in a similar way to hyperosmolar Tris-based saline. In experiments with a volume ratio of [Ag NP solution]:[experimental erythrocyte sample]=2:3 we used hyperosmolar Alen saline with the salt concentration increased by a factor of 5/3 in comparison to normal Alen saline, mM: 241.67 NaCl, 8.33 KCl, 1.67 $CaCl_2$, 1.67 $MgSO_4$, 6.67 Na_2HPO_4, 1.67 $NaH_2PO_4 \times 2H_2O$. The pH value in all Tris-based and Alen salines was adjusted to 7.4.

Phosphate buffer used for the hemolysis of erythrocytes was as follows, mM: 4 Na_2HPO_4,

1 $NaH_2PO_4 \times 2H_2O$. In experiments with isolation of cytosolic hemoglobin pH value was adjusted to 7.2 to be the same as in the cytoplasm of erythrocytes; in experiments with preparation of erythrocyte ghosts with membrane-bound Hb pH value was adjusted to 7.4.

All used chemicals were of the highest purity grade available and were purchased from Sigma (USA).

Preparation of erythrocyte ghosts with membrane-bound Hb

Blood plasma was separated from erythrocytes by triple centrifugation ($440 \times g$, 10 min) of blood in normal Alen saline, pH 7.4, at a temperature of 4^oC. The number of erythrocytes in the obtained erythrocyte suspension was calculated in the Gorjaev chamber and then the erythrocyte suspension was adjusted with Alen saline to the number of erythrocytes 10^7 cells/μl. Preparation of erythrocyte ghosts with membrane-bound Hb bound to AE1 exchanger (Hb_{AE1}) was done as described in [1]. 1 volume of adjusted erythrocyte suspension was diluted with 20 volumes of ice-cold phosphate buffer (pH 7.4) and centrifugated at 4° at $4000 \times g$ for 40 min. Supernatant with cytosolic Hb was removed and the precipitate with erythrocyte ghosts was resuspended in ice-cold phosphate buffer (pH 7.4) and centrifugated again at 4° at $4000 \times g$ for 40 min. The procedure was repeated 5 times. After that erythrocyte ghosts were concentrated in Alen saline (pH 7.4) by centrifugation at $10000 \times g$ for 30 min. The prepared erythrocyte ghosts had pinkish color due to Hb bound to AE1 exchanger of the ghost membrane (Hb_{AE1}). Obtained erythrocyte ghosts contained only membrane-bound Hb and did not have unbound cytosolic Hb.

Analysis of erythrocyte morphology

It is known, that the chemical composition of the erythrocyte membrane as well as the supramolecular organization and mechanical properties of its lipids and proteins determine morphology (or shape) in an erythrocyte [2–5]. In a blood stream erythrocytes constantly undergo changes of their shape, however after the isolation and fixation in glutaraldehyde it is possible to estimate amount of erythrocytes in a specific form [6]. Historically the first classification of erythrocytes according to their morphology was done by Bessis [7]. There are three major types of erythrocytes: discocytes, echinocytes and stomatocytes. A discocyte is a normal mature form of an erythrocyte having a biconcave discoidal shape. Under normal conditions the most number of erythrocytes have discoidal shape. Echinocyte is a crenated form of erythrocyte with spicules and stomatocyte is a cap-like form of erythrocyte. Both of these forms are usually considered as transformed, non-healthy forms of erythrocyte,

however, they present in normal blood in minor quantity [4]. The transformation of erythrocytes into echinocytes usually is reversible and is an evidence of the modification of the outer membrane layer and of the intercalation of exogenous molecules into the membrane [3, 4]. Transition of erythrocyte into stomatocyte is usually followed by its transformation into the sphere-like cell and its lysis. Therefore, study of the erythrocyte morphology under effect of Ag NPs helps to reveal any possible intercalation of NPs into the membrane and to trace hemolysis of erythrocytes. There are several methods of the quantitative estimation of erythrocyte morphology [4]. In the present study we employed the following technique: we calculated the number of erythrocytes taking the form of a discocyte, an echinocyte and a stomatocyte. The percentage of each form was estimated as a ratio of the number of cells in this specific form to the total number of counted erythrocytes [6].

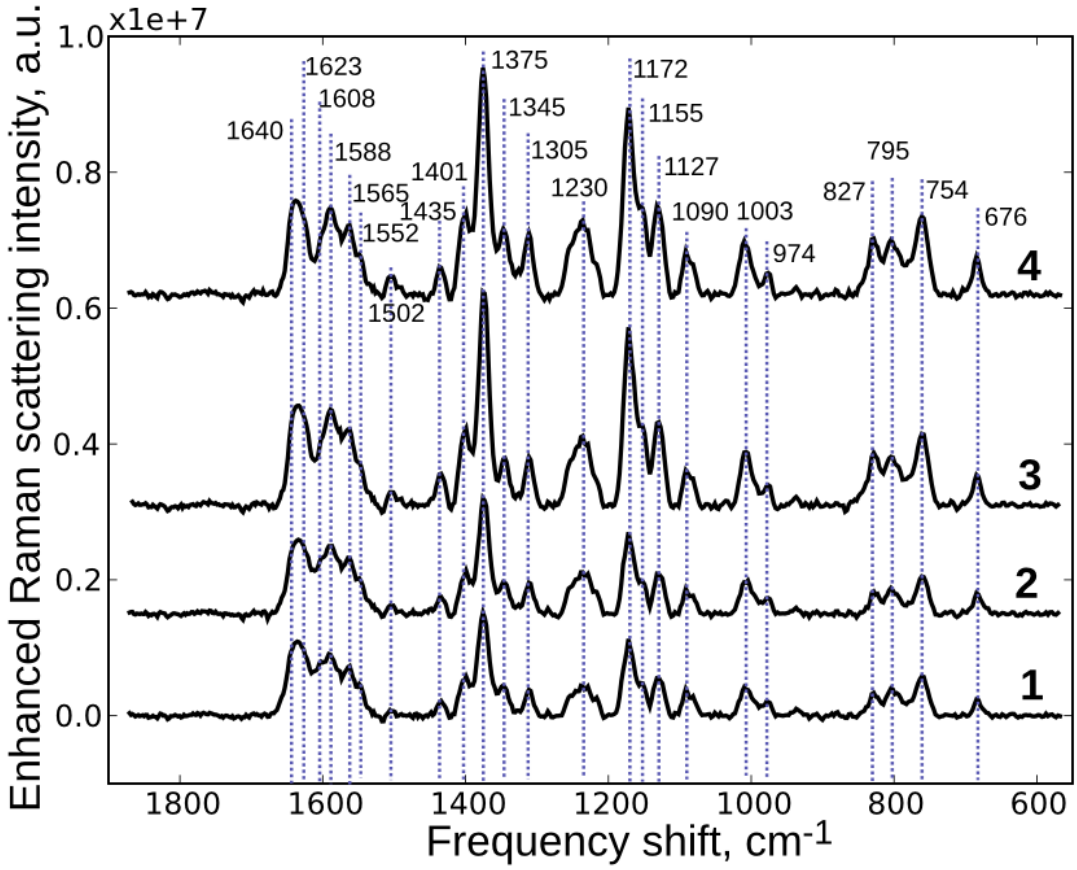

Figure 1: **Figure 1S.** SERS spectra of erythrocytes with different volume ratios of [Ag NPs]: [experimental erythrocyte sample]=2:8 (spectrum 1); 3:7 (spectrum 2); 2:3 (spectrum 3); 3:2 (spectrum 4). It is seen that the increase in the volume ratio of Ag NP solution results only in the increase in the intensity of the whole SERS spectrum of erythrocytes and does not affect a shape of peaks or relative intensities of peaks. Dotted vertical lines indicate position of peaks. Detailed peak assignment is shown in the Table 1.

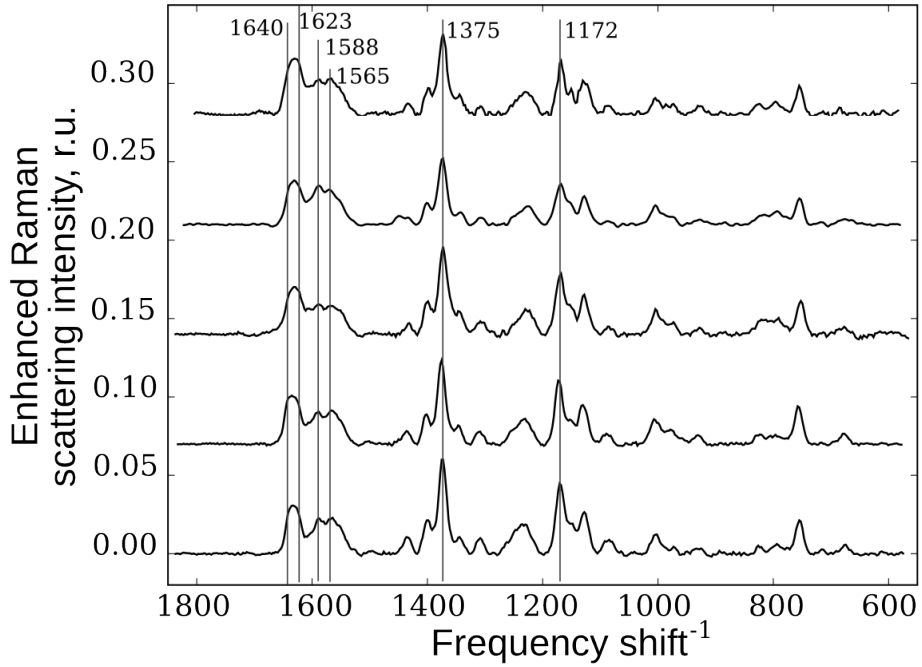

Figure 2: **Figure 2S.** SERS spectra of erythrocytes from five different blood samples with the volume ratio of [Ag NPs]:[experimental erythrocyte sample]=2:3. The similarity of spectra is an evidence of high stability of used Ag NP colloids and signal enhancement. Vertical lines indicate position of the main peaks.

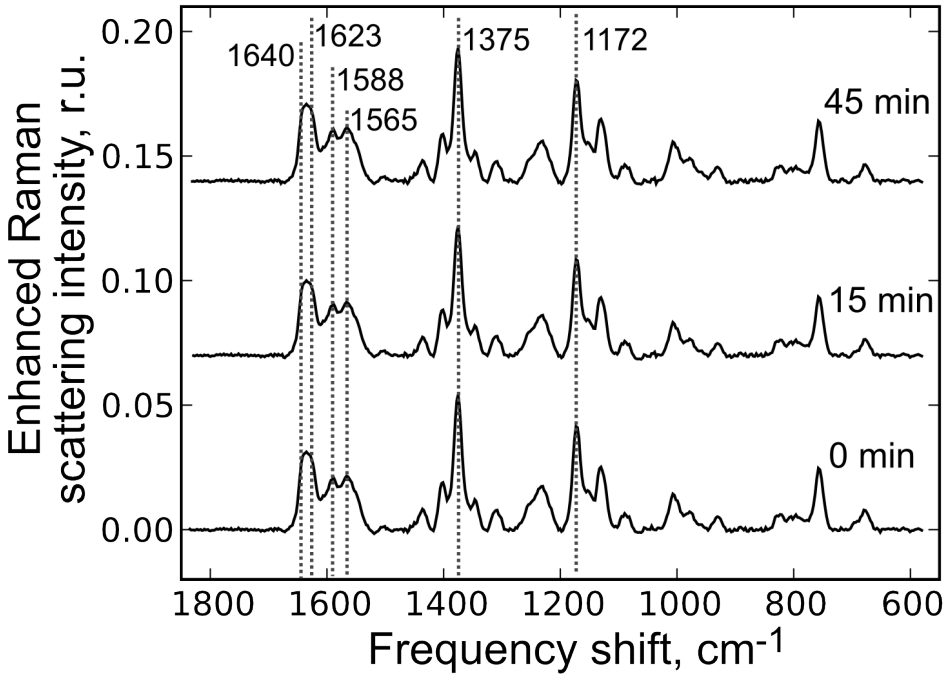

Figure 3: **Figure 3S.** SERS spectra of the same erythrocyte sample recorded at 0, 15 and 45 min. The volume ratio of [Ag NPs]:[experimental erythrocyte sample]=2:3. It is seen that spectra are identical to each other, that is an evidence of (i) the temporal stability of the signal enhancement; (ii) temporal stability of erythrocyte-Ag NP complex; and (iii) absence of the erythrocyte photodamage by laser light.

[1] Salhany, J.M., K.A. Cordes, and R,L. Sloan. 2007. Characterization of the pH dependence of hemoglobin binding to band 3. Evidence for a pH-dependent conformational changes within the hemoglobin-band 3 complex. *BBA* 1371: 107–113.

[2] Nakao, M. 2002 New insights into regulation of erythrocyte shape. *Curr. Opin. Hematol.* 9: 127–132.

[3] Gerald, L.H.W., M. Wortis, and R. Mukhopadhyay. 2002. Stomatocytediscocyteechinocyte sequence of the human red blood cell: Evidence for the bilayer couple hypothesis from membrane mechanics. *Proc. Nat. Acad. Sci. USA* 99: 16766–16769.

[4] Deuticke, B. 2003. Membrane lipids and proteins as a basis of red cell shape and its alterations. *in* Red cell membrane transport in health and disease, I. Bernhardt and C. Ellory, editors. Springer, Einbandgestaltung: Design&Production, Heidelberg. 27–60.

[5] Fischer, T.M. 2003. Human red cell shape and the mechanical characteristics of the membrane. *in* Red cell membrane transport in health and disease, I. Bernhardt and C. Ellory, editors. Springer, Einbandgestaltung: Design&Production, Heidelberg. 61–83.

[6] Gedde, M.M., E. Yang, and W.H. Huestis. 1999. Resolution of the paradox of red cell shape changes in low and high pH. *Biochimica et Biophysica Acta* 1417: 246–253.

[7] Living blood cells and their ultrastructure. 1973. Bessis, M. Springer-Verlag, Berlin.