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Short communication

CELL SEPARATION WITH HORIZONTAL CELL ELECTROPHORESIS UNDER NEAR-ISOPYCNIC CONDITIONS ON A "DENSITY CUSHION" [#]

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Abstract: This report describes an improvement made to the horizontal cell electrophoresis methodology. It involves using two liquid layers differing in density to produce an interface described as a "density cushion". The electrophoretic system that employed an anti-convective porous matrix to separate red blood cells (RBC) and charged dyes effectively was found to be unsuitable for some other mammalian cells. The "density cushion" method was found to be more versatile and applicable to studies on the separation of a variety of cell types. The experiments described show the differences between the electrophoretic mobilities of a human eosinophilic leukaemia cell line (Eol-1) and RBC, both with and without the modification of the cell surface properties.

Key words: Cell electrophoresis, Cell separation, Cell surface

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Abbreviations used: Eol-1 – human eosinophilic leukaemia cell line; PU – polyurethane; RBC – red blood cells

INTRODUCTION

Cell electrophoresis is a technique that permits the identification and measurement of the electrochemical properties of the cell surface. The analysis of the electrochemical features of cell surfaces is applicable to those changes which occur during the cell cycle and cell differentiation, and due to a variety of pathological states, including neoplastic transformation [1-4]. Cells from different species are similarly characterized by different electrophoretic mobilities [5, 6]. Differences in the physical properties of cell surfaces may also be used to separate cell subpopulations from cell mixtures via electrophoresis, especially when specific cell surface antibodies are unavailable and separation using flow cytometry is impossible.

The methods of microscopic cell electrophoresis were established for research on cell surface properties [7], and more recently, capillary cell electrophoresis was reported for analytical purposes [8, 9]. Preparative cell electrophoresis was achieved with the free-flow curtain apparatus [10], which is now commercially available [11].

Our earlier paper described a horizontal electrophoresis system which effectively separated human and chicken red blood cells (RBC), which differ by about 30% in their electrophoretic mobilities [5, 12]. The separation was carried out under near-isopycnic conditions, when cell sedimentation is negligible, within an anti-convective porous matrix of polyurethane sponge with a pore size over 300 μm. This method permitted a comparative electrophoretic study of several samples in parallel [12].

The polyurethane (PU) matrix was found to stabilize the electrophoretic system (system I), and is suitable for studies with red blood cells and lymphocytes. However, we observed that some cell types and cells with reduced or altered electrokinetic surface potential became immobilized because they attach to the PU. In an attempt to overcome this difficulty, we investigated the replacement of the anti-convective PU sponge with liquid phases described as a "density cushion". This approach was validated using RBC and the human eosinophilic leukaemia cell line Eol-1, with and without modified cell surface properties and horizontal cell electrophoresis carried out in the inter-zone between two liquid phases of different densities.

MATERIAL AND METHODS

Cell preparation and cell culture conditions

The preparation of human and chicken RBC was described in an earlier paper [12]. The human eosinophilic leukaemia cell line Eol-1 (DSMZ ACC386) was cultured in RPMI-1640 medium (Sigma, St Louis, MO/USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Lab., NY/USA), 10 μg/ml streptomycin (Polfa Tarchomin, Poland), 100 IU/ml penicillin (Polfa Tarchomin, Poland), and 10 μg/ml neomycin (Polfa Tarchomin, Poland). Cells were grown

in 25 cm^2 tissue culture flasks (Sarstedt, Newton, NC/USA) in a humidified atmosphere with 5% CO_2 at 37ºC. The cells were harvested and washed by centrifugation at 1000 rpm for 5 min at room temperature; the supernatant was discarded and the cells were resuspended in PBS (BioMed, Lublin, Poland). The cells in suspension were stained with 5 μM calcein acetyloxymethyl ester (calcein AM; Molecular Probes, Inc., Eugene, OR/USA) for 30 min at 37ºC, then washed twice with PBS by centrifugation. Pellets of RBC and Eol-1 cells were resuspended in PBS, counted using a Bürker haematocytometer (Superior, Marienfeld, Germany), and mixed with electrophoresis solution II in a 1:1 ratio.

Cell surface modification

A stock solution of ruthenium red (Fluka Chemika AG, Switzerland) at a concentration of 1 mg/ml in sterile water was prepared, and stored at 4ºC protected from light, as per Dwyer *et al.* [13]. RBC and Eol-1 cells pre-treated with calcein AM were suspended in PBS, and ruthenium red was added to the suspension at a concentration of 0.05 μM. The preparation was incubated for 15 min at room temperature, and excess dye was removed by centrifugation at 1000 rpm for 5 min.

The electrophoresis equipment and preparation of the system

Electrophoresis experiments were carried out in plexiglass apparatus made in the faculty workshop. The first system was designed for the separation of RBC in a stabilizing PU sponge; the apparatus and the preparative procedures were previously described [12]. The new apparatus was based on the previous system, with the main improvements being the depth of the separation chamber (10 mm instead of 4 mm) and the composition of the electrophoresis solutions being designed to provide a biocompatible "density cushion".

For this system, the electrophoresis chamber was filled with 40 ml of solution I, which was carefully covered with 15 ml of solution II to form layers with a total depth of 10 mm. The "density cushion" represents the interface between the stratified layers of the lower (I) and higher (II) density solutions. To equilibrate the whole system before cell electrophoresis, a voltage gradient of 7 V/cm (for separation with the PU sponge, i.e. system I) and 10 V/cm (for the "density cushion", i.e. system II) was applied for 20 min. All the cell electrophoretic separations were carried out at 4ºC in a refrigerator.

Electrophoresis solutions

The compositions of the solutions used for system I with the PU sponge were described earlier [12]. For the electrophoretic separation of cells on the "density cushion" (system II), the solutions were modified, but their compositions were also based on dextran MW 15000-20000 (MP Biomedicals LLC, Eschwege, Germany), sucrose (PoCh, Gliwice, Poland) and PBS (Biomed, Lublin, Poland); the reasons for these choices were also previously described [12]. The osmolarity, density, viscosity and conductivity were assayed for each solution used in the "density cushion" system, and Tab. 1 details their composition and

physical properties. The similar osmolarities of the two solutions ensure very slow mixing via diffusion.

Tab. 1. The composition and physical properties of the solutions used in the cell electrophoresis experiment.

Composition	Electrophoresis solution I	Electrophoresis solution II
	22.5 g dextran 15-20 TDa, 9 g sucrose/100 ml (90% H_20 & 10% 0.9% NaCl)	9 g dextran 15-20 TDa, 7 g sucrose/100 ml (90% H_20 & 10% 0.9% NaCl)
Osmolarity (mOsm/kg)	354	316
Density (g/ml)	1.106	1.06
Viscosity (cP)	10.1	3.0
Conductivity (mS)	1.68	1.99

Cell separation on the PU sponge (system I) and on the "density cushion" (system II)

The cell suspensions, i.e. RBC ($2 \cdot 10^6$/10 µl) and Eol-1 ($1 \cdot 10^5$/10 µl) and human and chicken RBC ($2 \cdot 10^6$/10 µl), were prepared as described and layered carefully on the PU sponge or "density cushion". Cells were separated by applying voltages (Gibco BRL, type Electrophoresis Power Supply LTI PS304, France) of 7 V/cm, 15 mA, and 10 V/cm, 34 mA, respectively for systems I and II. Control RBC ($2 \cdot 10^6$/10 µl) and Eol-1 cells ($1 \cdot 10^5$/10 µl) with and without modification with ruthenium red dye were electrophoresed for analysis in system II. The position of the RBC and Eol-1 bands was followed by photography with a digital camera (Camedia C-3040 ZOOM, Olympus Optical co., LTD., Tokyo, Japan) in all the experiments.

RESULTS

The comparative electrophoretic behaviour of human RBC and Eol-1 on a PU sponge

An analysis of the electrophoretic behaviour of RBC and Eol-1, which are both non-adherent and negatively charged cell types, was carried out in system I with the PU sponge for 60 min. Fig. 1 shows the positions of the cells before and after electrophoresis. The RBC are shifted toward the anode (+) (Fig. 2B), whereas the Eol-1 cells remained in their original position, presumably due to binding and attachment to the PU sponge. However, even after electrophoresis, the Eol-1 cells remained viable, as judged by viability tests with ethidium bromide (EB; Sigma, ST. Louis, MO/USA) and fluorescein diacetate (FDA; Sigma, St. Louis, MO/USA) [14]. Furthermore, no leakage of haemoglobin from the RBC was observed.

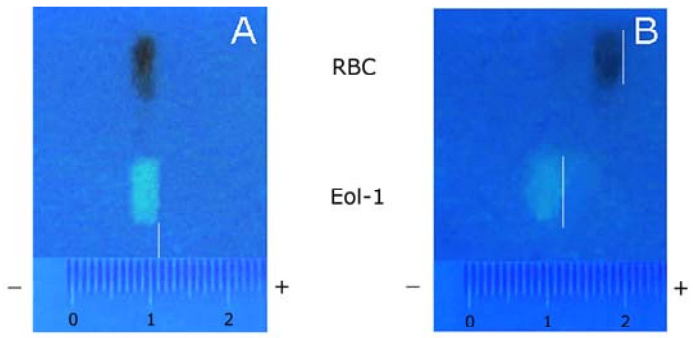

Fig. 1. A comparison of the electrophoretic mobility of human RBC and Eol-1 cells on a PU sponge (system I). Bands of cells at 0 h (A) and after 1 h (B) of electrophoresis are shown. The scale is in cm. The Eol-1 cells remained in their original positions. Bars mark the positions of the fronts of the cell bands.

The electrophoretic separation of human and chicken RBC on a "density cushion" (system II)

To overcome some of the limitations of using the PU sponge, a "density cushion" system (II) was applied and standardised with human and chicken RBC. Fig. 2 shows the electrophoretic mobilities of the two species of RBC before and after electrophoresis for 45 min. The human RBC have a greater electrophoretic mobility than the chicken RBC. The position of the separated bands corresponds with the control samples. The purity of the samples was checked with the Coulter counter (Beckman Coulter 2^{TM}, 2 Particle counter/size, FL/USA) and the Bürker haematocytometer (Tab. 2) [cf. 12].

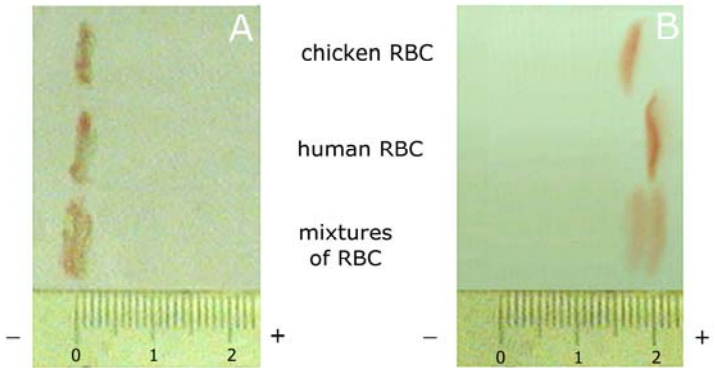

Fig. 2. The separation of human and chicken RBC is shown in horizontal, near-isopycnic electrophoresis on a "density cushion". Bands of chicken, human, and mixed human and chicken RBC at 0 h (A) and after 45 min (B) of electrophoresis. The effect of band stacking is visible. The scale is in cm.

Tab. 2. The purity of the erythrocyte fractions after 45 min electrophoresis under near-isopycnic conditions, as determined with a haematocytometer.

	Results from a Bürker haematocytometer	
	Human RBC number (%)	Chicken RBC number (%)
Faster band	97	3
Slower band	7	93

The electrophoretic behaviour of human RBC and Eol-1 on the "density cushion" (system II)

The effective RBC separation achieved on the "density cushion" provided the basis to test the separation of Eol-1 cells as representative mammalian nucleated cells. Fig. 3 shows RBC and Eol-1 cells stained with calcein AM before and after electrophoresis for 1 h. While the Eol-1 cells moved 5 mm towards the anode (+), the RBC moved about 15 mm in the same direction. Viability tests of the Eol-1 cells again proved very good, as described above.

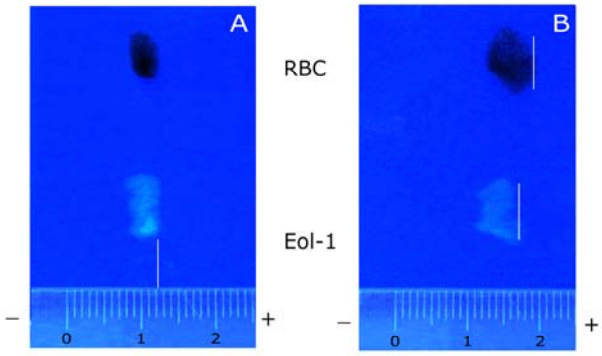

Fig. 3. The electrophoretic mobility of human RBC and Eol-1 cells in horizontal, near-isopycnic electrophoresis on a "density cushion" (system II). Bands of cells at 0 h (A) and after 1 h (B) of electrophoresis are shown. The scale is in cm. The bars mark the positions of the fronts of the cell bands.

The electrophoretic behaviour of human RBC and Eol-1 cells on the "density cushion" (system II) after surface charge modification

Ruthenium red (RR), a non-toxic, positively charged extracellular dye, was used to label both human RBC and Eol-1 cells prior to electrophoresis on the "density cushion" (system II). Fig. 4 shows that the RR-treated cells moved more slowly than the untreated cells. Moreover, both cell types showed good viability as judged by the tests described above.

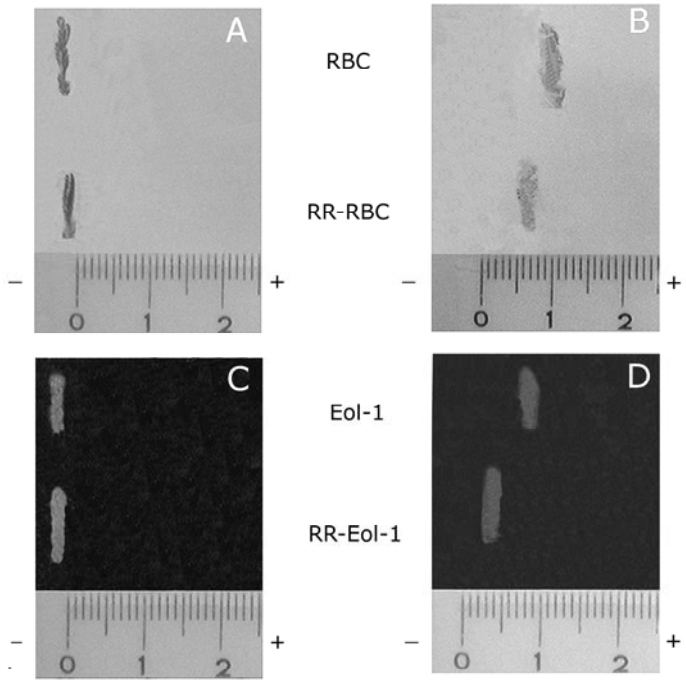

Fig. 4. The movement of ruthenium red-modified human RBC (RR-RBC) and Eol-1 (RR-Eol-1) compared to their unmodified counterparts in horizontal, near-isopycnic electrophoresis on a "density cushion" (system II). Bands of RBC and RR-RBC at 0 h (A) and after 1 h (B) of electrophoresis are shown. Bands of Eol-1 and RR-Eol-1 at 0 h (C) and after 1 h (D) of electrophoresis are shown on the "density cushion". The scale is in cm.

DISCUSSION

The aim of this research was to prepare a simple method for analytical and preparative cell electrophoresis which could be used for the separation of different cell types, together with the retention of good cell viability. Here we describe electrophoretic experiments in a system based on what is termed a "density cushion".

In an earlier paper, it was shown that when cell sedimentation is minimized, the horizontal electrophoresis of cells within an anti-convective porous matrix permits the separation of mixed human and chicken red blood cells according to their electrophoretic mobilities [12]. This was system I, and it was effective for the separation of RBC, but not for other cell types which were prone to adhere to the matrix material. Although the human eosinophilic leukaemia cell line Eol-1 did not move electrophoretically in the PU matrix, those cells were easily removed and shown to be viable after electrophoresis. This difficulty encountered with the Eol-1 cells on a PU sponge was overcome by carrying out horizontal cell electrophoresis at the interface of two liquid phases differing in

densities, termed a “density cushion” (system II). The higher density of the lower solution (containing isotonic 15-20 000 MW dextran and sucrose) was found to prevent the aggregation and sedimentation of cells. Under such conditions, chicken and human RBC moved in the interfacial liquid layers, and samples taken from the separated bands showed more than 90% purity. Similarly, the human eosinophilic leukaemia cell line Eol-1 and human RBC, when located on the “density cushion”, demonstrated different electrophoretic mobilities, with both cell types moving towards the anode (+). In the course of electrophoresis on the “density cushion”, the stacking (i.e. narrowing) of cell bands was observed [15].

Cells treated with the surface-modifying dye ruthenium red (RR) manifested a different electrophoretic mobility compared to untreated cells, and were effectively separated from the latter. We used ruthenium red, a hexavalent polycationic dye [16], which was reported to react strongly with the glycocalyx of eosinophils [16] and also to bind to RBC membranes [18]. Polycationic dyes were similarly found to reduce the electrophoretic mobility of both Eol-1 and RBC cells.

The modification of horizontal cell electrophoresis under near-isopycnic conditions to apply a “density cushion”, as described here, has facilitated the applicability of this method to research carried out with cells. We also noticed that the “density cushion” methodology permits the straightforward collection of separated cell fractions with good viability for further studies. The ability to compare the electrophoretic mobilities of several cell samples in one experiment suggests numerous applications for this method in the study of cell membrane electrochemical properties, both natural and modified [19]. With this method, cell electrophoretic mobilities can be assayed and compared, but it is more difficult if not impossible to calculate cell zeta potentials, since the dielectric constant of liquid in an inter-zone is difficult to determine (because of the presence of dextran macromolecules).

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REFERENCES

1. Abercrombie, M. and Ambrose, E.J. The surface properties of cancer cells: A review. **Cancer Res.** 22 (1962) 332-245.
2. Fürész, J., Pál, K., Budavári, I. and Lapis, K. The physico-chemical properties of tumor cells with different metastatic potential. **Neoplasma** 32 (1985) 689-693.
3. Walter, H. and Widen, K.E. Differential electrophoretic behavior in aqueous polymer solutions of red blood cells from Alzheimer patients and from normal individuals. **Biochim. Biophys. Acta** 1234 (1995) 184-90.

4. Rychly, J., Anders, O., Eggers, G. and Schulz, M. Electrophoretic mobility distribution of cells in leukaemia. in: **Cell Electrophoresis** (Schütt, W., Klinkmann, H., Ed.), Walter de Gruyter, Berlin (NY) 1985, 477-483.
5. Slivinsky, G.G., Hymer, W.C., Bauer, J. and Morrison, D.R. Cellular electrophoretic mobility data: A first approach to a database. **Electrophoresis** 18 (1997) 1109-1119.
6. Chaubal, K.A. Cell electrophoretic mobility as an aid to study biological systems, in: **Cell Electrophoresis** (Schütt, W., Klinkmann, H., Ed.), Walter de Gruyter, Berlin (NY) 1985, 515-526.
7. Mehrishi, J.N. Molecular aspects of the mammalian cell surface. in: **Progress in Biophysics and Molecular Biology** (Butler, J.A.V. and Noble, D., Ed.), Pergamon Press, Oxford, 1972, 3-70.
8. Lu, W.-H., Deng, W.-H., Liu, S.-T., Chen, T.-B. and Rao, P.-F. Capillary electrophoresis of erythrocytes. **Anal. Biochem**. 314 (2003) 194-198.
9. Omasu, F., Nakano, Y. and Ichiki, T. Measurement of the electrophoretic mobility of sheep erythrocytes using microcapillary chips. **Electrophoresis** 26 (2005) 1163-1167.
10. Eggleton, P. Separation of cells using free flow electrophoresis. in: **Cell Separation A Practical Approach** (Fisher, D., Francis, G.E. and Rickwood, D., Ed.), Oxford University Press, Oxford, New York, Tokyo, 1998, 213-252.
11. Mehrishi, J.N. and Bauer, J. Electrophoresis of cells and the biological relevance of surface charge. **Electrophoresis** 23 (2002) 1984-1994.
12. Wilk, A., Rośkowicz, K. and Korohoda, W. A new method for the preparative and analytical electrophoresis of cells. **Cell. Mol. Biol. Lett.** 11 (2006) 579-593.
13. Dwyer, D.S., Gordon, K. and Jones, B. Ruthenium Red potently inhibits immune responses both in vitro and in vivo. **Int. J. Immunopharmacol.** 17 (1995) 931-940.
14. Szydłowska, H., Zaporowska, E., Kuszlik-Jochym, K., Korohoda, W. and Branny, J. Membranolytic activity of detergents as studied with cell viability tests. **Folia Histochem. Cytochem.** 16 (1978) 69-78.
15. Heiger, D.N. Principles of Capillary Electrophoresis. in: **High Performance Capillary Electrophoresis – An Introduction** (Heiger, D.N., Ed.) Hewlett-Packard Company, France, 1992, 11-39.
16. Nakamura, F., Naka, M. and Tanaka, T. Inhibition of actin-activated myosin Mg (2+)-ATPase in smooth muscle by ruthenium red. **FEBS Lett.** 314 (1992) 93-96.
17. Pimenta, P.F. and De Souza, W. Ultrastructure and cytochemistry of the cell surface of eosinophils. **J. Submicrosc. Cytol.** 14 (1982) 227-237.
18. Candiano, G., Ghiggeri, G.M., Oleggini, R., Ginevri, F., Altieri, P. and Gusmano, R. Interaction between cationic dyes and erythrocyte membranes in minimal change nephropathy: an electrophoretic approach. **Pediatr. Nephrol.** 5 (1991) 173-178.
19. Korohoda, W. and Wilk, A. Cell electrophoresis - A method for cell separation and research into cell surface properties. **Cell. Mol. Biol. Lett.** 13 (2008) 312-326.