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PREPARATION OF UNIFORM HAEMOGLOBIN FREE HUMAN ERYTHROCYTE GHOSTS IN ISOTONIC SOLUTION

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Summary

A method is described for the preparation of haemoglobin free human erythrocyte ghosts in isotonic solutions using dielectric breakdown technique. In this single haemolytic procedure, almost complete removal of haemoglobin ($\leqslant 0.1\%$) was achieved by subjecting the erythrocytes suspended in phosphate buffered, isotonic KCl solution at 0°C to three consecutive electrical field pulses of 16 kV/cm in the presence of 10 mM EDTA; EDTA was used to prevent electrical haemolysis. Haemolysis is induced by subsequent dilution with isotonic and isoionic solution to lower the EDTA concentration. Haemolysis is complete after 5 min; the cells are centrifuged, washed and resuspended in a solution of the same composition and osmolarity containing 4 mM $MgCl_2$, but no EDTA. The resealing process, carried out at 37°C, was complete in about 1 h. Measurements of the size distribution of the ghost cells in the hydrodynamically focusing Coulter Counter at varying field strengths in the orifice revealed that the ghost population is nearly uniform. The mean (modal) volume of the ghost cells was 110—120 μm^3 when suspended in phosphate buffered NaCl solution. The apparent breakdown voltage was about 1.3 V.

Erythrocyte ghosts are important tools in membrane research and the improvement of preparation techniques continues to attract great interest. The most commonly used method of ghost cell preparation employs haemo-

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lysis in hypotonic solution [1—4]. However, there is a good body of evidence that haemolysis in solutions of low ionic strength results in significant alterations of the membrane integrity and composition [5,6].

These inherent problems of osmotic haemolysis have focused attention on preparation methods conducted in isotonic solutions. Zimmermann et al. [7—9] have reported, for the first time, an electrical method for obtaining erythrocyte ghost cells under isotonic, and isoionic conditions. More recently, Billah et al. [10] introduced another method for preparing ghost cells under isoionic conditions. These authors loaded intact erythrocyte cells with glycol and then diluted the suspension with a large volume of glycol free solution. Using the glycol loading method, several lytic steps are required to obtain nearly haemoglobin free ghost cells. In addition, this procedure involves the introduction of large concentrations of glycol which may alter the membrane integrity.

The physical method described by Zimmermann et al. [7,9] is based upon the dielectric breakdown of the cell membrane and the subsequent haemolysis of the intact erythrocytes [11,12].

Dielectric breakdown of the cell membranes occurs when intact erythrocytes are exposed for short time intervals, say microseconds, to high external electric field strengths.

In principle, the high electric field strengths can be generated for microseconds either by sucking the cells through a region of a high electric field strength, such as the orifice of a hydrodynamically focusing Coulter Counter, or by discharging a high voltage capacitor through the cell suspension [8,9,13,14]. When the first procedure is used, the haemoglobin content of the ghost cells obtained after dielectric breakdown and haemolysis can be lowered to about 1% due to the dilution effect in the orifice of the hydrodynamic focusing Coulter Counter [8].

In the second procedure, the haemoglobin content of the electrically prepared ghost cells depends, among other things, on the suspension density. With a 1:10 ratio of cell volume to buffer solution, the ghost haemoglobin content is about 10% when injecting an electric field pulse of 12 kV/cm for 40 μs in an isotonic solution containing predominantly univalent anions. In the discharge chamber experiment, the haemoglobin content can be lowered to 1—2% using a dilution ratio of 1:500. The remaining haemoglobin, presumably because of its association with the inner membrane surface in isotonic solutions, cannot be removed by further dilution or by applying several consecutive electric field pulses at 16 kV/cm at low suspension density (1:40) prior to dilution (unpublished data). The present communication describes a method for obtaining electrically homogeneous erythrocyte ghost cells which are essentially free of haemoglobin. The relatively haemoglobin-free ghost cells are obtained in a single lysis by applying several electric field pulses of 16 kV/cm to erythrocytes suspended in an isotonic solution containing predominantly univalent ions and EDTA. This step is followed by dilution in isotonic medium to lower the EDTA concentration. The property of EDTA to prevent haemolysis in isotonic solutions containing predominantly univalent ions [11,12] assists the induction of more localized breakdown centers (and of enlargement of the breakdown

centers) in response to several, very high electric field pulses. The subsequent dilution of the cells subjected to dielectric breakdown causes lysis due to the disappearance of the protective influence of EDTA as a result of its decreased concentration. Electrically homogeneous ghosts of optimum membrane properties can be obtained by the following procedure.

Fresh blood drawn from apparently healthy donors was used. The erythrocytes were washed several times with solution I (138.6 mM NaCl, 12.3 mM Na_2HPO_4, and 2.7 mM NaH_2PO_4) and then suspended in a solution II (111 mM KCl, 20 mM NaCl, 7.6 mM Na_2HPO_4, 2.4 mM NaH_2PO_4, 10 mM glucose) to which 10 mM EDTA was added. The calcium concentration in the solution was lower than 10^{-5} M since suprapure substances (Merck, G.F.R.) were used. The ratio of packed cells to solution was adjusted to 1:40, corresponding to $2 \cdot 10^8$—$3 \cdot 10^8$ cells/ml.

In order to obtain a high yield of haemoglobin free ghost cells, it is necessary to ensure a uniform application of the electric field pulse to the suspended cells. The discharge cell used consists of two flat, parallel platinum electrodes (3 cm × 3 cm), 1 cm apart, mounted in a rectangular well of a plexiglass chamber. Cell suspension is filled into the well until it is flush with the upper edge of the electrodes. 2 ml of cell free electrolyte is then layered over the cell suspension in order to cover the well of the chamber. Such an arrangement provides, one that the cells are exposed to the same electrical field strength between the electrodes and, two, that the field pulse is prevented from going partially through the air instead of completely through the suspension.

The temperature of the solutions was initially about 0° C. The suspension was subjected in the discharge chamber to a sequence of three electric field pulses of 16 kV/cm by discharging a high voltage capacitor (for experimental details, see ref. 14).

The duration of each pulse was 40 μs and the interval between two consecutive pulses was 30—40 sec depending on the time required for charging the capacitor. The temperature increased, after the third pulse, usually to 15—20° C. Immediately after the application of the electrical field pulses, the suspension was diluted by a factor of $\approx$ 25 to 250 ml, using solution II that was cooled to 0° C. The cells were centrifuged in the cold at 10 000 × g after haemolysis was complete (i.e. about 5 min). The centrifuged cells were washed with cold solution II in order to remove the adsorbed haemoglobin on the outer membrane surface, and resuspended in fresh solution II in which 4 mM $MgCl_2$ replaced an equiosmolar amount of KCl (ratio of packed cells to solution 1:40). The temperature of the solution was raised slowly to 37° C. The resealing of the cells was apparently complete in about 1 h. The cells were centrifuged and the very small "button" of red cells at the bottom of the centrifuge tube was removed by aspiration. The haemoglobin free ghost cells were transferred into solution I and the size distribution of the resealed ghost population was measured at increasing field strengths in the orifice of the hydrodynamically focusing Coulter Counter. The dimensions of the orifice of the Coulter Counter were 60 μm in length and diameter. Details of the experimental arrangement, evaluation of the critical electric field strength, and calculation of the corre-

sponding critical breakdown voltage of the membrane are described elsewhere (e.g. ref. 11). From Fig. 1 it is obvious that no skewness of the size distributions is observed with increasing electric field strength. Significant inhomogeneities in the ghost cell population, due to cells of different membrane properties or shape factors [9], would lead to a skewness of the size distribution in the supercritical electric field range, i.e. beyond the critical field strength where breakdown occurs (i.e. above 1.7 kV/cm, Fig. 1).

The mean (modal) volume of the ghost cell population calculated from the size distributions measured at subcritical field strengths (0.4—1.0 kV/cm) is on average 110—120 μm^3 and the breakdown voltage of the ghost cell membranes is about 1.3—1.4 V. It should be noted that the breakdown voltage within the size distribution is not volume independent as found for the intact red blood cells. The breakdown voltage for a volume of 90 μm^3 is 1.2 V, this is slightly smaller than the breakdown voltage for a volume of 147 μm^3 which is 1.5 V. This volume dependence of the breakdown voltage which is not predicted by the integrated Laplace equation used in the calculation of the breakdown voltage [11], is not completely understood; however, the occurrence of the volume dependence of the breakdown voltage within a size distribution is an indication that the membrane properties of the population exhibit slight inhomogeneities. This view is supported by the fact that the size distributions measured at higher electrical field strengths

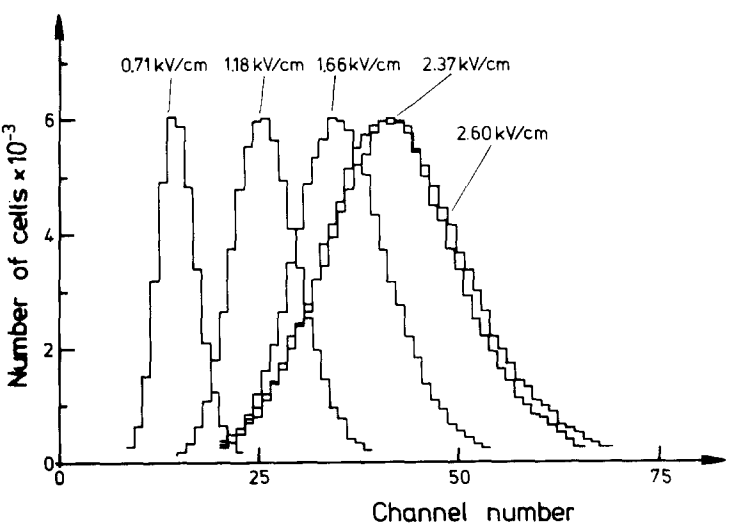

Fig. 1. Size distributions of haemoglobin free ghost cells measured at increasing electrical field strengths in the orifice of a hydrodynamically focusing Coulter Counter. The orifice was 60 μm in diameter and length. The ghost cells were prepared by electrical haemolysis in isotonic and isoionic solution. For details of the procedure, see text. The Coulter Counter measurements were conducted with ghost cells suspended in phosphate buffered NaCl solution (solution I). Note that increasing the electrical field beyond the critical value required for breakdown (1.7 kV/cm) the size distributions, measured as a function of the channel number, become coincidental. This is to be expected as the conductivity of the interior of the ghost cells, which is identical with that of the external solution, determines the resistance of the whole cell for super-critical field strengths [13]. As the current through the orifice is increased, the measured size distribution is broadening owing to an increased sensitivity of the system, a result of the fixed resolution of the Analogue to Digital Converter combined with the increased amplifier output.

do not correspond to a perfect Gaussian distribution compared with those measured at lower electrical field strengths. Since no real skewness is observed it can be concluded, however, that the ghost cell population is nearly uniform in respect to membrane properties and deformation. On the other hand, the findings of small variations in the breakdown voltage with volume for the same size distribution demonstrate that breakdown measurements are a very sensitive criterion for detecting inhomogeneities in the membrane properties of cells in a population. It should be also pointed out that the value of the breakdown voltage represents the upper limit of the real breakdown voltage due to the numerous time constants involved in the generation of the membrane potential in response to the external electrical field, and due to the time constants of the breakdown process itself [11,12].

The average haemoglobin content of the ghost cells so obtained, as measured by the pyridine haemochromagen method [1], was about 0.1% or less.

The haemoglobin free ghost cells are very fragile, unlike the ghost populations prepared in the usual manner by electrical haemolysis [14] which contains about 5% haemoglobin. Great care must be taken during the preparation procedure; for instance, rapid elevation of the temperature of the suspension for resealing or incubation of the resealed ghost cells at room temperature for a longer time (1 h or more) in isotonic phosphate buffered sodium chloride solution, leads to skewness of the size distributions at supercritical field strengths. On the basis of these results one may suppose that a small percentage of haemoglobin is essential for the stabilisation of the membrane [15].

In a second set of experiments the resealing process was carried out in the same solution in which the cells were haemolysed. The procedure of haemolysing and resealing was identical to that of the aforementioned experiment except that the ghosts were washed with isotonic KCl solution to remove the adsorbed haemoglobin from the outer membrane surface after resealing was complete.

Under these experimental conditions also uniform ghost cell size distributions were obtained. However, very often a slight skewness of the size distributions in the supercritical electrical field range was observed. The volume and the breakdown voltage were very similar to those experiments where haemolysis and resealing was conducted in different solutions. The haemoglobin content was on average slightly higher (0.2—0.3%) than in those ghost cells prepared by the aforementioned procedure. For that reason the procedure in which the haemolysed cells were washed prior to the resealing process, is preferred.

The mechanism of EDTA in protecting erythrocytes against electrical haemolysis is not completely understood. EDTA facilitates the removal of haemoglobin from erythrocyte ghosts due to its ability to chelate calcium and magnesium [16]. Yet the effect of EDTA observed in the present study may not be entirely related to metal ion chelation. The erythrocytes are treated with EDTA at 0°C, a temperature at which membrane calcium is not chelated [4]. During the pulsing of the cell suspension, the temperature is temporally raised to 15—20°C. It may be argued that the duration of the

temperature increase (≈ 2 min) is sufficient for chelation of membrane calcium with EDTA.

On the other hand, one must exercise caution when comparing the present observations with those of Bodemann and Passow [4], and Johnson [16]; their studies concerning the EDTA effect were conducted in solutions of low ionic strength and osmolarity whereas the experiments reported here were carried out under isotonic and isoionic conditions.

It seems more likely, as mentioned earlier, that EDTA compensates, up to high electric field strengths the osmotic pressure exerted by haemoglobin as EDTA appears to have a high reflection coefficient even when the critical breakdown voltage is reached [12]. In order to lower the reflection coefficient, very high electric field strengths and consequently more breakdown centers have to be generated. This would lead to a corresponding increase in the permeability and to an imbalance of the osmotic pressure exerted by haemoglobin; in turn, haemolysis occurs [12].

This interpretation is supported by preliminary studies in which the haemoglobin of the ghost cells could be lowered by subjecting the erythrocytes to several consecutive field pulses in solutions in which chloride was completely replaced by phosphate (Pilwat, G., Saleemuddin, M. and Zimmermann, U., unpublished data). Phosphate also prevents haemoglobin release and shifts the critical field strengths required for electrical haemolysis to higher values although it is not so effective as EDTA [11,12]. The haemoglobin content of the ghost cells so obtained could only be lowered to 0.4—0.5%, which corroborates the assumption that EDTA has an additional chelation effect.

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