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Short Communication

Modification of glass channel walls for separation of biological particles by gravitational field-flow fractionation

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Abstract

In the gravitational field-flow fractionation of complex samples, various interaction and adsorption phenomena can occur in separation channels that influence fractionation and complicate the explanation of resulting fractograms. To overcome these problems, the glass surface was modified to create charge-free, non-adsorbing hydrophilic media for the mild treatment of hydrophilic biological particles. The modification was carried out in two steps: (1) by a simple lacquering of the glass surface with polystyrene diluted in toluene and (2) subsequent adsorption of a detergent layer on polystyrene. Essential suppression of ionic interactions between soluble low-molecular-mass compounds and the channel wall and decreased adsorption effects were demonstrated in separations of blood samples by gravitational field-flow fractionation.

1. Introduction

Gravitational field-flow fractionation (GFFF) utilizes the Earth's gravitational field in conjunction with lift forces appearing intrinsically in shear flows as external force fields for the separation of particles [1]. In contrast to gravitation, the lift forces tend to drive particles away from the channel accumulation wall and to focus them into a narrow zone. This so-called focusing [2] elution mode is considered to be the most important for particles with diameters above 1 μm in GFFF. GFFF has been demonstrated to be suitable for the rapid separation and/or characterization of various kinds of particles, *e.g.* glass beads [1], polystyrene latex particles [3,4], red blood cells [5–8] and blood parasites [9,10].

There are several factors that may significantly influence the fractionation, *e.g.* attractive or repulsive forces, various kinds of interactions and adsorption. These may be observed for both low-molecular-mass, macromolecular and particulate samples. One has to consider the properties of the channel walls, the sample and the carrier liquid (containing salts, detergents or other additives) and the potential interactions among them. One has to minimize the sample–wall and sample–sample interactions to find the optimum separation conditions. Preparation of the suitable channel wall surface is an important step in this optimization. The influence of channel wall modification on the separation of polystyrene latex particles has been studied in GFFF [3]. Various surfactants were tested to find the best one to minimize interactions (particle–wall, particle–particle) and improve separation. How-

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ever, additives of this type cannot be used in the treatment of biological samples (*e.g.* viable cells) because they interfere with the natural conditions. Bories *et al.* [9] first observed and discussed the influence of the nature of biological particulate sample components and channel wall surfaces on elution in GFFF. In that work, the surface properties of uncoated glass and glass coated with biocompatible silicone (more hydrophobic) in the GFFF analysis of blood samples were compared. The study concerned the elution of biological particles exhibiting different degrees of hydrophobicity (red blood cells, parasitic cysts).

Because of problems with the strong adsorption of blood sample components on uncoated glass surface and because a siliconized surface exhibits various interactions with proteins and cells [11], we tried to broaden the choice of surfaces by another modification of the channel wall surface. This modification was aimed at eliminating ionic and hydrophobic interactions as much as possible, to create a mild hydrophilic medium for treating living cells and to show that the novel surface also exhibits a decreased adsorption of cells and low-molecular-mass compounds.

2. Experimental

2.1. Materials

Methylene blue (Basic Blue 9) was purchased from Ciba-Geigy (Basle, Switzerland), benzoic acid, sodium dodecyl sulphate (SDS), cetylpyridinium bromide (CPB) and other chemicals (all of analytical-reagent grade) from Lachema (Brno, Czech Republic) and Tween 60 from Fluka (Buchs, Switzerland). Fresh foetal calf blood was obtained from the Veterinary School in Brno (Czech Republic) and human blood samples were obtained from patients under hospital control. EDTA solution (30 mg/ml of dipotassium EDTA) was used as an *in vitro* anti-coagulation and calcium-complexing substrate. This solution was added to blood in the ratio 9.1:90.9 (v/v). The carrier liquid for GFFF of blood samples had the following composition: 8.5 g of NaCl, 1.1375 g of $Na_2HPO_4 \cdot 12H_2O$ and 0.135 g of KH_2PO_4 in 1000 ml of redistilled water, pH 7.2).

2.2. Methods and equipment

The laboratory-made FFF channel was constructed as follows. A channel was cut in an 80-μm Celluloid foil (spacer) which was placed between two plates of mirror-quality float-glass clamped between two Plexiglas blocks. The dimensions of channel were 360 mm (length) × 20 mm (width) × 0.08 mm (thickness). The inlet and outlet capillaries (0.5 mm I.D.) were connected to a laboratory-made sample injector and a Spectra 100 UV–Vis spectrophotometric detector (Spectra-Physics, San Jose, CA, USA), respectively. A fused-silica capillary (0.3 mm I.D.), connected directly to the channel outlet, was used as a flow cell. For a schematic representation of the experimental arrangement see, *e.g.* ref. 3. The void volume of the system (from the injection inlet to the flow cell) was determined to be 587 μl (mean value of ten measurements of an unretained peak of benzoic acid at a flow-rate 50 μl/min). The absorbance of the eluates was monitored at 290 nm. The response of the detector was recorded on a TZ 4620 chart recorder (Laboratory Instruments, Prague, Czech Republic). The flow-rates ranged from 0.02 to 1.0 ml/min. All experiments were carried out at room temperature.

The samples were injected into the channel directly through a septum-equipped injector using a 10-μl syringe (Hamilton, Reno, NV, USA). Both particulate (cells) and soluble samples were injected into the continuous flow of the carrier liquid. At low flow-rates (about 0.1 ml/min and less), red blood cells were eluted in a clearly distinguished particle peak without any relaxation because of the sufficient time for sedimentation and achieving an equilibrium position in the flow velocity profile.

The sample concentration was adjusted as follows: methylene blue 0.01 mg/ml, benzoic acid 0.2 mg/ml and potassium dichromate 0.1 mg/ml. As regards blood samples, 10 μl of

sample diluted in the carrier liquid (the ratio of the whole blood with EDTA to carrier liquid was 1:20) were applied to a channel.

Two different modifications of the glass channel wall surface were used, as follows.

(a) The channel was filled with 1.0% (w/v) solutions of Tween 60, SDS or CPB. After conditioning for 2 h at room temperature, the channel was washed with distilled water for 5 min at a flow-rate 0.5 ml/min, conditioned with an appropriate carrier liquid (5 min, 0.5 ml/min) and then the sample was injected.

(b) The glass plates were treated using a fine brush with a 1% solution of polystyrene (average molecular mass 100 000) in toluene. The volatile organic solvent was allowed to evaporate at room temperature for *ca.* 30 min prior to assembling the channel. The channel coated with the polystyrene film was subsequently treated in the channel with Tween 60. After conditioning for 2 h with 1.0% Tween 60 the channel was flushed with water (5 min, flow-rate 0.5 ml/min) and washed with an appropriate carrier liquid (5 min, 0.5 ml/min).

3. Results and discussion

3.1. Untreated glass walls

During our experiments with various biological samples in glass wall channels, we observed unexpected and unpredictable adsorption phenomena, especially at lower flow-rates, resulting in chaotic fractograms. With blood samples, we obtained reproducible results after several consecutive experimental runs, presumably after sufficient coating of the glass surface with sample components. These results were in good agreement with those published previously [6,8]. However, there is a danger of ageing and unpredictable changes with such an uncontrolled coating. While testing the samples containing cells and some basic cell stains (*e.g.* methylene blue [12]) without other additives, we observed a retained dye peak. We found that under certain conditions (low ionic strength, pH ranging from acidic to neutral) we can obtain a retained peak

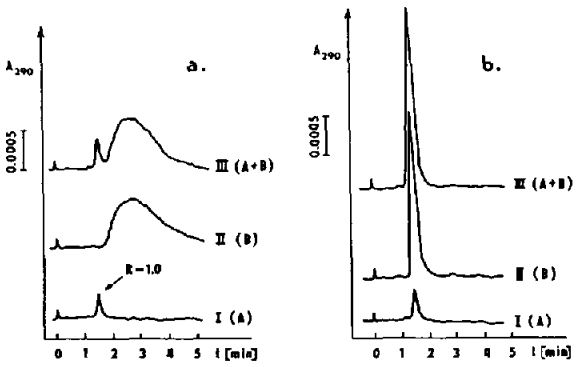

Fig. 1. (a) Comparison of fractograms of (I) benzoic acid (A), (II) methylene blue (B) and (III) a mixture of both (A + B). Untreated glass walls; flow-rate, 0.35 ml/min; injection without relaxation. (b) The same experiments as in (a) except that the walls were coated with a polystyrene–Tween 60 layer.

of the dye (see Fig. 1a). This is a result of ionic interactions between the positively charged basic dye and negatively charged glass surface [11,13]. The channel behaves like an open rectangular chromatographic column. This is an example of situation which could occur, *e.g.* in binding studies of soluble low-molecular-mass compounds and biological particulate matter.

3.2. Treated glass walls

We tried to modify and investigate the properties of the glass channel wall surface in two ways, as follows.

(a) By direct adsorption of detergents (non-ionic Tween 60, anionic SDS, cationic CPB) on unmodified glass walls [as described in Section 2.2, procedure (a)]. Among the detergents tested, Tween 60 showed the best results when the retention behaviour of methylene blue was monitored. However, the adsorption of the dye was not completely eliminated, *i.e.* the dye was never eluted in the void volume. In the first run after the modification channel, methylene blue was eluted at a retention ratio of 0.84 (at a flow-rate of 0.35 ml/min with water as a carrier liquid). Rapidly decreasing values of the methylene blue retention ratio and disappearance of the peak in subsequent runs indicated washing out of the detergent protective layer.

(b) By adsorption of a detergent (Tween 60) on pretreated (hydrophobized) channel glass walls [see Section 2.2, procedure (b)]. The procedure used for this treatment of the channel walls reflects two steps known in chromatography: (1) coating the silica surface with polymers to form a new homogeneous layer with well defined chromatographic properties (*e.g.* polystyrene coating of silica packings [14]) and (2) forming a surface by addition of soluble modifiers that meet the special conditions required for treatment of natural biological samples. Ideally, such a surface should be mechanically and chemically stable, hydrophilic and charge free. With polystyrene-based packing materials, a chemically bonded hydrophilic coating was created [15], but we chose a procedure analogous to that described by Chang [16] where Tween 60 was used to form a hydrophilic surface layer by hydrophobic interaction of its long alkyl chain with the diphenyl groups bonded on the silica surface. We took advantage of treating a smooth surface instead of a porous chromatographic packing to employ the simple modification technique of "lacquering" of the glass surface with polystyrene diluted in toluene. This technique enables a new homogeneous layer to be formed on the glass surface. When prepared with care, the layer retains the highly smooth character of the original glass surface. The Tween coating, formed as a result of strong adsorption of detergent on the polystyrene lacquer, can be easily renewed by washing out the residual Tween by a pulse of ethanol and re-adsorption according to procedure (b). In our experiments we renewed the Tween layer every day prior to starting the experiments. Other detergents can also be used in this way to form new surfaces. The surface properties can be checked, *e.g.* by observing the retention behaviour of suitable soluble marker compounds. No retention of methylene blue or benzoic acid was observed in the modified channel (Fig. 1b). No ionic interactions between soluble components and the channel surface were observed because nothing was eluted after a pulse of 2 *M* potassium chloride solution.

We also compared the behaviour of blood samples. In the polystyrene–Tween-treated channel even in the first run we obtained satisfactorily reproducible results. In the untreated channel, we obtained reproducible results only after several runs, which were necessary to form a suitable channel surface by adsorption of sample components, and the lowest acceptable linear flow-rate was 0.12 cm/s, because of strong sample adsorption at lower flow-rates. In the treated channel we could use a linear flow-rate of *ca.* 0.05 cm/s (with the same relaxation time of 1 min as in ref. 11) for both human and calf blood samples without observing undesirable adsorption effects (significant peak broadening and tailing). Moreover, we achieved reasonable results at the lowest flow-rate maintainable by our pump, which corresponds to a linear flow-rate of 0.035 cm/s without relaxation. Two fractograms recorded at low flow-rates (0.07 and 0.035 cm/s) are compared in Fig. 2. In both instances, the sample was injected into the channel without relaxation, because of a sufficient time for cells to settle and to achieve the equilibrium position in the velocity profile. Hence in this way we prevented flow-rate non-uniformities caused by switching the carrier liquid flow to the channel.

The modification of the glass channel walls by the above procedure allows reproducible results for the separation of biological particles to be

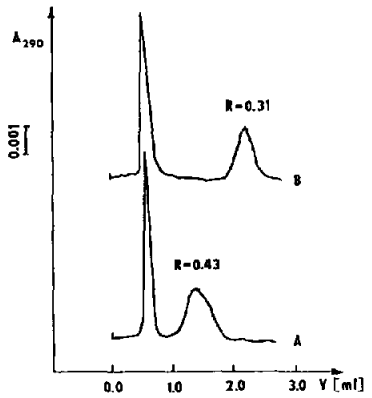

Fig. 2. Fractograms of calf foetal blood samples at two low flow-rates: (A) 0.07 and (B) 0.035 cm/s. Retention ratios of red blood cell peaks are indicated. Channel walls pretreated with polystyrene–Tween 60; injection of sample without relaxation.

obtained from the first run, undesirable ionic interactions of low-molecular-mass compounds to be minimized and very low flow-rates to be employed allowing elimination of relaxation time.

Moreover, the quality of the surface can be checked using suitable reagents (methylene blue and a pulse of concentrated salt), the detergent layer can be quickly renewed and also the polystyrene lacquer can be easily replaced in the event of damage (*e.g.* during channel dismantling), because the whole procedure is very simple.

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5. References

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