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HIGH-VOLTAGE CAPILLARY ZONE ELECTROPHORESIS OF RED BLOOD CELLS

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SUMMARY

The high-voltage wide-bore capillary zone electrophoresis of red blood cells was investigated. The reproducibility of the retention time (electrophoretic mobility) is excellent and the differentiation among various species is good. The peaks in the electropherogram describe the distribution of the size and/or surface charge of the cells and are therefore broad. The relationship between the peak height and the number of cells injected is good, with linear correlation coefficients better than 0.98. Details of the preparation of cell suspensions and support electrolytes are given, which is essential for obtaining reproducible results. The inner surface of FEP capillary tubing is degraded by the application of high voltage and a pause is necessary between successive experiments if good and reproducible peak shapes are to be obtained. The length of the pause increases with the number of experiments made, and finally the tubing becomes useless. Inspection of the inner surface by X-ray photoelectron spectroscopy showed the breakdown of CHF bonds, but the actual mechanism is not known.

INTRODUCTION

Since 1979[1,2], capillary zone electrophoresis (CZE) developed rapidly. With a capillary of 1 m or less long, both small and large molecules can be separated with efficiency of 10^4–*ca.* 10^6 theoretical plates. Whereas high-performance liquid chromatography (HPLC) suffers from a decrease in efficiency when dealing with large molecules, CZE features a high and increasing efficiency because the larger the molecules are the smaller is the diffusion coefficient. In previous work mainly small ions were studied. A few papers were devoted to protein separations[3]. It was found that in the separation of charged biomolecules by CZE, care had to be taken to avoid adsorption on the inner wall of the tubing, otherwise no peak is observed. Recently, CZE of proteins was accomplished with an efficiency of *ca.* $8 \cdot 10^5$ theoretical plates[3].

An attempt was made in our laboratory to explore the advantages of the CZE of cells or suspensions, which are several orders of magnitude larger than proteins or other biomacromolecules if a single cell or particle is counted as a molecule. Human,

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chicken, porcine and rabbit red blood cells (RBC) were electrophoresed and the mobilities of the RBC and their distribution thus determined were comparable to previously reported data. The high-voltage CZE of cells has advantages such as outstanding reproducibility, rapidity, simultaneous measurement of a swarm of cells and hence good statistical significance, automatic recording and relatively simple apparatus. The operating conditions were found to be critical and were systematically studied. The inner surface of the FEP (fluorinated ethylene–propylene copolymer) tubing was degraded during electrophoresis, leading to a steadily increasing relaxation time between two successive experiments until the tubing surface became permanently damaged.

EXPERIMENTAL

Chemicals

N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), morpholino-ethane sulphonic acid (MES) and other organic electrolytes were donations from Dojindo Laboratories (Kumamoto, Japan) and the FEP capillary was donated by Dr. Shigeru Terabe (Department of Industrial Chemistry, Kyoto University, Kyoto, Japan). Hydroxypropylmethylcellulose (HPMC) II was purchased from Sigma (St. Louis, MO, U.S.A.) and Tris, glucose (chemically pure) and the inorganic salts (analytical-reagent grade) were purchased from Beijing Chemical Works (Beijing, China). Triply distilled water was prepared in this laboratory.

Electrolytes

As a medium for RBC, the support electrolyte must be isotonic and of the same density as the blood. The electrolytes used in this study are listed in Table I. The use of glucose instead of sucrose will be discussed elsewhere.

Apparatus

The Model RHR 40 PN high-voltage power supply (0–40 kV, 0–3 mA) was purchased from Spellman (Plainview, NY, U.S.A.) and the UV Model 238 detector was from LKB (Bromma, Sweden), in which a filter of 206 nm was mounted. The sensitivity range was set at 0.005 a.u.f.s. The laboratory-made CZE apparatus is shown

TABLE I
COMPOSITIONS OF THE SUPPORT ELECTROLYTES

Code	*Ingredients*[a,b]	*pH adjuster*
H1	0.012 *M* HEPES, 5.14% G	Tris
H2	0.012 *M* HEPES, 4.70% S, 2.50% G	Tris
M	0.0055 *M* MES, 5.30% S, 2.50% G	Tris
P1	$2.2 \cdot 10^{-4}$ *M* KH_2PO_4, $4.0 \cdot 10^{-4}$ *M*, KCl $1.14 \cdot 10^{-4}$ *M* $MgCl_2$, 5.30% S, 2.50% G	Na_2HPO_4
P2	$4.4 \cdot 10^{-4}$ *M* KH_2PO_4, $8.0 \cdot 10^{-4}$ *M*, KCl $2.3 \cdot 10^{-4}$ *M* $MgCl_2$, 7.60% S, 1.00% G	Na_2HPO_4

[a] The pH and the percentage of the additive (HPMC) are specified in the text.
[b] S = Sucrose; G = glucose.

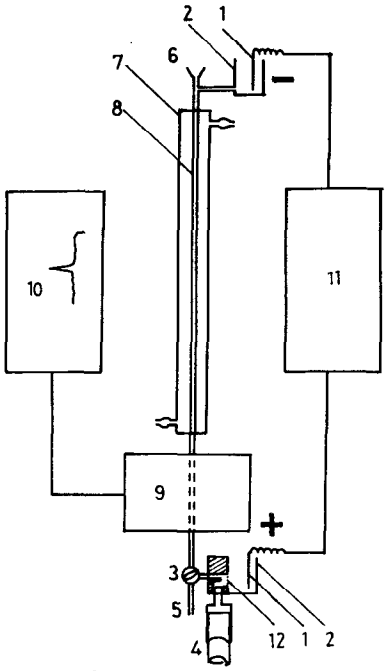

Fig. 1. Schematic diagram of the CZE apparatus. 1 = Pt electrode; 2 = electrolyte reservoir; 3 = three-way valve; 4 = 20-ml plastic syringe; 5 = drain; 6 = injection port; 7 = water cooling jacket; 8 = FEP capillary; 9 = UV detector; 10 = recorder; 11 = high-voltage power supply; 12 = semipermeable membrane.

schematically in Fig. 1; an FEP tube (8) of 0.45 mm I.D. was fitted vertically. The electrolyte solution in the capillary was cooled by a water-jacket (7), with a few exceptions when air cooling was used as specified in the text. A semipermeable membrane (12) at the outlet of the tubing formed a seal to prevent the solution from flowing by gravity. A small hole was drilled at the top of the UV detector housing as the inlet passage for the FEP tubing, which protruded out from the front panel and the part of it in the housing served as the flow cell. The upper and lower electrolyte reservoirs (2) were LKB injection block 2127-007 and membrane block 2127-008, respectively. The three-way valve (3) was a built-in part of the latter. The lower electrode was grounded.

Filling the tubing with electrolyte

Valve 3 (fig. 1) was turned so that the electrolyte solution in the upper reservoir was sucked into the capillary by drawing the plunger of the 20-ml plastic syringe (4). The valve was then turned to connect the capillary and the lower reservoir and 30 s later the sample was injected with a microlitre syringe. The high-voltage power supply was turned on to start the electrophoresis.

Renewal of electrolyte

After the electrophoresis had ended, valve 3 (Fig. 1) was turned to drain the liquid in the capillary and the inside of it was washed successively three times with

distilled water and twice with the electrolyte before it was refilled. If the electric current differs considerably from the normal value while the voltage remains the same, the electrolyte in the reservoir must be renewed. One filling of the reservoir sufficed for four CZE experiments provided that the organic electrolyte was used. If the electrolyte is to be replaced with another the whole flow system should be washed carefully with distilled water.

Preparation of cell suspensions

Fresh arterial blood from chicken and pig, venous blood from rabbit and human blood from the finger tip were taken and the anticoagulant trisodium citrate was added. They were washed four times with 100 volumes of phosphate-buffered physiological saline (PBS) and then diluted to 40 times the original volume (*ca.* 10^5 cells mm^{-3}) in the support electrolyte; they were then ready for the CZE experiment. If lysis happened to occur, the suspension was centrifuged at 2500 rpm for a few minutes and the settled cells were diluted as above.

Fixation of cells

CZE of fixed RBC was also studied. The blood with anticoagulant was allowed to settle and the cells were washed four times with 20 volumes of PBS and then mixed with 8 volumes of 3% (v/v) formalin in PBS. The mixture was kept at 4–6°C with occasional tapping for 4 h, then 2 volumes of cooled 37% (w/v) formalin were added and thorougly mixed and the mixture was kept consecutively at 4–6°C and room temperature each for 24 h with occasional tapping. The fixed cells were washed 4–5 times with 0.9% (w/v) saline and diluted with 9 volumes of the saline and stored in a refrigerator.

If glutaraldehyde was used instead of formalin, the blood with anticoagulant was first washed five times with 10–20 volumes of 0.9% saline, the cells and 1% (v/v) glutaraldehyde in saline were first separately cooled to 4°C and 10 volumes of the 1% glutaraldehyde were added to the cells slowly while tapping. The reaction was allowed to proceed for 30–45 min and the fixed cells were washed five times with physiological saline, diluted with 9 volumes of water and then stored in a refrigerator.

RESULTS AND DISCUSSION

Addition of HPMC

Previous work met with difficulties when proteins were electrophoresed in a capillary because proteins were adsorbed on the inner wall of the tubing no matter what material was selected for the tubing. The problem was solved by increasing the pH of the electrolyte to more than 8.25[3] so that both the protein molecules and the wall of the tubing were negatively charged. This approach does not seem to be widely applicable. At the beginning of this work the adsorption of the cells was also of concern and bovine serum albumin was tried as an additive to the electrolyte to alleviate the adsorption. Reasonable peaks could be observed but the reproducibility was poor. HPMC, an additive for suppressing unwanted electroosmotic flow, was unexpectedly found to be excellent in obtaining good CZE peaks. It is the linear agglomeration of the RBC rather than adsorption which spoils the CZE of erythrocytes. HPMC presumably acted as a surface-treating agent which broke down the linear agglomerates.

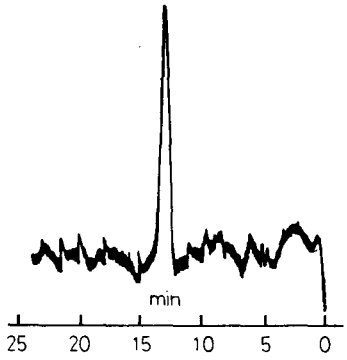

Fig. 2. Electropherogram of human RBC. Electrolyte: H1 containing 0.10% (w/v) HPMC, pH 7.18. V = 20 kV; I = 120 μA; T = 19°C. Q (injection volume) = 1 μl.

Without HPMC, there were many spikes superimposed on the peak of RBC which stemmed from the agglomerates of irregular size and charge. The RBC peak also tailed slightly.

Effect of inner diameter of the tubing

No peak could be observed with tubing of I.D. $\leqslant$ 0.3 mm and good results were obtained with I.D. = 0.45 mm. Tubing of 0.5 and 0.6 mm I.D. gave similar results, but the current was high, the baseline became too noisy and spurious peaks frequently emerged. Hence tubing of 0.45 mm I.D. was employed in this study through-out. The total length was 85 cm and the distance from the injection block to the detector was 60 cm.

The CZE of RBC

A typical electrophorogram of the RBC of a healthy man is shown in Fig. 2. The distribution of electrophoretic mobility can be directly seen from the curve and the most probable mobility can easily be calculated from the retention time. As

$$V = V_E + V_{os} \tag{1}$$

where V, V_E and V_{os} are the velocities of the apparent migration of the cells, the electrophoretic migration due simply to the electric field and the electroosmotic flow, respectively, the effective electrophoretic mobility, m (cm^2 s^{-1} V^{-1}), of RBC is obtained as

$$m = \frac{(L/t_R) - V_{os})}{E} \tag{2}$$

where L is the length of the tubing from the injection point to the detector (cm), t_R the retention time (s) and E the electric field strength (V cm^{-1}). The sedimentation of the cells in the vertical tubing is negligible (*ca.* 1 cm h^{-1}) and is not included in eqn. 1 because the electrolyte was so prepared that its density was nearly identical with that of the RBC. By injecting pyridine into the lower end of the capillary, V_{os} was

TABLE II
RETENTION TIMES OF VARIOUS RBC ELECTROPHORESED INDIVIDUALLY AND MIXED WITH OTHERS

RBC	*CZE conditions*	*Retention time (min)*	
		Individual	*Mixed*
(a) Chicken, formalin fixed	Electrolyte M1 + 0.010% HPMC, pH 7.33; V = 15 kV; I = 110 μA; T = 17°C (air cooled)	28.3	28.3 (a + b)
(b) Porcine, glutaraldehyde fixed		32.8	32.5 (a + b)
(c) Rabbit	Electrolyte H1 + 0.10% HPMC, pH 7.18; V = 20 kV; I = 120 μA; T= 18°C	26.6	26.9 (c + d)
(d) Human		14.0	14.1 (c + d)

measured[4] to be 25 cm in 36.5 min, *i.e.*, 0.0114 cm s^{-1}. From the retention time of 13.9 min of the peak in Fig. 2, m is calculated to be $-3.55 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1}. This agrees with the result of $-4.3 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1} (refs. 5 and 6) if the difference in the viscosity and the isotonic additive of the electrolytes used are taken into consideration[7]. The base width of the peak measured with the laser Doppler technique, $0.65 \cdot 10^{-4}$ cm^2 s^{-1} V^{-1} (ref. 8), is twice that in Fig. 2, and the latter is close to the value resulting from microscopic cell electrophoresis[5]. This serves to illustrate the high efficiency of the CZE technique.

When human and rabbit RBC and a mixture of them were electrophoresed successively, no distortion of the peak or shift of the peak maxima was observed in the electropherogram of the mixture, which indicates the absence of interaction between the RBC of the two species. Similar results were obtained for the formalin-fixed chicken RBC and glutaraldehyde-fixed porcine RBC. The retention times are listed in Table II. The differentiation of RBC from different sources or the cells of patients from those of normal persons might therefore be possible.

The number of cells in a single injection was *ca.* $1 \cdot 10^5$, hence the peak shape was a good approximation to the distribution of the mobilities, which could be obtained in less than 30 min, while the microscopic cells electrophoresis measured the cell one after another. Even modern computerized apparatus such as the Parmoquant II (G.D.R.) took more than 1 h to measure 100 cells, which was less than sufficient for describing the distribution of the mobilities.

Reproducibility of the determination of mobilities

The retention times (mobilities) of various cells under different conditions are compared in Table III. The coefficients of variation were always below 2.0%, and injections with different concentrations (Table III, No. 4) or different sample volumes (Table III, No. 1) did not impair the reproducibility. With such a high precision, minor changes in surface charge (and hence mobility) due to disease could then be detected with ease. The reproducibility of microscopic cell electrophoresis was bad and it would consume too much time if a host of cells (say, more than 500) are measured in order to give results with sufficient statistical significance.

TABLE III
REPRODUCIBILITY OF THE RETENTION TIME OF VARIOUS RBC
Injection volume 1 μl unless stated otherwise.

RBC	*Electrolyte and CZE conditions*	*Retention time (min)*	*n*	*Coefficient of variation (C.V.) (%)*
(a) Chicken, formalin	P2 + 0.008% HPMC, pH 7.40; V = 15 kV; I= 140 μA; T $\approx$ 20°C (air)	23.9	5	1.8
(b) Chicken, formalin fixed[a]	P1 + 0.020% HPMC, pH 7.40; V = 15 kV; I = 110 μA; T $\approx$ 16°C (air)	27.5	5	1.6
(c) Chicken, formalin fixed	M1 + 0.010% HPMC, pH 7.33; V = 15 kV; I = 110 μA; T $\approx$ 16°C (air)	28.2	4	0.3
(d) Chicken	M1 + 0.010% HPMC, pH 7.33; V= 15 kV; I = 130 μA; T = 20°C	23.5	5	1.2
(e) Human[b]	H1 + 0.10% HPMC, pH 7.40; V = 20 kV; I = 150 μA; T = 18°C	15.1	5	0.5

[a] Different concentrations, $1.1 \cdot 10^5$, $0.64 \cdot 10^5$, $0.89 \cdot 10^5$ and $0.5 \cdot 10^5$ cells μl^{-1}, were injected.
[b] Different volumes, 0.2, 0.5,1.0, 1.5 and 2.0 μl of the RBC, were injected.

Another example is the retention times of the RBC of healthy male and female persons, as shown in Table IV. The coefficient of variation of the six measured mobilities was 1.32%, which was no worse than measuring the RBC from a single person.

Linearity

The peak height varied linearly with the sample size as in chromatographic analysis. Table V shows the linearity at different pH values and with different ranges of

TABLE IV
RETENTION TIMES OF THE RBC OF HEALTHY INDIVIDUALS
Electrolyte H1 + 0.10% HPMC, pH 7.18; V = 20 kV; I = 120 μA; T = 19°C.

No.	*Age*	*Sex*	*Retention time (min)*
1	20	Male	14.0
2	28	Female	13.6
3	23	Male	13.7
4	30	Male	13.6
5	21	Female	13.8
6	27	Male	14.1
			13.8
Mean			(C.V. 1.32%)

TABLE V
EQUATIONS OF REGRESSION LINES UNDER DIFFERENT CONDITIONS

Experiment	*CZE conditions*	*Regression line*[a]
A	Electrolyte H1 + 0.10% HPMC, pH 7.40; V = 20 kV; I = 150 μA; T = 18°C; injection volumes, 0.5, 1, 2, 3 and 4 μl	$H = 2.91Q + 2.19$ ($r = 0.9835$)
B	Electrolyte H1 + 0.10% HPMC, pH 7.18; V = 20 kV; I = 120 μA; T = 19°C; injected volumes, 0.2, 1.0, 1.5 and 2 μl	$H = 9.06Q + 0.93$ ($r = 0.9903$)

[a] H = peak height (cm); Q = injection volume (μl); r = correlation coefficient.

sample size. The good linearity might be the basis of quantitative analysis or a means of cell counting. At pH 7.40 (Table V, A) the current passed was considerably higher than at pH 7.18, which might be responsible for the regression line deviating from the origin. However, it is not clear why the slope of the regression line at pH 7.40 was less than one third of that of the line at pH 7.18.

The pause between two experiments

When a piece of FEP tubing had been subjected to a high electric field strength a number of times, the CZE results became irreproducible. The initial performance could be restored if a pause from a few minutes up to 1 h was allowed before the next experiment was started. It seems that the inner surface of the tubing was subjected to certain physical/chemical changes which necessitated a relaxation period. Careful study showed that the necessary minimum pause for reproducible results depended on the history of the tubing, as outlined in Table VI.

It can be seen from Table VI that the necessary pause time increased steadily with the number of experiments made, which led to the conclusion that an irreversible breakdown process was taking place. When the tubing had been used more than 200–250 times, it was no longer useful. The upper end (high voltage end) of this useless tubing was cut off and the inner wall surface was examined by X-ray photoelectron spectroscopy (XPS). Fig. 3 shows that the peak of binding energy 285.9 eV (C_{1s} electrons) almost disappeared (curve b) which implies that the CHF[9] structure was broken under a high electric field strength. What really happens is not clear. A field strength below 300 V cm^{-1} as in this study is unlikely to be able to cleave the chemical bonds, but an uneven distribution of the electric field might be a cause. If a gas bubble is formed in the vicinity of the tubing wall, the high field strength across the bubble would induce a spark discharge that is sufficient to destroy the linkages among the various atoms on the surface of the wall. It should be pointed out that the so-called

TABLE VI
RELATIONSHIP BETWEEN THE NECESSARY PAUSE TIME AND THE HISTORY OF THE CAPILLARY TUBING

Number of CZE experiments already made	0–10	10–50	50–100	>100
Minimum pause time needed (min)	10	15	30	>40

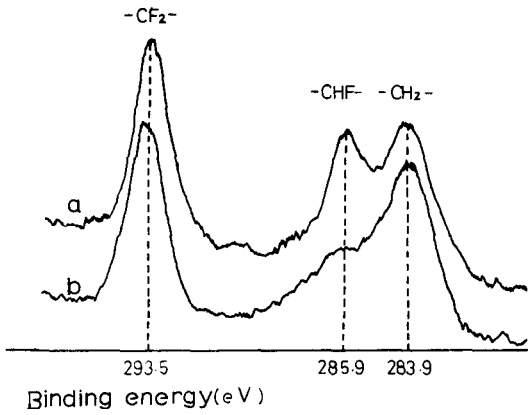

Fig. 3. XPS spectrum of inner surface of FEP tubing. (a) New tubing; (b) used tubing (electrophoresed more than 200 times).

useless tubing behaved well if small molecules were electrophoresed. This again serves to demonstrate the difficulty in electrophoresing proteins, which are abundant in the cell membrane.

Washing of cells

The RBC must be carefully and thoroughly washed so that correct peak shapes and accurate and reproducible results are obtained. Experiments showed the PBS was the most suitable washing solution, and 4–6 washings gave the best results. Washing less than three times led to much longer retention times, and after washing more than six times lysis was liable to occur.

Storage of RBC suspension

Conventionally the RBC were dispersed in 50 volumes of physiological saline and kept at 4°C. However, the peak of the RBC was difficult to observe when the

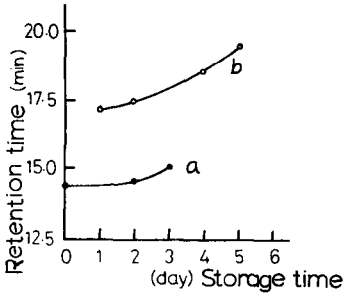

Fig. 4. Effect of storage time on the retention time of human RBC. (a) Electrolyte, H1 + 0.10% (w/v) HPMC, pH 7.40; V = 20 kV; I = 150 μA; T = 20°C. (b) Electrolyte, H2 + 0.10% (w/v) HPMC, pH 7.30; V = 20 kV; I = 110 μA; T = 17°C.

storage time exceeded 2 days. The electrolyte for CZE containing HPMC was found to be a satisfactory medium for the storage of RBC and good electrophoretic peaks resulted after storage for 7 days. However, the retention time of RBC increased with storage time, although slowly, as shown in Fig. 4. Large errors in the retention times could result of RBC are stored for more than 3 days, whereas the peak shape improves considerably.

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