CELL PARTITION IN TWO PHASE POLYMER SYSTEMS

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Aqueous phase-separated polymer solutions can be used as support media for the partition of biological macromolecules, organelles and cells. Cell separations using this technique have proven to be extremely sensitive to cell surface properties but application of the systems are limited to cells or aggregrates which do not sediment significantly while the phases are settling. Partition in zero g in principle removes this limitation but an external driving force must be applied to induce the phases to separate since their density difference disappears. We have recently shown that an applied electric field can supply the necessary driving force. We are proposing to utilize the NASA FES to study field-driven phase separation and cell partition on the ground and in zero g to help define the separation/partition process, with the ultimate goal being to develop partition as a zero g cell separation technique.

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INTRODUCTION

Much of modern biomedical research is directly aimed at defining and elucidating the normal and pathological activity of living systems. A problem in such work is frequently encountered when attempts are made to prepare the specific cell population of interest in a pure state. Non-specific preparation techniques based on cell size or density are seldom sufficiently sensitive, as the total range of these parameters encountered among biological organisms is relatively narrow. Separation methods based upon cell surface properties hold more promise, however, since a cell's function and its ability to interact with other cells in its immediate environment appear frequently to be reflected in characteristics of the cell membrane. One such characteristic which is being exploited for preparative purposes is cell surface charge as detected by electrophoresis. Free-flow electrophoresis is capable of spatially distributing a cell population on the basis of the net charge density located on the exterior of the cell membrane. An even more sensitive separation technique is available, however, which also depends partly on the surface charge but which has been shown to be capable of separating cell populations which are electrophoretically indistinguishable.

When aqueous solutions of two different polymers are mixed above certain concentrations they usually form immiscible, liquid, two-phase solutions. Each of these phases generally consists of more than 90 percent water and can be buffered and made isotonic by the addition of low molecular weight species. If a cell or particle suspension is added to such a system, then shaken, the cells--upon re-equilibration--are frequently found to have partitioned unequally between one of the phases and the interface. This preferential

partition behavior can be used as the basis of a separation procedure for differing cell populations since partition in these systems is determined directly by cell membrane properties (1,2).

Cell populations which have related, but not identical, surface properties seldom exhibit sufficiently different partition behavior to be separated in a single extraction. In such cases, sequential partitions are carried out via countercurrent distribution (CCD) to effect the separation. CCD in phase systems derived from dextran/polyethylene glycol (PEG) mixtures has proven to be an extremely sensitive and valuable preparation technique in cell biology. Erythrocyte populations can be separated on the basis of cell age by CCD, for instance (3). This separation cannot be accomplished by preparative electrophoresis (4). The leukocyte fractions from various mammalian species have been fractionated via CCD (5,6) as have mouse spleen cells (7) and a mouse leukemic cell line (8,9). Human lymphocytes can be sub-fractionated this way into sub-populations which vary dramatically in their T:B:null cell ratios and in their responses to various mitogens (10). Cells from other organs also distribute into sub-populations according to maturity after CCD. Rapidly regenerating rat liver cells, for instance, have a higher partition coefficient than normal liver cells (11). Similarly, the relative position of rat epithelial cells in a CCD curve depends strongly on cell age and location in the epithelium (crypt or villus) (12). CCD has therefore produced some very interesting and useful cell separations.

The effectiveness of CCD as a separation procedure resides in the fact that the partition coefficient, K, is sensitive to a variety of cell surface characteristics. Moreover, K should depend <u>exponentially</u> on the relevant surface properties, as may be seen from the approximate thermodynamic expression (13):

$$K = n^{t}/n^{if} = C \exp(1/kT(\Delta G_{el}-(\gamma_{cb}-\gamma_{ct}-\gamma_{tb})^{2}A/4\gamma_{tb}))$$

where: n^{t} = number of cells in top phase
n^{if} = number of cells adsorbed at interface

C = constant, independent of cell properties

ΔG_{el} = difference in electrostatic free energy of cell when adsorbed at interface and when in top phase

γ_{cb} = interfacial free energy of cell in bottom phase

γ_{ct} = interfacial free energy of cell in top phase

γ_{tb} = interfacial tension between top and bottom phases

A = cell surface area

k = Boltzmann's constant

T = absolute temperature

The electrostatic free energy term occurs because in the presence of some salts a stable Donnan potential exists between the bulk phases (vide infra) which affects the distribution of charged particles. ΔG_{el} will in general be directly proportional to the cell surface charge density although its exact form will depend on the shape of the potential profile across the interface, which is complex. The partition coefficient therefore depends exponentially on the cell surface charge, in contrast to the linear dependence of electrophoretic mobility on charge density. This fact accounts in part for the relatively higher sensitivity of CCD over preparative cell electrophoresis.

The above expression also indicates that cell charge is not the only parameter determining K. The interfacial free energy of the cell/solution interface also can play a strong role. This free energy will be determined by the intrinsic nature of the interaction between the cell membrane and the phase polymers and the degree to which each of them adsorbs to the cell surface. This adsorption will lower the free energy between the polymer-coated cell and the phase in which that polymer predominates. The competitive adsorption of the two polymer species depends in turn on the chemical nature of the polymers and on a variety of cell membrane properties. Few of these membrane properties have been identified as yet, but in a PEG/dextran system having no phase potential difference there is good evidence that PEG-membrane interactions become stronger and partition into the PEG-rich phase higher as cells with a greater ratio of polyunsaturated to mono-unsaturated fatty acids in the membrane lipid are partitioned (2). The chemical composition and

structure of the membrane—independent of surface charge—can, therefore, determine partition behavior as well.

A strength of the partition approach to cell separation is that the conditions which determine the value of K are mainly under experimental control. Hence, the cell characteristic on which the separation is to be based can be made the dominant determinant of K by appropriate choice of operating conditions. The potential difference which appears between the two bulk phases, referred to earlier, is caused by the slight preference of some salts (sulfates, phosphates, citrates) for one phase over the other. By manipulating the ionic species and concentrations in the system, then, the magnitude of this potential difference can be controlled. Likewise, by varying the chemical character of the phase polymers themselves, γ_{ct}, γ_{cb} and γ_{tb} can be changed and the interfacial free energy term made dominant. In particular, if appropriate polymers are used, affinity ligands such as antibodies or haptens can be covalently bonded to one of the phase polymers. Cells bearing the specific structure to which the affinity ligand is directed will be preferentially coated with the substituted polymer and the cells will partition into the phase rich in that species. The phase can, therefore, act as a support medium for the affinity ligand, one which has much greater access to the cell surface than the gel beads commonly used. Separation on the basis of an extremely wide variety of membrane characteristics is therefore possible depending on the choice of phase system.

LIMITATIONS OF PARTITION ON EARTH

Countercurrent distribution of cells has been applied with great success to relatively small biological cells, such as erythrocytes and lymphocytes. However, there are a variety of cell types such as megakaryocytes, many tumor cells and tissue culture lines which are too large and/or dense to be separated successfully on earth. Such cells do not remain in suspension long enough to allow the phases to separate and permit a transfer along the countercurrent train.

In a one g environment, the partition of cells in phase-separated aqueous polymer systems is not an equilibrium process. Although the phases themselves can readily be brought to equilibrium, the distribution of partitioned material is time dependent due to sedimentation. Only after all the cells have sedimented to the phase boundary or the bottom of the container will the distribution be stationary—and then of no use. The degree to which the non-stationary nature of the distribution affects the usefulness of partition depends on the sedimentation rate of the cells in the appropriate phase, that is, on the cell size, shape, and density and on the phase density and viscosity. Since cell partition usually occurs between the top, PEG-rich phase and the interface, large cells or cell clumps will sediment into the interface from the top phase during the time it takes for the phases to separate, distorting the true, equilibrium distribution. If CCD is performed on such suspensions, the material transferred as the top phase will not contain all the cells that belong in that phase and the bottom phase will contain the cells that have sedimented into the interfacial region as well as those that are truly adsorbed there. This gravity-driven accumulation has two related effects on the CCD. First, it reduces the resolution of separation for all cells with a finite sedimentation rate; the CCD curves obtained for most cell types will therefore represent only a fraction of the separation in principle possible. Secondly, it eliminates the use of this separation technique entirely for cells which are so large they sediment as rapidly as the phases settle. Nominally, the critical cell diameter for this effect is 24 microns. There is a variety of cell types of clinical and fundamental interest which are larger than this, however. Carrying out partition studies and CCD under low gravity conditions would eliminate the cell size constraint because sedimentation would be insignificant.

PHASE SEPARATION AND PARTITION IN A REDUCED GRAVITY ENVIRONMENT

Countercurrent distribution with two phase aqueous polymer systems can be successfully applied on earth because phase separation occurs sufficiently rapidly that the 60 to 120 transfers necessary for many separations can be accomplished in a reasonable length of time. Under the best conditions, using

a thin layer CCD apparatus to minimize the phase thickness, it takes about five minutes for dextran/PEG phases to separate, resulting in run times of up to 10 hours. Phase separation at one g is driven by two mechanisms:

1. convective forces caused by the density difference between the phases;
2. interfacial free energy which tends to minimize the interfacial area between the phases.

In a low g environment the convective forces will be too small to produce phase separation in an acceptable length of time. Moreover, it is known that the interfacial tensions developed in these systems are also too small (10^{-2}-10^{-4} dyne cm^{-1}, (14)) to be effective in driving separation. This conclusion is based on the behavior of dextran/Ficoll phase systems which can be made with phases of equal density; such systems take many hours to separate after mixing. An external driving force must therefore be applied if partition experiments are to be carried out in zero g.

The approach we have taken to producing phase separation by an external force involves the application of a small electric field across the system. This technique was suggested by the observation (15) that droplets of one phase suspended in the other had an easily measurable electrophoretic mobility. Moreover, the mobility was found to be a linearly increasing function of droplet size, up to at least 15 μm in diameter. The phase systems therefore follow to some extent the behavior predicted by Levich (16) and Levine's (17) theory of the electrophoresis of mercury drops in ionic solutions which predicts such a size dependence, although the mechanisms of charging must be different in the two systems. The sign of the mobility inverts depending on which of the two phases is dispersed (i.e., depending on which side of the interface is externalized).

The effect is illustrated in Figure 1 where a phase system made up of 5% dextran (M_w = 5 x 10^5) and 4% PEG (M_w = 6 x 10^3) in 0.2M K_2SO_4 has been examined. The system was allowed to equilibrate then a small volume of top, PEG-rich phase was dispersed in a large volume of bottom phase. The

sizes of individual top phase drops were measured visually and their electrophoretic mobilities (T/B) determined at 25°C via analytical particle electrophoresis. The phase volume ratios were then reversed and the mobilities of bottom phase, dextran-rich drops suspended in top phase (B/T) also determined as a function of drop diameter. The results, plotted in Figure 1, show that the mobilities of both types of drops vary linearly with diameter. If the slopes of the mobility-diameter plots for T/B and B/T drops are compared their ratio is 1.28. If it is assumed that the magnitude of the surface potential is equal and opposite for each type, Levich's theory predicts this ratio ought to be given, under our conditions of relatively low conductivity, by:

$$(2\eta_t + 3\eta_b)/(2\eta_b + 3\eta_t)$$

where η_b = bottom phase viscosity
η_t = top phase viscosity

Substituting in the appropriate values gives a value of 1.39, in reasonable agreement with experiment.

It should be clearly pointed out that the electrophoretic mobilities of T/B and B/T drops are of opposite sign. Application of an electric field across a phase system which has been mixed to form an emulsion ought to induce phase separation, therefore, since drops of the two phases will move in opposite directions.

The principle of electric field driven phase separation has recently been demonstrated. A phase separation chamber across which a known electric field can be applied was constructed by the Advanced Technology Operations Division of Beckman Instruments. The apparatus consists of two electrode chambers containing bright Pt electrodes separated from a phase chamber (5 cm L x 0.5 cm W x 0.2 cm deep) by two Amicon XM-100 membranes. Feeder ports give access to the chamber for filling and drainage. Electrode rinse buffer is circulated through the upper and lower electrode chambers to remove electrode reaction

products. The chamber and electrode assembly is made of poly(methyl methacrylate) lapped and polished to provide a good seal between the upper and lower halves. The optical system used to follow phase separation turbidimetrically consists of a small ruby laser whose beam diameter is limited to 0.03 cm by an entrance aperture. The beam traverses the width of the phase chamber at a vertical position which can be adjusted relative to the midline. The beam intensity is measured with a solid state detector and amplified after traversing the chamber and an 0.03 cm diameter exit aperture.

When a mixed turbid phase system is introduced into the chamber most of the light is scattered off the optical axis and the photodetector output is low. As the phases separate, the upper and lower phases clear, the scattering decreases and the detector output increases with time, reflecting the kinetics of separation. Experiments may be run in the presence or absence of the electric field and the kinetics readily compared.

Using this apparatus we have demonstrated field-driven phase separation in a system consisting of 7.5% dextran (M_w = 4 x 10^4), 4.5% PEG (M_w = 6 x 10^3) and 0.1M K_3 citrate. The lower molecular weight dextran was used to reduce the phase viscosity and increase mobility. With an applied field of E = 0, at a phase volume ratio of 9:1 (top:bottom) the initial rate of clearing is about 0.12% per minute. With an applied field E = 6 V cm^{-1} the rate increases to 1-2.5% per minute. Phase separation therefore takes place about an order of magnitude more rapidly in the presence of the field than in its absence at this ratio of bottom to top phase volume.

Applying an electric field to a system containing cells to produce phase separation in the absence of gravity will also result in motion of the cells, in this case by electrophoresis. In all the phase systems studied to date, including all those used in cell separation work, the sign of the phase drop mobilities are such that top phase drops bear a negative surface potential so the anode is placed at the top of the chamber. Since cells in physiological media all bear a net negative surface charge, they will also migrate upwards—away from the interface—when the field is applied. Cells will therefore tend

to move out of that region above the interface which is retained with the bottom phase when a CCD transfer is made. This motion will therefore have the opposite effect on the partition coefficient to that of cell sedimentation and, in principle, effective partition coefficients approaching the ideal values should be realized.

While most of the principles discussed above have been demonstrated in terrestrial experiments, examination of phase separation, electric field effects and cell partition in a reduced gravity environment has yet to be carried out. It is hoped that through the use of the Fluids Experiment System these concepts can be tested and developed sufficiently fully that low g partition separations of cell populations of biomedical interest will become feasible.

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FIGURE LEGENDS

Figure 1. Absolute values of electrophoretic mobilities of phase drops as a function of drop size. See text for description of system. Mobilities of drops of top phase suspended in bottom phase (T/B) have a negative sign while those for bottom phase drops in top phase (B/T) are positive. T = 2.5.0°C.