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Carel J. Van Oss[a]
[a] DEPARTMENT OF MICROBIOLOGY, STATE UNIVERSITY OF NEW YORK AT BUFFALO BUFFALO, NEW YORK

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SYMPOSIUM ON NEW METHODS OF SEPARATION (*continued*)

Electrokinetic Cell Separation

CAREL J. VAN OSS

DEPARTMENT OF MICROBIOLOGY
STATE UNIVERSITY OF NEW YORK AT BUFFALO
BUFFALO, NEW YORK 14214

Abstract

Particles of the size of living cells are too big to be separated in stabilizing media such as gels. The separation thus has to be done while they are freely suspended in a buffer contained in a vessel of appropriate shape. The major two disturbing factors under these conditions are cell sedimentation and electroosmotic backflow along the walls of the vessel.

Cell sedimentation can be overcome (a) by measuring very small cell transport paths, along which the cells have no time to sediment, using a microscope; (b) by applying a continuous liquid flow perpendicular to the direction of separation; (c) by stabilizing the cells in a density gradient; (d) by ascending electrophoresis, in the direction opposite to that of gravity; (e) by using a column packed with beads of a homogeneous size; and (f) by doing the separation at zero gravity, in outer space.

Electroosmotic backflow. This can (a) be overcome by coating the vessel-walls with a material of 0 ζ potential; and (b) be managed by forcing it into a completely uniform flow by coating the vessel walls with a material of non zero ζ-potential and by providing both ends of the vessel with porous plugs made of the *same* material.

INTRODUCTION

Only the electrophoresis of the largest units that can undergo this process to any useful purpose has to contend with the detrimental action of both sedimentation and electroosmosis.

All particulate and soluble materials of a dimension smaller than a few hundred nanometers can be stabilized by gels (*1*). The use of gels or

porous blocks obviates all sedimentation and most convection problems, while the proper choice of gel pore size can generally eliminate the retardation of particles or molecules due to the exiguity of the gel's pores (*2*). Electroosmotic backflow is, of course, by no means eliminated by the use of gels or porous blocks, but the electroosmotic flow in gels or blocks is completely uniform and is thus of small importance in preparative separations, while it can easily be quantitatively taken into account in analytical electrophoretic determinations.

Thus only those particles that are too large to be stabilized by gels are subject to the twin disturbing actions of sedimentation and electroosmosis. The most important of such particles in the field of biology undoubtedly are living cells. In microscopic electrophoresis of living cells, sedimentation is no problem, due to the fact that cells need only be observed for a few seconds, during which they migrate only a few micrometers and have no opportunity to sediment to any extent. In the classic microelectrophoretic method, however, electroosmotic backflow along the walls of the capillary is the great drawback. On the other hand, in preparative cell electrophoresis, although electroosmosis may play a certain role, the major impediment is the strong tendency of all cells to sediment to the bottom of the electrophoresis vessel within the time required for the separation.

SEDIMENTATION

Microelectrophoresis

As already mentioned above, cell electrophoresis during very brief time spans over very short pathways does not give rise to a noticeable sedimentation. The great problem here is the electoosmotic backflow, which is treated below.

Continuous Flow Electrophoresis

Continuous flow electrophoresis is a fairly complicated process in which cells are suspended in a steadily flowing stream of liquid, perpendicular to which an electric field is applied. When all parameters are well controlled, a steady state can be reached, under which the cells separated by the electric field while being transported in the flowing liquid vein, continuously arrive at the same exit ports, according to their particular electrophoretic mobility. Electroosmosis plays a considerable role in this method, which makes it only possible to focus sharply one fraction at a

time (see below). Nevertheless, by this method some important cell separations have already been accomplished (*3–7*). Endless belt electrophoresis, developed by Kolin (*8*), is another variant of continuous flow electrophoresis.

Stabilization in a Density Gradient

It is, of course, theoretically possible to levitate any object heavier than water by adding solutes to the water until a density is attained that is close to that of the object to be levitated. In practice, a density *gradient* rather than a uniform high density is more generally utilized, because such a gradient also has a stabilizing power against minor thermal and other convections. Nevertheless, and in particular for cells, which need stabilization most, the presence of high concentrations of solute in the water unfortunately also tends to give rise to strongly hypertonic osmotic pressures. In order to obviate this, high-molecular-weight materials have been proposed for the elaboration of density gradients, e.g., by Boltz et al. (*9*), who after thorough analysis of the available macromolecules have chosen a cross-linked dextran of molecular weight 400,000 for the purpose (Ficoll, Pharmacia, Piscataway, New Jersey). For some cells, however, such high-molecular-weight polymers cannot be used, as they give rise to severe cell aggregation. However, we have found that lymphocytes, which are among the more important cell types that are in need of electrophoretic separation, will not aggregate when the lowest available molecular weight of dextran is used, i.e., a linear dextran of a molecular weight of 10,000 (Dextran T–10, Pharmacia, Piscataway, New Jersey) (*10*). Nevertheless, even with the lower molecular weight of polymers, their concentration in a density gradient is generally such that a considerable amount of polymer adsorption onto the cell surface must be feared, giving rise to a change in ζ-potential which, of course, complicates the intended electrophoretic separation.

Ascending Electrophoresis

We recently observed that in the total absence of a density gradient, ascending electrophoresis of cells can be practiced in vertical tubes (*10*). By this method it has proved possible to separate human lymphocytes into three groups of different electrophoretic mobilities. Upon once or twice repeated reelectrophoresis of the fastest group, virtually pure *T* cells could be obtained in quantities of the order of 10^7 cells. Isolation of the elec-

trophoretically slower B cells is somewhat more complicated because the slowest moving fraction is also the one that remains most contaminated by the few cells that form aggregates and thus are heavier and tend to remain close to the bottom of the tube. Fortunately, B cells are not only the slowest ones electrophoretically speaking but also have the greatest tendency to aggregate. Nevertheless, upon aggregation some T cells apparently remain entrapped. The electrophoretic purification of B cells therefore is strongly linked to the problem of complete avoidance of cell aggregation.

Columns Packed with Beads of a Homogeneous Size

To separate cells that are all of virtually identical size but that consist of groups of different electrophoretic mobilities, such as erythrocytes, tubes packed with glass beads of homogeneous size can be used with advantage. To prevent the cells from sticking to the glass beads, the beads have to be treated with polyoxyethylene. In order to avoid the large interstices that tend to occur between the glass beads and the wall of the tube, the wall of the tube should be coated with a gel (agarose, for instance) in which the glass beads, after being packed into the column, can partly imbed themselves. An additional advantage of such an inner gel coating is that after the electrophoresis the entire column can be extruded and sliced in order to obtain the various fractions. We found that the optimal diameter of the glass beads for the separation of human erythrocytes is approximately 100 μm (or 100–140 mesh). In a homogeneously packed tube the diameter of the smallest spherical particle that can just be retained in the beads' interstitial spaces is approximately $15\frac{1}{2}\%$ of the diameter of the glass beads themselves (*11*). However, for cells that occur in families of various sizes, this method cannot be used, as the larger cells will remain entrapped in the interstitial spaces while the smaller cells will too readily be able to sediment unhindered through these spaces.

Electrophoresis at Zero Gravity in Outer Space

When gravity can be entirely abolished, sedimentation no longer occurs. That condition can only be maintained for any length of time in outer space. For that reason a series of experiments has been done, and others are in advanced stages of preparation, for electrophoresing cells in outer space. An experiment in Apollo 16 with two different sorts of latex particles showed that sedimentation was indeed entirely abolished at zero gravity

(*12*), although electroosmosis still was an important problem in that experiment. More recently the electrophoretic separation of red blood cells by isotachophoresis has been attempted in Skylab 3 (*13*), while further electrophoretic separations of erythrocytes as well as of lymphocytes and other cells are projected for the impending Apollo-Soyuz flight.

ELECTROOSMOSIS

Zero ζ-Potential Coatings

Glass walls generally have a considerable ζ-potential. But there exist various techniques for covalently binding a variety of compounds of much reduced or even of zero ζ-potential to glass (*14*). In particular, the use of proteins of the appropriate isoelectric point seems promising. Proteins can be covalently bound to glass (*15*). A treatment for agarose has recently been published that reduces its ζ-potential to zero (*16*). With such treated agarose gels, glass tubes may be coated and in that fashion their electroosmosis can be abolished. Tubes made of various plastics frequently will be found to have a much lower ζ-potential than glass and can often be used without any further coating or precaution. When, however, material such as protein is present in solution together with the cells, it must be kept in mind that most plastics quite readily adsorb protein (*17*) and many other materials and will thus acquire the ζ-potential of the material that they adsorb. However, in a protein-free medium, plastics as construction material for electrophoresis tubes may be quite satisfactory, at least for preparative purposes.

Uniformization of the Electroosmotic Backflow

In microelectrophoresis with closed capillaries the particular drawback of electroosmosis is that it is very strong along the side of the wall of the capillary, so that the mobilities found for cells or particles differ tremendously according to the exact place inside the capillary. Numerous electrophoretic mobilities of particles must therefore be plotted as a function of the depth of focusing in the capillary. On the parabola thus obtained, the true electrophoretic mobility can be found by interpolation (*18*). This process is not only lengthy but also gives rise to various inaccuracies.

We have succeeded in abolishing this inconvenience by coating the capillary with a gel of a given ζ-potential and by occluding both ends of the capillary with the same gel. This creates an electroosmotic backflow

that is entirely uniform throughout the remaining lumen of the capillary. The magnitude of this backflow can be determined with great precision by the simple electrophoresis of an uncharged substance in a slab of the same gel. Thereupon the electrophoretic mobilities of the cells inside such capillaries can be determined at *any* level of focusing and will, after simple addition of the electroosmotic correction factor, directly yield the electrophoretic mobility (*19*). Microelectrophoresis using uniformized electroosmosis as described above can therefore be done without any special apparatus except for a microscope, a dc power supply, and a few capillary tubes. This diminishes the time it takes to determine the electrophoretic mobility of a given family of cells considerably.

In preparative electrophoresis the uniformization of electroosmotic flow can be made use of to increase the effective electrophoretic path length, in what actually can be a fairly short tube, by arranging a strong, uniform electroosmotic backflow in one direction.

Effects of Electroosmosis in Continuous Flow Electrophoresis

In continuous flow electrophoresis, electroosmotic flow perpendicular to the main direction of liquid flow causes the electrophoretically separated bands to assume crescent-shaped cross sections which, according to the electrophoretic mobilities, are either concave or convex with respect to the anode (*20*). It is, however, possible to have *one* of the electrophoretic fractions migrate as a flat band so that that fraction can be collected with optimum purity (*21*). For the precise definition of *all* electrophoretic fractions, the crescent phenomenon must be abolished, which can only be done by a zero ζ-potential coating.

BUFFERS

Zeta potentials (*22*) and thus, generally speaking (for further precision, see Refs. *23* and *24*), electrophoretic mobilities of cells and particles are higher at low ionic strength. In the same electric field the Jouleian heat development is lower at low ionic strength. There is thus every advantage in electrophoresing cells in low ionic strength buffers which, however, need to be made osmotically isotonic by the addition of nonelectrolyte solutes. One such buffer, pH 7.7 and ionic strength $\Gamma/2 = 0.01$, with an osmolality of 256 *m* Osm./liter and consisting of 0.18% Na_2HPO_4, 0.02% KH_2PO_4, and 4.32% glucose, can be used in most cases.

COOLING

Both in preparative and in microelectrophoresis, cooling arrangements are indispensable for the dissipation of the Jouleian heat.

In preparative electrophoresis, even at an ionic strength of $\Gamma/2 = 0.01$, a field of 5.5 V/cm necessitates a total potential difference of 500 V at 6 mA, generating 3 W. The most appropriate cooling method is by circulating cold water in a water jacket surrounding the electrophoresis tube (*10*).

In microelectrophoresis the main reason for cooling lies in the need for keeping the viscosity and dielectric constant unchanged. A constant temperature is easily attained in the microelectrophoresis method described above by placing the capillary in a trough (made from a longitudinally split plastic tube) through which water (at 20°C) from a higher placed reservoir on the left is allowed to stream horizontally and to run out to a sink on the right (*19*).

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