Porcine blood cell separation by porous cellulose acetate membranes

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Abstract

Leukocytes were separated from whole porcine blood using laboratory prepared polymeric asymmetric porous membranes from cellulose acetate (CA) and by applying standard blood cell separation methods: centrifugation in a Ficoll solution gradient and in sucrose solution concentration gradient. Leukocytes, obtained by different separation methods were characterised by their quantity, type, viability and growth ability. Membranes prepared by a wet phase inversion process from different cellulose acetate/acetone/water and magnesium chlorate VII systems, were characterised according to: permeability to deionised water, surface morphology and by the determination of the flux of the permeate during the whole porcine blood separation. Cellulose acetate membranes prepared from 300 μm thick cast solution (14.8 wt% of cellulose acetate, 19.9 wt% of water, 2.3 wt% of Magnesium perchlorate, and 63.0 wt% of acetone), have separation characteristics comparable with the standard separation methods; in the dead-end mode filtration, 21.3% of leukocytes from porcine whole blood are separated. The leukocyte number in peripheral blood before separation was $450,000\text{ ml}^{-1}$; the number passed through after was $95,000 \pm 6620$. The main interest of the study was to introduce the CA membrane filters for the continuous technological separation of the leukocyte/lymphocytes from animal (= porcine, bovine, horse..) blood.

Introduction

White blood cells (leukocytes) can be used as a source of interferons and other cytokines useful in human and veterinary medicine (Boedeker et al., 1984; Filipič et al., 1990, 1994). For this purpose they should be purified and concentrated from whole blood. Leukocytes can be isolated from whole blood by standard methods like haemolysis in ammonium chloride (Mizrahi et al., 1990) or centrifugation in Ficoll or sucrose solution concentration gradients (Cutts, 1976; Almici et al., 1993). These methods are relatively time consuming and some are rather expensive. In addition, they do not seem to be appropriate for continuous large-scale separation processes. In the search to find a more efficient way for blood cell separation, the membrane filtration systems could offer a possible solution.

Since their discovery (Loeb and Sourirajan, 1962) polymeric asymmetric porous membranes have

enabled the development of many new separation processes, among which microfiltration (separation of cells and viruses) can be viewed as a 'classical' membrane separation process (Hitzler, 1993; Harris, 1990). Various kinds of dialysis using different polymeric membranes have also been developed (Mulder, 1991a; Strathmann, 1992). Artificial kidneys, incorporating polymeric membranes as their essential separation part, are widely used for removal of toxic metabolic waste from the blood of patients with chronic or acute kidney failure (Ward et al., 1985; Broek et al., 1993).

Cellulose acetate (CA) was the first material used for preparation of polymeric asymmetric porous membranes (Loeb and Sourirajan, 1962) which were used for desalination of seawater by a reverse osmosis. They were prepared by the so called wet phase inversion process (Rouvers, 1987; Rouvers et al., 1987; Rouvers and Smolders, 1987; Stropnik et al., 1996).

For the membrane forming system CA/acetone/water

it is well known that asymmetry of the membrane is pronounced; relatively thick skin on the polymer solution/nonsolvent interface (on the upper part of the membrane) is formed (Rouvers, 1987; Rouvers et al., 1987; Rouvers and Smolders, 1987; Stropnik et al., 1996). One of the important characteristics of such membranes, is their relative impermeability to water. The problem of greater permeability was solved by Loeb and Sourirajan using water solution of magnesium chlorate VII (Mg perchlorate, $\text{Mg}(\text{ClO}_4)_2$) as solvent for CA and acetone to make the solution less viscous (Loeb and Sourirajan, 1962; Loeb, 1981). Membranes thus prepared have greater water permeability. Further research showed that a great variety of cellulose acetate membranes can be prepared changing the composition of the polymer solution (Manjikian, 1967; Srathmann et al., 1971), the composition of the coagulation bath (Kutowy et al., 1978) and the conditions of the solvent evaporation step (Kunst and Sourirajan, 1970). As a suitable material for membrane filters CA-membranes were introduced into the fields of medicine and biology with their reasonable biocompatibility and non-toxicity, mainly for the needs of haemodialysis, haemofiltrations and plasmapheresis (Malchesky, 1994; Weliky, 1965; Broek, 1993; Ward et al., 1985).

The present work deals with the problem of leukocyte/lymphocyte separation from the whole porcine blood by means of polymeric asymmetric porous membranes consisting of cellulose acetate. They were prepared by wet phase inversion process from the ternary system CA/acetone/water with the addition of the fourth component – magnesium chlorate VII.

A comparison of the CA membrane blood separation and standard blood cell separation methods, such as the centrifugation in Ficoll and sucrose solution concentration gradients, was made. Separated leukocytes were characterised by their quantity, type, viability and growth ability.

Materials and methods

Membrane preparation and characterisation

Three different solutions (CA1, CA2 and CA3) with various CA compositions and two different cast knife dimensions (250, 300 μm) were used for membrane preparation (see Table 1).

Cellulose acetate (Aldrich, St.Louis, USA) and acetone (Kemika, Zagreb, Croatia) were stirred in an

Table 1. Compositions of the solutions for membranes preparations (weight%); slit dimensions on knives (μm)^a

Cellulose acetate solution	Cellulose acetate (CA)	Acetone	Deionised water	$\text{Mg}(\text{ClO}_4)_2$
CA1	14.8	63.0	19.9	2.3
CA2	17.0	63.1	19.9	0.0
CA3	17.0	69.2	12.4	1.43
Slit dimensions on knives (μm)				
250	300			

^a Ten cellulose acetate membranes were prepared from each cellulose acetate solution.

Erlenmeyer flask until a homogeneous solution was formed (12–24 h). Deionised water with magnesium VII chloride (magnesium perchlorate, Fluka Chemie, Buchs, Switzerland) or deionised water alone (in the case of CA2 solution) were slowly added and stirred for another 4–6 h. Clear solution was cast on the precisely grinded glass plate (unevenness of the plate was around 5 μm) with special quadric stainless-steel knife with the exactly dimensioned slit (unexactness of the slit dimension was under 5 μm). As soon as possible (in less than five seconds), the thin layer of cast solution together with the glass plate was immersed into the coagulation bath consisting of pure water at 4–8 °C. Formed (proto)membrane was left in the coagulation bath for approximately 10 min and then transferred to the second ice-cold coagulation bath where it was left for another 2–3 h. Membranes were kept in the dark in the deionised water until they were characterised and used for porcine blood cells separation.

Round, 41.8 cm^2 surface area membranes were characterised by the determination of their permeability to deionised water in the standard 250 ml Amicon 8400A dead end filtration cell with stirrer (150 min^{-1}). The applied pressure of nitrogen (max. 6 bar) was adjusted with the 0.1 bar sensitivity and standard volumes of permeated water were measured in the definite time intervals.

The size and shape of the pores on the membrane surface and their distribution were determined under an optical microscope.

Porcine blood collection and leukocytes separation

Sterile collected porcine blood obtained from the slaughter house was put into plastic flasks containing

1/5 of the final volume of 8.3% of sodium citrate in water to prevent coagulation. Leucocytes were separated from the citricised blood using: CA membranes, Ficoll solution gradient, sucrose solution gradient and haemolysis.

CA membranes separation

250 ml of porcine citrated blood was poured into the standard Amicon 8400A stirred cell set up, pressure of nitrogen (2 bar) was applied and the flux of the permeate was determined by measuring the standard volumes of permeate in the definite time intervals. 200 ml of porcine blood was allowed to pass through each membrane (CA1, CA2 and CA3). Mostly leukocytes were collected in the permeate, while erythrocytes and some of the leukocytes and trombocytes retained on the membrane.

Ficoll solution gradient separation

Aliquots of 6 ml of the whole citricised porcine blood were poured onto Ficoll (Sigma, St.Louis, USA) solution and centrifuged at 400 g for 20 min as described elsewhere (Cutts, 1976; Lindl and Bauer, 1989). Lymphocytes only were collected from the glass tube with a syringe.

Sucrose solution gradient separation

Leukocytes were also separated by the standard procedure of centrifugation of the citricised porcine blood in the sucrose solution concentration gradient (Cutts, 1976; Ridge, 1978). 50 ml of the citricised porcine blood was added to the water solution of sucrose with the concentration gradient formed from the 40 wt% and 45 wt% sucrose solution in the sterile glass tube and centrifuged at 400 g for 20 min. Leukocytes, located in the 40/45 wt% concentration gradient's intermediate phase, were collected with a syringe.

Haemolysis

Blood cells from 50 ml of whole citricised porcine blood were obtained by centrifugation. Plasma was discarded. The erythrocytes in the sediment were lysed by adding of 9 volumes of 0.83% ammonium chloride as described in the protocol of Mizrahi et al. (Mizrahi et al., 1990) and leukocytes were collected by the centrifugation at 300 g for 10 min.

Leukocytes characterisation

The total number of leukocytes in 1 cc of whole citricised porcine blood and in the leukocyte enriched

Table 2. Deionised water flux and porcine blood permeate flux ($\text{l/m}^2 \text{ h}$) through various membranes from cellulose acetate, $\Delta p = 2 \text{ bars}^a$

CA membrane cast solution	Solution thickness (μm)	Deionised water fluxes ($\text{l/m}^2 \text{ h}$)	Blood permeate fluxes ($\text{l/m}^2 \text{ h}$)
CA1	250	206.40 ± 4.383 (6)	8.9 ± 0.50 (7)
	300	99.00 ± 3.252 (6)	4.6 ± 0.08 (5)
CA2	250	18.50 ± 0.350 (6)	6.6 ± 0.27 (5)
	300	11.20 ± 0.466 (6)	3.8 ± 0.21 (7)
CA3	250	nd	0.0
	300	7.25 ± 0.360 (5)	0.0

nd: Not done.

^a Means $\pm$ standard deviation (SD) of the indicated number of measurements (in parentheses) are shown.

suspensions obtained by the centrifugation in concentration gradients, haemolysis or by the membrane separation was determined by haemocytometer.

The types of cells (differential leukocytes count) were determined by Wright stain. Viability was determined by the trypan blue exclusion method, using 0.1% trypan blue solution in phosphate buffer saline (PBS).

The leukocytes number in fractions obtained by each separation method was adjusted to 1×10^5 cells/ml and cells were cultivated afterwards in siliconised glass Erlenmeyer's flask for 72 h at 37°C , using Eagle's medium, supplemented with 10% foetal calf serum and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). The number of leukocytes and their viability was determined every 24 h for 3 days.

Results and discussion

Permeability of various CA membranes to deionised water and to whole citricised porcine blood is shown in Table 2. Over 30 prepared CA membranes show the reproducibility of the fluxes in the frame of 35%. By increasing the cellulose acetate content, and by decreasing water and magnesium chlorate content in the cast solution for membrane preparation, the permeability to water and to whole porcine blood is decreased.

The deionised water and porcine blood fluxes decrease also when the nominal thickness of the cast solutions is increased from 250 to 300 μm . With rel-

atively small changes in the preparation conditions membranes with rather different permeability characteristics were prepared.

Membranes, prepared from the 'pure' (CA/acetone/-water) system (cast solution consists only from polymer and solvent, and coagulation bath consists only of pure nonsolvent (water), without fourth component) have very low permeability to water. Another characteristics of this 'pure' system is also the delay in the appearance of the turbidity. Turbidity is the result of the scattering of the light on the forming and growing nuclei of the polymer phase, which appears in the membrane forming a ternary system as a consequence of phase inversion. In the case of the (proto) membrane formation from the 'pure' CA/acetone/water system, strong syneresis (shrinkage) in the direction perpendicular to the coagulation bath-cast solution interface takes place during the turbidity delay period. Relatively thick skin is formed with the gelation and/or glass transition (vitrification) of the ternary system on the upper part of the membrane. During the turbidity appearance period, nuclei of the polymer lean phase are formed and grow to constitute the porous part of the membrane after the solidification of the surrounding polymer rich phase; entrapped nuclei form the pores responsible for the mass transport through the membrane. The skin on the upper part of the membrane is responsible for a very low water flux. Any modification of the 'pure' membrane forming CA/acetone/water system which leads to the shorter period of the perpendicular syneresis on the upper part of the system will bring about the quicker appearance of the nuclei of the polymer lean phase, and consequently, the quicker appearance of the turbidity. Instantaneous turbidity appearance means that nuclei of the polymer lean phase are formed just on the upper part of the membrane forming system. Such membranes have very open pores structure instead of compact and dense skin. Fig. 1 shows a surface of the membrane prepared from the 300 μm thick CA1 cast solution.

Surface pores have an asymmetric structure with a uniform size between 80–100 μm in diameter.

From the data given in Table 2, we can conclude that the addition of magnesium chlorate VII and water to the CA-acetone solution 'opens' the pores structure on the 'top' of the formed membranes, and that the addition of water has stronger effect than the addition of magnesium chlorate VII. It has been known that the addition of water as the nonsolvent for polymer to the cast solution leads to the membrane forming ternary system CA/acetone/water due to instantaneous

Table 3. Number of leukocytes per ml of samples obtained by different separation methods^a

Treatment	Leukocytes/ml	Recovery (%) ^b
CA1 (300 μm)	95 000 $\pm$ 6.620 (5)	21.3
CA2 (300 μm)	280 $\pm$ 8.900 (6)	0.5
CA3 (300 μm)	0	0
Ficoll	200 000 $\pm$ 1.143 (5)	46.0 ^c
Sucrose	300 000 $\pm$ 1.110 (6)	55.0
Haemolysis	400 000 $\pm$ 0.286 (4)	89.0

^a Means $\pm$ SD of the indicated number of separations (in parentheses) are shown.

^b Percentage of recovery was determined due to the initial leukocyte number in peripheral blood before separation (450 000 leukocytes/ml).

^c Mainly lymphocytes.

demixing and, consequently, to a membrane with very open pore structure (Rouvers, 1987; Rouvers et al., 1987; Rouvers and Smolders, 1987). The same effect on pore opening is achieved with the low temperature (ice-cold water) during the membrane formation process. So it is possible to prepare membranes with very different rates of permeability by the variation of the casting solution composition and the temperature of the coagulation bath.

Data from the Table 2 also show that during whole porcine blood separation in the standard Amicon dead end filtration cell the flux of porcine blood permeate is dramatically declined in comparison to the flux of the pure deionised water. In the case of membranes, prepared from the CA3 solution, no measurable flux of the permeate under the pressure difference of two bars was observed. This very well known and to membrane separation processes important phenomenon is the consequence of the concentration polarisation and/or fouling of the membrane (Mulder, 1991b). So any membrane which is planned to be used in some separation process cannot be selected only on its nominal permselective performances. It should be tested within the frame of the particular separation process conditions (Kim et al., 1994).

In Table 3, the number of leukocytes per ml of the samples obtained by the membrane separation and by the 3 standard blood cells separation methods (Ficoll and sucrose concentration gradient centrifugation and haemolysis) are presented.

During porcine blood cell separation with membrane prepared from the nominally 300 μm thick CA1 cast solution, 21.3% of leukocytes were found in the permeate. The yield of leukocytes obtained by

Figure 1. The surface of the membrane prepared from the 300 μm thick CA1 cast solution (magnification 100X).

this membrane is about 30% of the standard separation method. Membrane prepared from the 250 μm thick CA1 cast solution has the highest permeate flux (8.9 l/m² h) during porcine blood separation and the greatest number of leukocytes in the permeate. On the other side also a large number of erythrocytes are found in the permeate. The optimisation of the membrane preparation conditions for the permselective properties most suitable for leukocytes separation from the whole porcine blood is still open, as well as the testing of the system in the cross flow mode of membrane separation. This will be the next step in our experiments.

Figure 2 shows that after membrane separation of porcine blood cells, erythrocytes are prevailing blood constituents in the retentate.

Wright staining of permeated fractions showed that lymphocytes, monocytes, basophilic and neutrophilic granulocytes are the major leucocytes population, while we did not find acidophilic granulocytes.

Erythrocyte retention and leukocytes permeation through the membrane from CA during the separation of the citricised porcine blood is just the opposite of what we were expecting and what so far has been published (Hitzler, 1993). For the reason mentioned above, the prognostication of the membrane separation ability should not be based only on the blood cells size since leukocytes (8–12 μm) that pass through membranes

are about the same size as erythrocytes (9 μm) (Hitzler, 1993; Alberts et al, 1989). The great ability of leukocytes to adapt their form to the membrane pore shape during the separation process on the one hand, and CA membrane and blood cell surface charge as well as erythrocyte swelling in the citricised blood and their rigidity on the other hand are possible reasons for the described cell separation phenomenon.

Although the permeate fluxes at the whole porcine blood separation by membranes prepared from the CA1 and from the CA2 cast solutions are of the same order of magnitude, membrane prepared from the nominally 300 μm thick CA2 cast solution retained almost all leukocytes. These two facts (permeation of leukocytes and retention of erythrocytes; very low correlation between permeate fluxes at porcine blood separation and membrane blood cell separation ability) once again point out that the membrane separation abilities have to be tested within the frame of the particular separation process conditions (Kim et al., 1994).

Leukocytes rich fractions obtained by different separation methods were cultivated for 72 h at 37 °C without stirring. At the beginning the number of cells per ml was adjusted as it is shown in Table 4, and initial cell viability was 99% for all different leukocytes rich fractions.

The comparison of different cultures shows a pecu-

Figure 2. Surface of the membrane prepared from the 300 μm thick CA1 cast solution after the citricised porcine blood separation; the arrow indicates erythrocytes that retained on the membrane surface (100X magnification).

Table 4. Survival of leukocytes in growth cultures prepared from samples obtained by various isolation procedures^a

Isolation procedure	Initial cell number ($\times 10^5$)	Initial cell viability (%)	Leukocyte survival in growth cultures during the three days cultivation (% viability)		
			24 h	48 h	72 h
CA1 (300 μm)	4.5	99	45	60	67
Ficoll	5.0	99	95	82	70
Sucrose	2.0	99	95	81	69
Haemolysis	1.0	99	98	93	80

^a Means of two replicates are shown.

liar viability of leukocytes obtained through membrane separation. Their number dropped drastically after 24 h, although leukocytes did not show any noticeable damage under the optical microscope at the beginning of the cultivation period. It is possible that during the membrane separation leukocytes suffered from certain shock or that some elements essential for cell growth were removed. Still, these assumptions do not give an adequate answer to the growth expansion after the 24 h lag period. It will be of interest to test a presence of essential growth factors in leukocyte-containing fraction after the membrane filtration, as well as to grow separated leukocytes in different defined mediums.

Conclusions

From the results obtained in the present work it can be concluded that leukocytes can be separated from whole blood by means of porous asymmetric membranes prepared from the CA.

The described system could provide a useful tool for leukocytes separation from mammalian blood, since similar results were obtained with human blood as well (data not shown). With variation of composition and thickness of the cast solution and by its precipitation in the ice-cold water it is possible to prepare membranes with different permselective properties. Since

membranes technologies are already mature this can be the way to the large scale technology for various blood cells separations.

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