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Highly Selective Protein Separations With Reversed Micellar Liquid Membranes

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CORRESPONDENCE

Highly Selective Protein Separations with Reversed Micellar Liquid Membranes

Sir: The separation and purification of proteins continue to be areas of great interest and intense investigation. This area of separation science has received impetus from the expanding fields of biotechnology and genetic engineering (*1-10*). Over the last decade, liquid chromatography (LC) has emerged as an important technique for the analysis of biologically important molecules. It has been used for separating closely related as well as disparate proteins, determination of structural form, peptide mapping, and so on (*11*, *12*). The various theoretical and empirical shortcomings of LC have been documented as well (*7*, *8*, *13*). From a practical standpoint, LC is a fine analytical tool for the analysis of whole proteins but is often inadequate for quantitation and preparative scale separations. Some of the problems result from the nature of the solute (i.e. the protein) while others result from the nature of the technique (i.e. LC). For example, problems of protein denaturation and irreversible adsorption are well-known and much work has been done to minimize these (*7-13*). Also, LC is a batch process, which requires large amounts of support and mobile phase for moderate-sized preparative separations. An ideal system for the preparative separation of proteins would be compact, have the efficiency and selectivity of LC, the ability to operate in a continuous mode and easily handle large quantities of protein with little or no denaturation. Currently no such technique or system exists.

In the late 1970s Luisi and co-workers (*14*, *15*) and Menger and Yamada (*16*) demonstrated that proteins could be solubilized in nonpolar organic solvents without adversely affecting their conformation or enzymatic activity. This was done by using certain reversed micelle forming surfactants. Recently there have been attempts to utilize reversed micelles in the separation and purification of proteins. Göklen and Hatton took advantage of this phenomenon and demonstrated that significant amounts of protein could be separated and purified with no denaturation by using solvent extraction with reversed micelle containing organic solvents (*17*, *18*). The apparent selectivity of this method was impressive as was the ability to maintain the activity of the protein.

Membrane-based separations are easily configured to operate in a continuous, preparative-scale mode. Unfortunately, most membrane techniques lack the selectivity necessary for many important separations including that of proteins. In this work we examine reversed micellar liquid membranes and show that they can be used to efficiently separate proteins while maintaining selectivities currently found in better-known methods. Problems of denaturation during isolation and purification often are minimal.

EXPERIMENTAL SECTION

Materials. Aerosol OT (AOT), also known as sodium dioctylsulfosuccinate, was obtained from American Cyanamid Co. The proteins cytochrome *c* (horse heart), myoglobin (horse heart), and lysozyme were obtained from Sigma and used as received. Bovine serum albumin (BSA) was obtained from Advanced Separation Technologies. HPLC grade water and *n*-hexane were obtained from Fisher Scientific.

Methods. A schematic of the membrane chamber used in the protein transport studies is shown in Figure 1. The chamber was filled with 4.0 mL of 0.1 M NH_4OAc (adjusted to the desired pH) and different concentrations of KCl (from 0.05 to 0.20 M). The liquid membrane (0.2 mL) was injected into the connecting tube (Figure 1) with a syringe connected to a 5-cm piece of microbore Teflon tubing. The reversed micellar liquid membrane was made by dissolving the desired amount of AOT in *n*-hexane and presaturating this with the protein solution to be separated. Each experiment began by adjusting the KCl concentration as desired and placing 10 mg of protein in one side of the chamber (henceforth referred to as the mixture side). The protein concentrations on the receiving side were determined by UV spectrophotometry, using a Perkin-Elmer 559 instrument. BSA and lysozyme were quantitated at 280 nm and cytochrome *c* and myoglobin were quantitated at 408 nm. Equilibration times of 24 h were used in this study. More intensive data on transport rates and large scale competitive separations are to be presented in a more extensive future article. Both UV and circular dichroism (Jasco 20) spectrophotometry were used to monitor all proteins for conformation changes and denaturation.

RESULTS AND DISCUSSION

There are at least three basic phases that must be consid-

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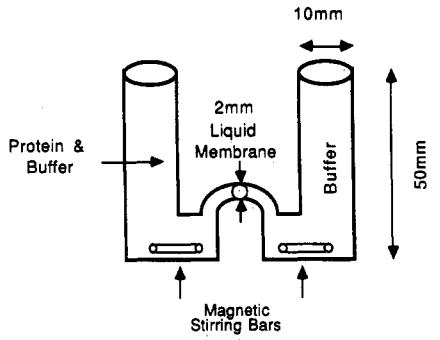

Figure 1. Illustration of the glass diffusion cell for the separation of proteins with reversed micellar liquid membranes.

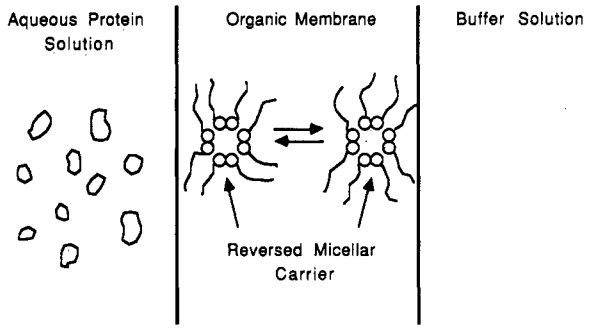

Figure 2. Simplified illustration of the reversed micellar liquid membrane through which the selective permeation of proteins occurs.

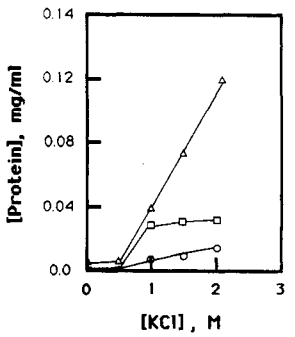

Figure 3. Plots showing the effect of KCl concentration on the permeation of cytochrome *c* through a reversed micellar liquid membrane at pH 7.0. Each point represents a measurement after 24 h of equilibration. The *x* axis gives the concentration of KCl on the receiving side. The concentration of KCl on the "mixture" side is 0.05 M (△), 0.1 M (□), and 0.2 M (○).

ered and controlled in any membrane separation process. One is the solution of the impure mixture, second is the membrane, and the last is the receiving solution to which the desired component is transferred (Figures 1 and 2). There are at least seven chemical factors that control the selectivity and efficiency in the reversed micellar membrane system. They include, the ionic strength of both the mixture and receiving sides, the pH of the mixture and receiving sides, the size of the reversed micelle, the charge of the reversed micelle, and the nature of the membrane solvent. Other factors such as membrane thickness, temperature, and so on are also important but will not be considered in this particular communication.

Figure 3 shows the effect of ionic strength (at constant pH) on the penetration of cytochrome *c* through a reversed micellar membrane. The concentration of KCl was varied on the receiving side (*x* axis of Figure 3) and on the mixture side. It is evident that the greater the difference in ionic strength across the membrane, the greater the protein transport. Protein transport against or in the absence of KCl gradient is small but finite, particularly at the higher salt concentrations

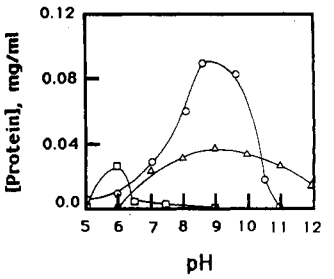

Figure 4. Plots of the effect of pH on the permeation of cytochrome *c* (○), lysozyme (△), and bovine serine serum albumin (□) through a reversed micellar membrane. The [KCl] was 1.0 M on both sides of the membrane. Each point represents a measurement after 24 h at equilibrium. The plot for myoglobin is not shown because all of the experimental points fall in the crowded region of this figure between pHs 5 and 7 and below 0.04 mg/mL proteins.

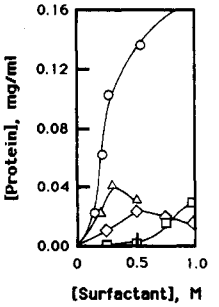

Figure 5. Plots showing the effects of AOT concentration in the liquid membrane on the permeation of cytochrome *c* (○), lysozyme (△), myoglobin (◇), and bovine serum albumin (□). The pH (7.0) and [KCl] (1.0 M) were held constant on both sides of the membrane. Each point represents a measurement taken after 24 h of equilibration.

(Figure 3). Finally at very high concentrations of salt in the receiving side, protein transport eventually becomes constant. Analogous behavior was seen for all of the proteins in this study.

Figure 4 shows the effect of pH on the transport of four different proteins when the salt concentration is held constant. The ability of different proteins to penetrate the membrane is highly pH dependent. The maximum transport of bovine serum albumin occurs at pH of about 5.8 while that of lysozyme occurs at pH 9. At pH values >11 lysozyme will transport across the membrane while all other proteins are relatively retained. Conversely, myoglobin and BSA traverse the membrane in significant quantities only at pHs between 5 and 7. Each protein has a unique pH profile. This profile can be maximized, shifted, and further optimized by adjusting the ionic strength as discussed previously.

Figure 5 shows the effect of AOT concentration in the membrane on protein transport. The amount of surfactant present is one of several factors that control the size of the reversed micelle (*19*). When other factors are held constant (e.g. pH, ionic strength, and membrane solvent), the amount AOT in the membrane controls protein transport. For example, bovine serum albumin will not permeate the membrane in significant amounts unless the AOT concentration is well over 0.5 M (Figure 5). Both lysozyme and cytochrome *c* can permeate the membrane at AOT concentrations (0.25 M) that inhibit the transport of myoglobin and BSA. There also appears to be an optimum concentration of AOT, above which protein permeation decreases (Figure 5).

Several things are noteworthy about the reversed micellar membrane system. The ability to selectively transport one protein while excluding all others is particularly encouraging. Also important is the fact that the selectivity can be changed and controlled in a logical manner (vide supra) to effect the separation of all species. Interestingly, membrane transport

was observed for larger proteins such as bovine serum albumin even though these species could not be separated via liquid–liquid extraction in a related system (*17*, *18*). This may be due to the fact that membrane-based systems can utilize facilitated or coupled transport. Under the conditions of this study, no significant protein conformational changes or denaturation was observed for solutes on either side of the membrane (see Experimental Section). Further work is currently under way involving more complex protein mixtures and matrices as well as on the use of different membrane configurations and types.

Registry No. AOT, 577-11-7; cytochrome *c*, 9007-43-6; lysozyme, 9001-63-2.

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Simple Jet Electrodes for Kinetic and Synthetic Purposes

Sir: A jet electrode (JE) consists of a stationary working electrode with hydrodynamic mass transport achieved by impingement of a jet stream normal to the electrode surface. The theory and use of jet electrodes (wall jet electrode, wall tube electrode) has been well-documented (*1–9*). Techniques involving JE's are analogous to those involving rotating disk electrodes. Jet electrodes with nozzle diameters greater than the electrode diameter have uniform accessibility and are thus suitable for mass-transport-controlled analytical applications.

Previous accounts describe the use of JE's that have employed elaborate pump systems and sealed electrochemical cells. We describe a JE that employs simple and inexpensive equipment. We have used this JE to perform kinetic measurements on semiconductor electrodes (*10*). The pump is a commercial veristaltic pump. The electrochemical cell used was nonsealed and of low volume. The cell geometry could be easily adapted for use with almost any solid electrode. The system might be of interest to electroanalytical laboratories for routine use as an efficient stirring device or, with modifications, as an analytical device.

EXPERIMENTAL SECTION

Materials. Acetonitrile (ACN, Burdick and Johnson, UV grade, dried by refluxing over phosphorus pentaoxide) and 0.1 M tetrabutylammonium fluoroborate (TBAFB, Southwestern, ground and dried under vacuum for 2 days) was the electrolyte solution. Ferrocene (Aldrich) was purified by sublimation.

Equipment. The core of the system was a veristaltic pump (Junior Model, Manostat) fitted with $^1/_4$- or $^3/_8$-in.-o.d. silicone rubber pump tubing. Nozzle and exit tubes to the cell were made of Teflon tubing. Plumbing made use of HPLC fittings. A glass tubing to HPLC fitting connector (Alltech, no. 20060) was modified with a raised ridge and inserted into the silicone tubing. Heat-shrink tubing was used to keep the tubing from slipping off. No pulse dampener was used in our studies.

Two cells of different sizes were used. They were both of similar design, consisting of a glass beaker (50 or 100 mL) with a Teflon electrode cap (Figure 1). The total cell volumes (including tubing) for the two cells were about 10 or 25 mL. The larger cell, which had a diameter of 3.6 cm and a solution depth of 1.5–2.0 cm, was used to obtain the voltammetric and electrolysis data reported here.

Electrodes were all constructed with 6-mm glass tubing. The tubing fit snuggly into the holes of the Teflon cap to prevent rotation of the electrodes during jet operation. The reference electrode was $Ag/AgNO_3$ (0.01 M), TBAFB (0.1 M), ACN. The frits were made of Vycor disks. The inlet and outlet tubes were also 6-mm glass tubing. The nozzle inlet tube had a 90° bend on the end so the jet streamed horizontally into the electrode. The nozzle of the jet was the unmodified end of the Teflon tubing.

Nozzle Velocity Determination. Mean flow rates (cm^3/s) for the veristaltic pump obtained with exit tubes of diameters 0.086, 0.17, and 0.27 cm were measured with a graduated cylinder and stop watch while using water as a solvent. Mean nozzle velocities were calculated from the flow rates divided by the cross sectional area of the nozzle.

Voltammetry and Electrolysis. All electrochemical experiments utilized a PAR 173/179 potentiostat/coulometer and positive *iR* compensation.

Current–potential curves were recorded by using a sealed platinum-wire working electrode having a radius (*R*) of 0.067 cm. The wire was sealed in the end of a bent glass tube as depicted in Figure 1 and polished with alumina.

Bulk electrolysis were performed with a platinum-gauze working electrode with dimensions of 1.5 × 4.5 cm. The current was monitored with an x–y recorder (Houston, 200), and the total charge was recorded when the current decayed to 1% of its initial value.

The jet was aimed directly at the working electrode with the pump at the highest speed for both the I/E curve and the bulk electrolysis.

RESULTS AND DISCUSSION

The most remarkable feature of the system is the nonsealed, low solution volume cells employed. The solution did not vortex head gas except in instances where the nozzle stream was directed at the outlet. It was possible to position the outlet to within 1 mm of the electrode without vortex problems. The cell and pump geometry is shown in Figure 1.

Flow rates were measured with water for the different tubing diameters, *d*, listed in Table I and range from 12 to

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