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The surface charge of human glutaraldehyde-fixed erythrocytes

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We measured the number of charged residues at the surface of fresh human erythrocytes after fixation with glutaraldehyde by polyelectrolyte titration using polycations of different chemical composition and various molecular weights. Independent of the reagents used, the number was $(8.5 \pm 1.5) \cdot 10^8$ negatively charged residues per cell. The surface charge density of 6.3 e/nm^2 is considerably higher than that calculated from the electrophoretic mobility for which the surface charge density is calculated to be 0.09 e/nm^2.

Introduction

The colloid stability of human erythrocytes is determined by sterical and by electrostatical factors at the cell surface. Conveniently, free-flow electrophoresis is used to determine the electrophoretical mobility as a measure for the zeta potential [1–3]. This, however, is only an indirect measure for the charge density of the cell surface. Tentatively, the zeta potential may be considerably lower than the surface potential due to the three-dimensional structure of the cell surface.

Recently, we observed that polyelectrolyte titration is a simple method to measure the surface charge of biopolymers. We observed excellent agreement between experimental and calculated data for proteins [4], mucopolysaccharides [5] and phospholipid liposomes [6]. The investigations with lipoproteins suggested that the method may be successfully applied for measurements of composites of biopolymers [6,7]. We are not aware about corresponding investigations with intact cells. Therefore, we would like to report about our studies with human erythrocytes.

Materials and Methods

Reagents and sample preparation. The principle of polyelectrolyte titration was recently described [5,8]. We used an automated titrator equipped with a two-wavelength detector system developed from BASF-Aktiengesellschaft, Ludwigshafen/Rh.

Polydimethyldiallylammonium chloride (PDDA) (M_r $(1–2) \cdot 10^6$, Chemviron, Brussels, Belgium), *N*-methylglycolchitosan iodide (MGC) (M_r 32 000, ICN Pharmaceuticals, Plainview, U.S.A.) and *N*-methylated polyethyleneimine (Polymine 10 (M_r 440), Polymine 35 (M_r 2000), Polymine 50 (M_r 3000) and Polymine 100 (M_r 6000), BASF-Aktiengesellschaft) were used as polycations. Potassium polyvinyl sulfate (KPVS) (M_r 320000) was obtained from Serva GmbH, Heidelberg, F.R.G. The metachromatic indicator *ortho*-toluidine blue was ordered from Fluka-GmbH, Neu-Ulm, F.R.G. The reagents were used without further purification as their applicability

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was confirmed in previous experiments.

1 ml of blood was drawn from a healthy donor by venipuncture into 1 ml of isotonic citrate solution. After centrifugation for 2 min at 3000 rpm, the supernatant was decanted. The erythrocytes were suspended in 10 ml of phosphate-buffered saline (pH 7.4) and recentrifuged at 3000 rpm for 1 min. After the removal of the supernatant, the washing was repeated six times. The concentration of protein in the final supernatant was less than 50 μg/l. The erythrocytes were suspended at 4°C in a solution (1.5% paraformaldehyde/1.5% glutaraldehyde/2.5% polyvinylpyrrolidone (M_r 4000)) in 0.09 M sodium phosphate buffer (pH 7.4). The mixture was gently mixed for 15 min and left for 2 h at 4°C. Thereafter, the fixative was removed and the cells washed four times with 0.15 M saline containing 0.045 mol/l sucrose (pH 7.4). After the final washing, the cells were investigated by light microscopy to confirm their intact structure. The suspension was diluted to about $5 \cdot 10^4$ cells/ml. The exact cell concentration with their mean particle size and size distribution was determined with a Contraves Autolyzer AL 800.

Polyelectrolyte titration of erythrocytes. $(0.5–5) \cdot 10^6$ fixed erythrocytes were incubated with a defined amount of polycation (PDDA, MGC or polyethyleneimine of different molecular weight) at pH 7.4 in an aqueous solution containing 0.045 mol/l of sucrose. On the basis of charge equivalence, the amount of polycation exceeded that of the erythrocytes. After 30 min of incubation, 100 μl of an aquous solution of 20 mg/l *ortho*-toluidine blue was added. The volume was adjusted with 0.045 mol/l of sucrose solution to give a final volume of 3 ml. Thereafter, the excess of polycation was re-titrated in the presence of the cells with an aqueous solution of KPVS (21 mg/l). The number of titratable charge equivalents of the polycation in the cell suspension was subtracted from the number of charge equivalents titratable in the absence of erythrocytes in order to obtain the number of charged centers neutralized by the cells. Based on the assumption that ionic stoichiometric 1 : 1 complex formation occurs between the cell surface and the polycation, respectively, the polycation and the polyanionic titrant [9–11], this number was regarded as the number of counterionic charge centers on the surface of the cells. The precision and accuracy of the determination was proved by measurement of a dilution series of erythrocytes under identical analytical conditions.

Results and Discussion

The application of polyelectrolyte titration for the evaluation of the surface charge of intact cells is primarily restricted as a consequence of the necessary isotonicity of the incubation medium for the maintenance of the cell integrity. Such a salt concentration interferes with the metachromatic indicator reaction [11]. However, the titration is effective in NaCl solutions with a molarity of less than 0.1. Glutaraldehyde fixation and addition of sucrose prevents the lysis of erythrocytes in such a medium. According to measurements of the electrophoretic mobility, the fixation of erythrocytes with aldehyde does not change the surface charge density [1].

The re-titration of the unbound portion of the polycation after removal of the erythrocytes by centrifugation gave inconsistent results. In these preliminary experiments, the number of charge centers per cell was 3–4-times higher than in the presence of the cells. The difference was caused by elimination of polyelectrolyte bound to but only partially neutralized by negative charge centers at the cell surface. Therefore, we re-titrated the excessive amount of polycation in the presence of the erythrocyte suspension.

The measurement of concentration series of cell suspensions (Fig. 1) resulted in a proportional

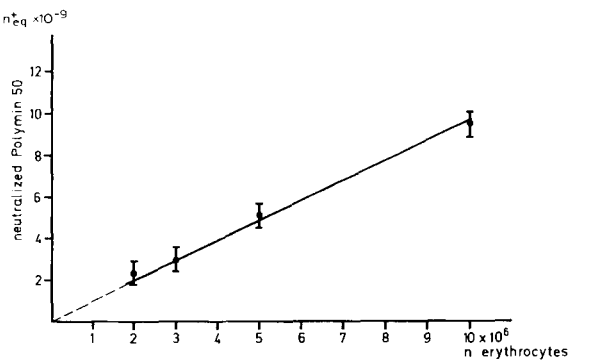

Fig. 1. Neutralization of quaternized Polymin 50 by glutaraldehyde-fixed erythrocytes as determined by polyelectrolyte titration. (n^+_{eq}, number of neutralized sites of the polybase.)

increase of neutralization of polycation with an increasing number of cells in the titration medium. Furthermore, extrapolation of the curve revealed no neutralization of polycation in the absence of cells. Incubations with $(0.5-2)\cdot 10^6$ cells resulted in a linear increase of neutralization of polycation, indicating a stoichiometric interaction. At higher cell concentrations, the degree of neutralization was relatively less. The effect was caused by agglomeration of cells after addition of the polycation, as confirmed by light microscopy. Therefore, the number of surface charges per cell was analyzed in the linear range of the curve.

To exclude pretended neutralization of counterionic polycation as a consequence of the chemical structure of the polyelectrolyte, we used linear quaternary polycations (MGC and PDDA) differing in their molecular weight $(32\cdot 10^4$ vs. $(1-3)\cdot 10^6)$, as well as different molecular weights of the branched quaternized polyethyleneimine ranging from 440 to 25 000.

The results of the titration experiments (Table I) clearly demonstrate that the measured number of negatively charged residues of the erythrocytes is almost constant and independent of the chemical structure and molecular weight of the polyelectrolyte. Obviously, the excessive portion of charged centers of the polyelectrolyte bound to the cell surface but not accessible for re-titration, is negligible. Previous observations indicate that the ammonium polymers used for this investigation interact by electrostatic binding to synthetic and biological polymers [8]. Since there is no evidence from microscopical and electromicroscopical analysis that aldehyde-fixed erythrocytes are lysed by polycation near their point of charge neutralization [12], the data support the conclusion that stoichiometric electrostatic interaction occurs between polycations and erythrocyte surface charges. The point of charge equivalence as determined by polyelectrolyte titration is similar to that observed in flocculation experiments with erythrocytes and polycations [12]. Furthermore, it is identical with results from electrophoretic studies in the presence of polylysine [13].

TABLE I

TITRATION OF THE SURFACE CHARGE OF GLUTARALDEHYDE-FIXED ERYTHROCYTES BY POLYELECTROLYTE TITRATION WITH VARIOUS POLYCATIONS

Polycation	Number of charged residues/erythrocyte
PDDA	$9.3\cdot 10^8$
MGC	$8.4\cdot 10^8$
Polymin 10	$9.8\cdot 10^8$
Polymin 35	$10.2\cdot 10^8$
Polymin 50	$6.8\cdot 10^8$
Polymin 100	$6.8\cdot 10^8$
Mean ± S.D.	$(8.5\pm 1.5)\cdot 10^8$

Dividing the number of charged residues per cell by the mean surface area of $0.135\cdot 10^9$ nm^2/erythrocyte (with a diameter of 7.5 μm and a thickness of 2 μm [14]), the surface charge density is 6.3 ± 1.0 e/nm^2. In view of the three-dimensional structure of the cell surface, the surface charge density of the outer layer determining the zeta potential will be less. The value is in the same order as observed for human very-low-density lipoproteins (with 1.1 e/nm^2 [6]) or for pepsin (with 2.3 e/nm^2 at pH 7.4 [4]). However, it is considerably higher than the surface charge density calculated from the electrophoretic mobility.

For a simplified two-dimensional charge-layer model of a spherical erythrocyte with: radius $(a) = 3.75$ μm, mobility $(\mu\text{m}) = 1.09\cdot 10^{-4}$ cm$^2\cdot$V$^{-1}\cdot$s^{-1} in an aqueous solution, viscosity $(\eta) = 0.89\cdot 10^{-3}A\cdot$V$^{-1}\cdot$s$^{-2}\cdot$m^3 and $(\varkappa) = 1.273\cdot 10^{-7}$cm^{-1} [15], the Helmholz-Smoluchowsky equation gives a value of $1.27\cdot 10^7$ charged residues. This results in a surface charge density of 0.094 e/nm^2, a value which is of the same order as for hydrophobic proteins [6].

Comparative determinations of the surface charge of biopolymers by measurement of the electrophoretic mobility and by hydrogen ion titration revealed an underestimation of the surface charge with the former method [16] by a factor of 2. This difference still does not explain the 67-fold difference as compared with polyelectrolyte titration or with free-flow electrophoresis in the presence of polylysine. Tentatively, the electrostatic binding of polycations to the highly hydrated surface of erythrocytes causes dehydration without affecting the cell membrane. This would point to the importance of the microenvironment of the three-dimensional cell surface to bind counterions

which, as a consequence, may cause a drastic reduction of the electrophoretic mobility of the normal (or aldehyde-fixed) erythrocyte.

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References

1 Heard, D.H. and Seaman, G.V.F. (1961) Biochim. Biophys. Acta 53, 366–374
2 Brooks, D.E. and Seaman, G.V.F. (1972) J. Colloid Interface Sci. 43, 670–686
3 Valet, G., Bamberger, S., Hofmann, H., Schindler, R. and Ruhenstroth-Bauer, G. (1979) J. Histochem. Cytochem. 27, 342–349
4 Horn, D. and Heuck, C.C. (1983) J. Biol. Chem. 258, 1665–1670
5 Heuck, C.C., Schiele, U., Horn, D., Fronda, D. and Ritz, E. (1985) J. Biol. Chem. 260, 4598–4603
6 Heuck, C.C., Daerr, W., Haberbosch, W., Horn, D. and Lüddecke, E. (1983) J. Biol. Chem. 258, 8317–8322
7 Horn, D. (1980) Polyethyleneimine – Physicochemical Properties and Applications. Polymeric Amines and Ammonium Salts (Goethals, E.I., ed.), pp. 333–356, Pergamon Press, Oxford
8 Josephs, R. and Feitelson, J. (1963) J. Polym. Sci., Part A-I, 3385–3394
9 Kokufuta, E., Shimizu, H. and Nakamura, I. (1980) Poly. Bull. 2, 157–162
10 Katayama, T., Takai, K.I., Kariyama, R. and Kanemasa, Y. (1978) Anal. Biochem. 88, 382–387
11 Horn, D. (1978) Progr. Colloid Polym. Sci. 65, 251–264
12 Grunze, M., Heuck, C.C. and Forssmann, W.G. (1984) Biomed. Biochim. Acta 43, 9–11
13 Nevo, A., De Vries, A. and Katchalsky, A. (1955) Biochim. Biophys. Acta 17, 536–547
14 Emmons, W.F. (1927) J. Physiol. 64, 215–222
15 Hiemenz, P.C. (ed.) (1977) Principles of Coloid and Surface Chemistry, p. 370, Marcel Dekker, New York
16 Brown, R.A. and Timasheff, S.N. (1959) in Electrophoresis (Bir, M., ed.), Vol. 1, pp. 318–322, Academic Press, New York