

Immobilization of Human Red Blood Cells

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Human red blood cells were immobilized in an alginate network which was cross-linked with Ca^{2+} ions. The immobilized cells were stored for longer than 5 weeks at 4°C in an isotonic buffered NaCl solution containing glucose, inosine, adenine, and guanosine for energy supply. The immobilized cells were released from the alginate network by dissolving the matrix with citrate. Both the immobilized and released cells retain their biconcave shape over the storage period. Measurements of the released cell population in a hydrodynamically focussing Coulter Counter demonstrated that the mean size of the size distribution, the breakdown voltage, and the internal conductivity have not changed in contrast to control measurements on red blood cells stored conventionally in suspension indicating that immobilization preserves cellular functions.

Introduction

The immobilization of cells has attracted increasing interest as to their potential industrial use [1, 2]. From an economic standpoint the use of immobilized cells in the catalysis of single or multi-enzyme reactions on an industrial scale is very promising in comparison with the conventional methods of enzyme isolation and subsequent immobilization which are both time consuming and expensive. A point of more general importance in the field of cell biology is the observation, that immobilized cells are much more stable than cells suspended in a liquid [1, 2].

Cells can be immobilized by adsorption, covalent bonding, by cross-linking or by entrapment in a polymeric matrix.

In this communication we report on the immobilization and stability of erythrocytes (as an example of wall less cells) using the entrapment technique. Immobilization of erythrocytes has been achieved to date by entrapment in collagen [3] and by adsorption

[4]. The ionotropic gelation technique first developed by Klein and coworkers [5, 6] and recently adopted by others [7, 8] seemed to be especially advantageous for our study with respect to mild immobilization conditions and reversibility of network formation.

Immobilization of Red Blood Cells

Blood was withdrawn from healthy human donors and stored in ACD buffer for no longer than one day. The erythrocytes were centrifuged and washed several times with a solution I of the following composition (in mmol/l): 155 NaCl, 5 CaCl_2 , 1 Tris, 10 glucose, 5 inosine, 0.3 adenine, 0.3 guanosine, and 20 ml of a penicillin/streptomycin mixture (10000 U/10 mg/ml). For the immobilization of the erythrocytes, Na-alginate (Alginate Industries Ltd., London) was used.

A number of commercial alginate polymers (Manucol LD, DH, and DM, molecular weights 40000, 90000, and 150000, respectively) were tested for their suitability for the immobilization of erythrocytes. The experiments showed that Na-alginate Manucol DH had the best properties for the entrapment of erythrocytes. Other cross-linked alginate compounds were shown to have slight haemolytic activity (see below) or not have sufficient mechanical stability. The cross-linkage of Na-alginate through ionic bonds was achieved with Ca^{2+} ions. Other multivalent ions such as Al^{3+} or Mg^{2+} either induced haemolysis of the entrapped erythrocytes or did not give rise to the formation of a network. The rate of the cross-linking reaction is dependent on the concentration of Ca^{2+} ions. At concentrations of about 110 mM CaCl_2 the reaction is completed within 5 min under given experimental conditions (see below), whereas it takes about 30 min at a concentration of about 10 mM CaCl_2 . Since high concentrations of calcium (say above 5 mM) can lead to irreversible changes in the erythrocyte membrane, care must be taken to ensure that the calcium is rapidly bound in the cross-linkage reaction in order to avoid a prolonged action on the membrane. A Ca^{2+} concentration of 10 mM was found to present the optimum cross-linking conditions for erythrocytes. In order to achieve an optimum rate of cross-linkage erythrocytes suspended in an isotonic solution of NaCl buffered with 1 mM Tris and 4% Manucol DH (ratio of packed cells to solution, 0.2)

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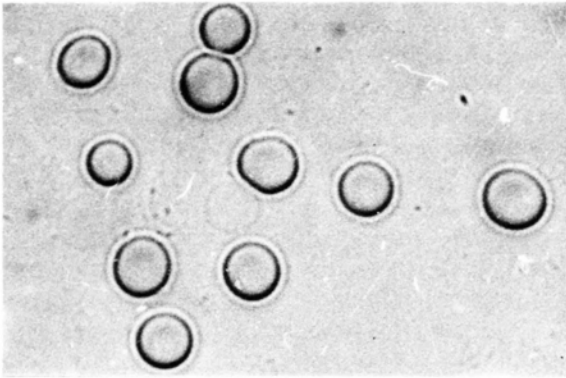


Fig. 1. Typical light micrograph of immobilized human erythrocytes in a Ca^{2+} cross-linked alginate matrix after a storage time of five weeks. The biconcave shape of the erythrocytes is clearly recognisable.

was added dropwise to a stirred isotonic NaCl-solution with 1 mM Tris and 10 mM CaCl_2 , pH 7.4, or to a solution of 110 mM CaCl_2 , pH 7.4. The resulting beads of about 2–3 mm in diameter were decanted, washed several times in solution I and subsequently stored in the same solution at 4 °C. The storage solution was renewed every four days in order to supply energy to the cells.

Fig. 1 illustrates immobilized erythrocytes, as seen under the light microscope. The erythrocytes were stored for a period of 5 weeks. The biconcave shape of the entrapped erythrocyte is clearly recognisable. During a period of five weeks no haemoglobin could be detected in the supernatant photometrically at a wave length of 415 nm, when the cross-linkage of the alginate was performed with 10 mM Ca^{2+} ions. In the case of 110 mM Ca^{2+} ions haemoglobin was detected in the supernatant after about 3 weeks.

Biophysical Characterization

The erythrocytes were released from the cross-linked matrix at certain intervals. For this purpose the cross-linked matrix was transferred to the stirred solution I in which a part of the NaCl was displaced by 35 mM sodium citrate. Since the affinity of Ca^{2+} for citrate is substantially higher than for alginate, the network is dissolved. At room temperature the reaction lasted about 2 h. The erythrocytes were subsequently isolated by centrifugation and resuspended in an isotonic NaCl-solution buffered with 1 mM Tris. The cell volume, the internal conductivity

and the cell membrane integrity of the released red blood cells were studied using a hydrodynamically focussing Coulter Counter to measure the size distribution of the red blood cells as a function of the external electrical field strength in the orifice of the Coulter Counter [9]. Above a certain critical external field strength dielectric breakdown of the cell membrane is induced. This is apparent as an underestimation of the cell volume [5, 6]. The electrical breakdown voltage which can be calculated from the external electrical field strength provides information on the integrity of the membrane and on its electro-mechanical properties [10, 11]. In addition, the underestimation of the size distribution in the supercritical field range also yields information on the internal conductivity and thus on the intracellular ion composition [12].

Fig. 2 shows the size distributions of the erythrocytes which were immobilized for about five weeks in the alginate network. The size distributions determined at both low and high external electrical field strengths (*i. e.* beyond the level at which electrical breakdown of the membrane occurs) are homogeneous, indicating that the size distribution is electrically homogeneous [13]. If there had been any structural change in the membrane or any geometrical change of the cell the size distribution would have been skewed, at least in the high electrical field ranges [13]. The apparent underestimation of the size distribution when measured beyond the critical field strength (due to breakdown of the cell membrane) becomes obvious when the pulse height of the mean size is plotted against the external field strength as shown in Fig. 3 (curve a). For comparison, the corresponding data for red blood cells immediately investigated after collection are also given (curve b). The discontinuity in slope of the function pulse height versus electrical field strength reflects the breakdown of the cell membrane. The breakdown voltage can be calculated from the discontinuity in slope using the integrated Laplace equation and a shape factor of 1.09 [10]. Within the limits of accuracy the value of 1 V is in agreement with the value quoted for erythrocytes in the literature [10, 13] and calculated from curve b in Fig. 3. The mean volume of the erythrocyte population (calculated from the size distribution measured at low electrical field strengths) has a value of $83\mu\text{m}^3$. This is equal or slightly larger than that recorded for cells that had not been immobilized (see ref. [13], see also Fig. 3).

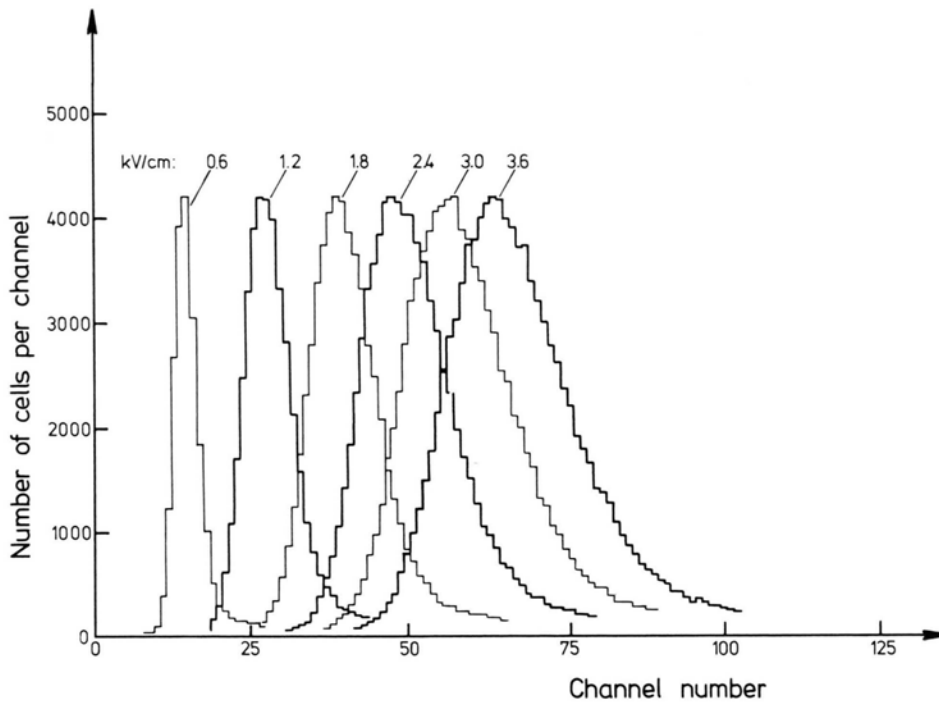


Fig. 2. Size distribution of human erythrocytes which had been immobilized for five weeks in a cross-linked alginate matrix as a function of the external electrical field strength in the orifice of a hydrodynamically focussing Coulter Counter. The distributions (measured at low and high electric field strengths, respectively) show normal distribution which is a clear indication that no changes of the cell membrane properties have occurred (10).

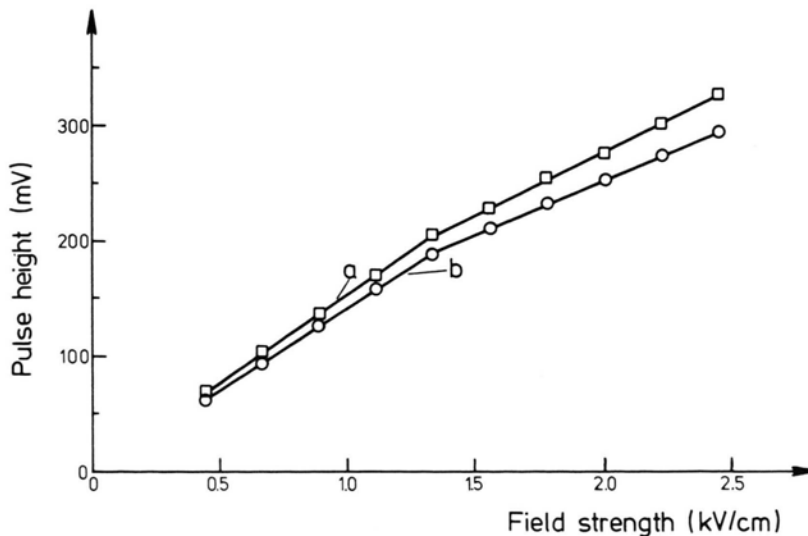


Fig. 3. Pulse height of the mean of a red blood cell size distribution versus increasing field strength in the orifice of a hydrodynamically focussing Coulter Counter. Curve a represents the measurement performed on red blood cells immobilized in an alginate matrix for 5 weeks. Curve b represents the control measurement performed on red blood cells immediately after collection of the blood.

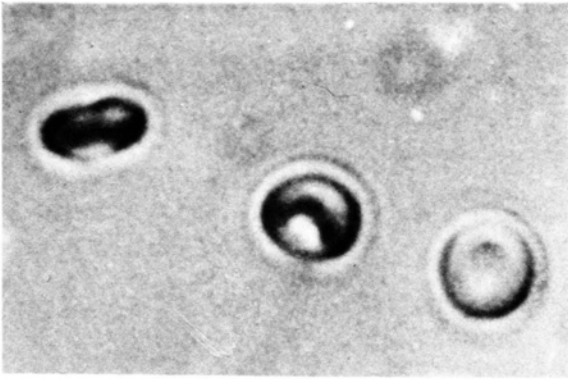


Fig. 4. Typical light micrograph of human erythrocytes after dissolving the cross-linked alginate matrix in which they had been immobilized for 3 weeks.

The degree of underestimation after breakdown is directly related to the internal conductivity [12]. For erythrocytes immobilized for about 5 weeks the underestimation is on average about 30%. This value is within the limits of accuracy or deviates only very slightly in some cases from the values measured for erythrocytes that had not been immobilized (see also Fig. 3, curve b).

Under the light microscope the released erythrocytes were seen to have retained their biconcave shape (Fig. 4). Since the shape of the erythrocytes is dependent on the metabolism of the cells [14, 15], one is lead to the conclusion that there are no significant changes in the erythrocytes during the period of entrapment in the cross-linked matrix, either from a biophysical point of view or with respect to their metabolism.

Discussion

The immobilization of erythrocytes in a cross-linked matrix offers a number of attractive applications both in membrane research and from a clinical and technical point of view. Since the erythrocytes are surrounded by a mechanically stable envelope, substantially higher pressure gradients can be set up across the membrane in hypotonic solutions than in erythrocytes that have not been mechanically stabilized. According to measurements carried out by Rand and Burton [16] a maximum pressure gradient

of only $2-3 \times 10^{-4}$ bar can be set up in a human erythrocyte before it will burst. Indeed, recently, using plant protoplasts immobilized in an alginate matrix (unpublished results) it could be demonstrated that pressure gradients in the order of a few hundred mbar can be built up. Studies of membrane transport and structure in response to pressure gradients across the membrane of animal cells might therefore be a promising way both to elucidate pressure gradient (*i. e.* cell turgor pressure) dependent processes in walled cells and to close the gap between osmoregulatory processes in turgescient plant cells and bacteria and in the almost-turgor-less cells of animals. Experimental evidence is available, that changes of pressure gradients of about 0.3 bar result in significant changes in the electrical membrane parameters and membrane transport of algal cells [17, 18]. In particular, it is well accepted that a certain cell turgor pressure is required for plant cell growth, cell enlargement and cell division [19-21]. The molecular processes underlying these phenomena are still far from being understood.

The investigations reported here have shown that the erythrocytes are easily stored for prolonged periods when they have been immobilized. Although these studies will have to be extended for longer periods of time (in the order of two to three months) including measurements of intracellular enzyme activity, previous experience with immobilized microorganisms leads one to expect that this procedure might have great advantages for the storage of blood in blood banks. Microorganisms immobilized in various cross-linked networks may be stored for substantially longer periods than would otherwise be possible if they were kept in suspension [1]. The physico-chemical mechanisms for this effect are not fully understood. However, the entrapment of wall-less cells provides improved experimental conditions for the study of membrane structure and transport since the disturbing influence of the cell wall arising in such measurements when performed on whole cells is eliminated.

In principle, the procedure described here can be applied to all wall-less cells, such as tumor cells, plant protoplasts, vacuoles, and lipid and bacterial vesicles, so that in general the possibility exists with this technique of immobilization for the storage, or even the transport, of cell and vesicle cultures and suspensions.

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