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NASA CASE NO. MFS-26049-1NP

PRINT FIGURE 2

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TECHNICAL ABSTRACT

NASA Case No.

MFS-26049-1-NP

CONTROLLED METHOD OF REDUCING ELECTROPHORETIC MOBILITY OF VARIOUS SUBSTANCES

The present invention is directed to a method for quantitatively reducing electrophoretic mobility of macromolecules, particles, and cells in a controlled, concentration-dependent manner by interacting the substances with neutral, polymer-linked affinity compounds. The method will be useful in achieving electrophoretic separation for a large number of substances or particles whose normal electrokinetic charge structure would not allow separation under past systems. It has potential for both preparative and analytical use.

The method of the invention comprises interacting the substances of interest with a two-component system consisting primarily of a hydrophilic neutral polymer and a second affinity component consisting of a hydrophobic compound, and immunocompound, or a ligand. It is preferred that the hydrophilic neutral polymer used in the invention compricse polyethylene glycol (PEG), however, other suitable polymers such as polyvinyl alcohol, polyvinyl pyrrolidone or polypropylene glycol may also be employed. The hydrophobic compound used in the two-component system is preferably a fatty acid alkyl derivative, or acyl group, e.g., a hexadecane or octadecane tail. The immunocompound in the system can include antibodies or active fragments

derived therefrom, e.g., F(ab) or Fc, or similar macromolecules with binding specificity, e.g., Protein A, Protein G, Avidin, or Concanavalin A. The ligands useful in the invention include hormones, antigens, enzyme inhibitors, and haptens. Electrophoretic separation can be accomplished in a variety of different manners (i.e., specific/reversible, specific/irreversible, non-specific/reversible, etc.) depending on the particular two-component system selected to interact with the desired macromolecules, particles, or cells.

The novelty of the invention appears to lie in the use of the two-component system to achieve separation in a variety of macromolecules, cells, etc. in a controlled, concentration-dependent manner unobtainable in the prior art. The mobility of the treated substance is reduced in a concentration-dependent manner by masking the "effective" or "apparent" surface charge using the neutral polymer. The reduction in electophoretic mobility will be directly proportional to the amount of the two-component affinity compound employed in the assay. The technique can be employed using standard laboratory electopheresis equipment.

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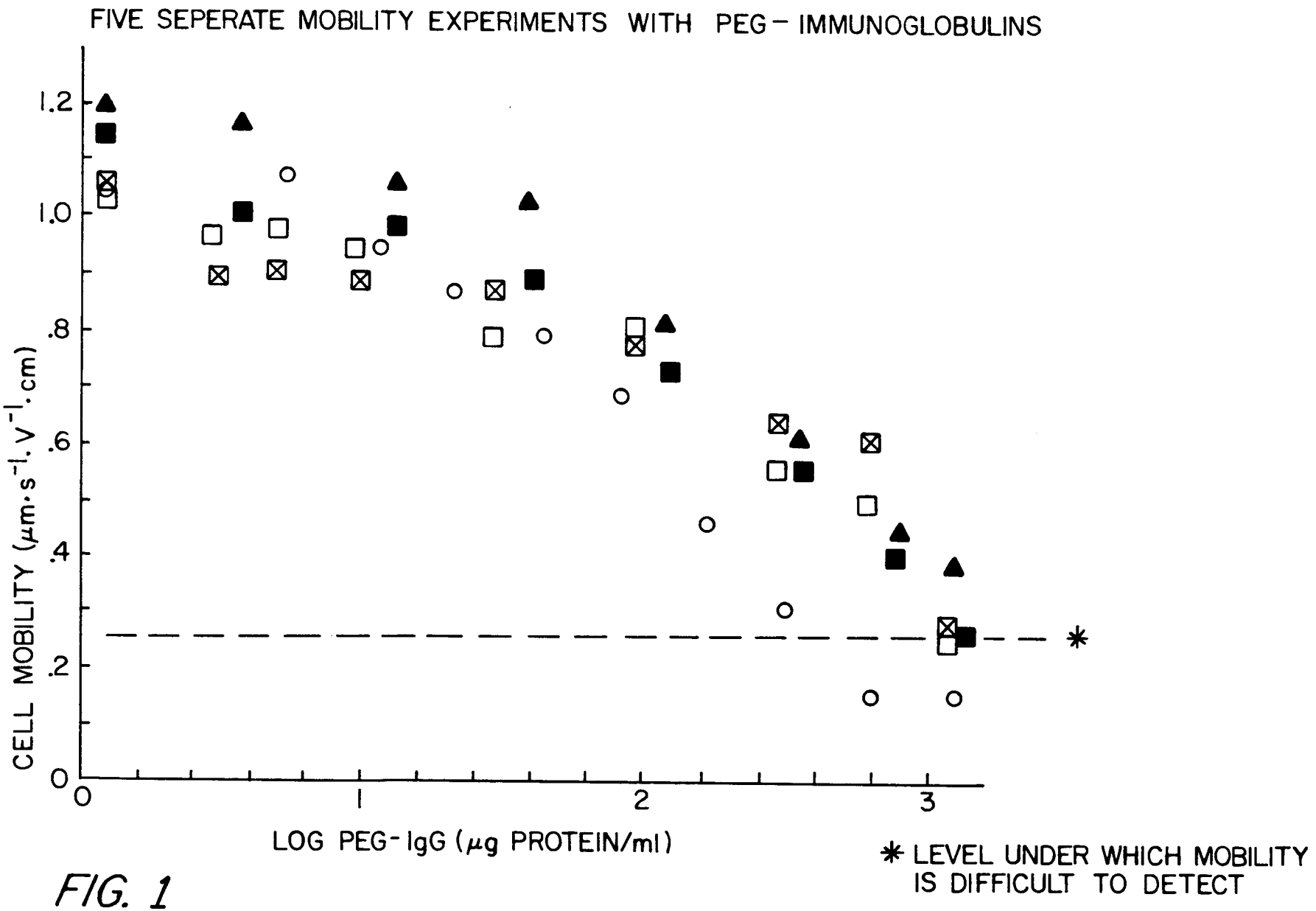
FIVE SEPERATE MOBILITY EXPERIMENTS WITH PEG – IMMUNOGLOBULINS
CELL MOBILITY (μm·s⁻¹.V⁻¹.cm)
1.2
1.0
.8
.6
.4
.2
0
0
1
2
3
LOG PEG-IgG (μg PROTEIN/ml)
* LEVEL UNDER WHICH MOBILITY IS DIFFICULT TO DETECT

FIG. 1

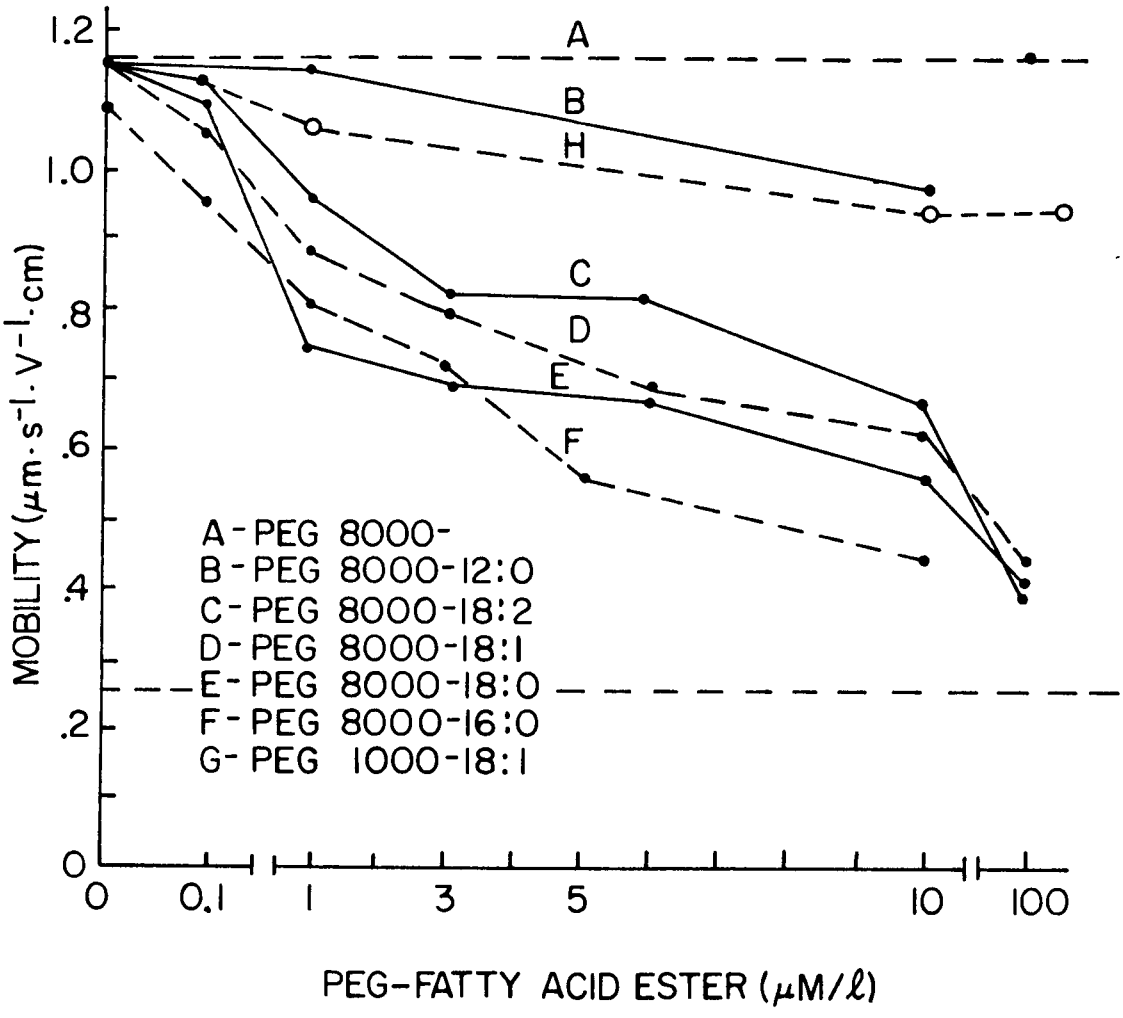
1.2
1.0
.8
.6
.4
.2
0
MOBILITY (μm·s⁻¹·V⁻¹·cm)
A
B
H
C
D
E
F
A - PEG 8000-
B - PEG 8000-12:0
C - PEG 8000-18:2
D - PEG 8000-18:1
E - PEG 8000-18:0
F - PEG 8000-16:0
G - PEG 1000-18:1
0
0.1
1
3
5
10
100
PEG-FATTY ACID ESTER (μM/ℓ)

FIG. 2

CONTROLLED METHOD OF REDUCING ELECTROPHORETIC MOBILITY OF PARTICLES, CELLS, AND THE LIKE

ORIGIN OF THE INVENTION

The invention described herein was made in the performance of work under a NASA contract and is subject to the provisions of Section 305 of the National Aeronautics and Space Act of 1958, Public Law 85-568 (72 Stat. 435; 43 U.S.C. 2457).

FIELD OF THE INVENTION

The invention relates in general to a method for quantitatively reducing electrophoretic mobility of particles, cells and macromolecules, and specifically to a method of reducing electrophoretic mobility in a concentration-dependent manner by interacting substances with neutral polymer-linked affinity compounds.

BACKGROUND OF THE INVENTION

For years, electrophoresis has been used to separate and isolate macromolecules and particles, due to differences in apparent "surface" charge. When two substances have the same charge but different mobilities, the faster moving substances will overrun and migrate ahead of the slower one. This is usually due to substance geometry and other hydrodynamic considerations. In general, the affinity of one substance to another will affect the mobility of that substance when both are subjected to electrophoresis. It is thus theoretically possible to maximize separation of particles with the same or similar charges through the use of specific affinity compounds. For a general review of affinity electrophoresis (of macromolecules) , see Takeo, Electrophoresis 5:187-195 (1984). Previous affinity electrophoresis techniques have been usually directed toward altering substance charge.

Affinity electrophoresis techniques are of enormous potential value in a variety of medical biochemical areas (e.g., research, diagnostics). Of particular importance will be techniques for isolating or separating very large molecules or cells such as cancer cells, blood cells, DNA molecules, lymphocytes, proteins, etc. New techniques are needed since, in a great number of cases, the cells or particles of interest are not amenable to presently known electrophoretic separation techniques. This is particularly true for cells which are easily damaged by the extreme conditions (temp, pH, salt concentration, fluid shear) of many (electrophoretic) separation techniques. Unfortunately, it is also true that most cells have reasonably similar electrophoretic mobilities (approximately 0.9 ± 0.3 $\mu m s^{-1} v^{-1} cm$). Electrophoretic separation methods for such substances need to be specific as regards their physiological significance. It is extremely important that the range of electrophoretic mobility be controlled, preferably in a concentration-dependent manner.

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The use of affinity agents such as antibodies to enhance the specificity of electrophoresis, and develop a useful system by which a large number of particles and cells could be separated was theorized as early as the 1920's. However, antibody-induced cell aggregation, and a demonstrated lack of cell-antibody interaction appreciably altering substance electrophoretic mobility kept the goal at arms length. Previous research in this field attempted to use immuno-compounds and other agents to affect

electrophoretic mobility of various cells. For example, Seaman, pgs. 1135-1229 in The Red Blood Cell, Vol. 3 (1975), discloses modifying red blood cell surfaces with such materials as antisera, enzymes, and neutral polymers. In the first two cases (antisera and enzymes), problems were reported including aggregation, limited reductions of mobility of the cells, and a general lack of specifity in the treatments. It was found that the use of neutral polymers actually increased particle electrophoretic mobility. Effects of unmodified antibodies on red blood cells (mentioned above) has also been reported by Sachtleben, in Cell Electrophoresis, pgs. 100-114 (1965). The use of a double antibody system yielding up to about a 40% reduction in electrophoretic mobility in epithelial cells and lymphocytes was reported in Cohly et al in Cell Electrophoresis, pgs. 611-616 (1985). (In an attempt to alter net cell surface charge, rather than cell surface electrohydrodynamics, these authors exposed cells covered with antibodies to secondary anti-antibodies whose net positive charge, they hoped, would partially neutralize the cells native net negative charge.) Thus previous attempts in reducing mobility generally suffered from aggregation, and large, controlled reductions in electrophoretic mobility have not been achieved.

In the patent art, many attempts at separating biological cells and other compounds through electrophoresis are known. For instance, U.S. Patent No. 4,474,886 (Willard) relates to the use of SDS polyacrylamide gel in gel electrophoresis wherein the SDS gel binds to proteins to aid in separation by mass. The SDS is charged and uniformly coats the

macromolecule creating uniform charge per unit area. Thus separation is on the basis of surface area which is related to size. SDS kills cells, which regardless cannot be subjected to such gel electrophoresis. Independent of these blocks, this method would not aid in separating two molecular samples of similar mass which have different charges.

As indicated above, the use of neutral polymers in affecting electrophoretic mobility has been examined. It has been observed that such polymers typically cause an increase, and not a decrease, in the electrophoretic mobility (see Brooks, J. Colloid Interf. Sci., 1971 and Seaman, above). Various researchers have employed charged polymers in methods to obtain decreases in the mobility of macromolecules but not particles. Examples of these techniques are described in references such as Shimura et al., Electrophoresis 8:135-139 (1987) (charged polylysine added to ligand to aid biomolecule separation), and Vestermark, German Patent No. 2,040,091 (electrophoretic separation achieved through use of charged polyethylene glycol ions). These techniques do not aid in "masking" the native surface charge of macromolecules, particles or cells, because the polymers in these cases are of opposite charge and will neutralize, not remove or mask, native charges on the macromolecules. What has not been accomplished, therefore, is the development of a system by which hydrophilic, uncharged polymers, such as polyethylene glycol (PEG), can be used to substantially reduce electrophoretic mobility by altering the hydrodynamic nature of the "surface" of cells, particles and macromolecules in a concentration-dependent manner and/or in a manner based on their specific antigenic characteristics.

SUMMARY OF THE INVENTION

According to the present invention, a method of reducing electrophoretic mobility of macromolecules, particles, cells and the like in a controlled manner is provided which comprises interacting the macromolecules, particles or cells with a polymer-linked affinity compound comprised of a hydrophilic neutral polymer, such as polyethylene glycol, and a component selected from the group consisting of a hydrophobic compound, an immunocompound, such as an antibody or active antibody fragment or similar macromolecule with binding specificity (e.g., Protein A, Avidin, Protein G, Concanavalin A), and a ligand. The reduction in electrophoretic mobility achievable in the present invention is up to approximately 100% for particular substances, and the mobility reduction achieved is directly proportional to the concentration of the interacting affinity compound employed. The present invention is advantageous in that electrophoretic separation is now possible for substances or particles whose normal electrokinetic charge structure would not allow separation. This separation can be achieved with specifity, and in a controlled, analytical, concentration-dependent manner. Furthermore, the interaction of the affinity compound with the substance of interest can be tailored in the range of reversible to irreversible.

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BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1 is a graphic representation of the effects of immuno-affinity compounds of the present invention in reducing electrophoretic mobility of cells.

Figure 2 is a graphic representation of the effects of hydrophobic affinity compounds of the present invention in reducing electrophoretic mobility of cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present invention, a hydrophilic neutral polymer such as polyethylene glycol is used in a two-component system to reduce electrophoretic mobility in a wide variety of macromolecules, particles and cells. The use of polyethylene glycol has been known in the separation of cells, such as described in Karr et al, J. Chromatography 354: 269-282 (1986), in which it is employed in a phase partitioning system. However, phase partitioning, a liquid-liquid extraction technique which is relatively three-dimensional and energy independent, is quite unrelated to electrophoresis procedures, which are energy dependent and involve linear separation of materials based on differences in apparent surface charge. It has also been disclosed in a previous U.S. Patent, No. 4,690,749 (Van Alstine et al), to employ PEG in controlling electroosmosis. However,, in that patent, the PEG is permanently (i.e., covalently) linked to the surface of nonliving particles so as to produce test particles, e.g., glass beads, of defined mobility. In the present invention, the PEG-linked affinity compounds do not permanently bind to the particle or cell separated, and the technique disclosed herein can be used specifically for separating desired or target cells, after which the affinity ligand is released from and/or metabolized by those cells.

In the embodiments of the present invention, the polymer-linked affinity compound employed to reduce electrophoretic mobility in a controlled manner will be comprised primarily of a hydrophilic neutral polymer and a second component consisting of a hydrophobic compound, an immuno-compound, including

active antibody fragments or similar macromolecule or with binding specificity (e.g., Protein A, Avidin, Protein G, Concanavalin A), or a ligand. For all of the embodiments, it is preferred that the hydrophilic neutral polymer neutral polymer employed comprise polyethylene glycol (PEG). However, other hydrophilic neutral polymers which can be covalently coupled to the second component of the polymer-linked affinity compound may be employed and may be advantageous under certain specific applications. Other neutral hydrophilic polymers usable in the invention include polyvinyl alcohol, polyvinyl pyrrolidone, polypropylene glycol, polyethylene glycol-polypropylene glycol co-polymers, and polypropylene glycol-polyethylene glycol co-block polymers.

As discussed previously, it had been observed that adsorption of neutral polymers typically increased the electrophoretic mobility of particles and cells. This is most likely due to the fact that the particles or cells non-specifically adsorb the polymers at multiple polymer contact points on their surface (see Brooks, above and Seaman, above). In the present invention, the hydrophilic neutral polymer-containing compounds adsorb at the "surface" of the macromolecule, cell or particle via demonstrably specific interactions. As a result, these adsorbate neutral affinity compounds alter particle or molecular electrohydrodynamic interaction with the suspending medium (increase local viscosity) at the particles or macromolecule's "surface", lowering cell/particle mobility. In the case of macromolecules, the affinity compounds of the present invention similarly contact the macromolecule at its outer periphery and alter its hydration, and

the effect in reducing electrophoretic mobility is analogous to the effect of the compounds on the outer layers of cell or particle surfaces.

In one of the preferred embodiments of the invention, an immunocompound or fragment thereof, is employed as the second component of the polymer-linked affinity compound. By immunocompound is meant antibodies or fragments thereof, or similar compounds (such as Concanavalin A, Protein G, Protein A, Avidin) which can bind to specific "antigens". Particularly useful in the present invention are immunoglobulins (e.g., IgG) and their active fragments (e.g., F(ab) or Fc) derived therefrom. However, any antibody of human or animal derivation may be successfully employed in the invention. It has been shown by a group of authors including Karr, Harris, Van Alstine, et al., that PEG derivatized Protein A (which binds to antibody molecules) can be used to effect Immunoaffinity Partition. This technique may also work for Immunoaffinity electrophoresis using PEG-Protein A or PEG-Protein G and unmodified antibodies or PEG-Avidin and Biotin-conjugated antibodies, etc. Affinity compounds containing an immunocompound as a second component are particularly advantageous in that they can be made very specific to a particular cell, particle, or macromolecule, and can be used successfully to reduce mobility in a large number of substances having a surface or outer periphery charge structure which normally would not allow them to be separated by electrophoretic means. Immuno-affinity compounds are thus effective in reducing electrophoretic mobility of specific particles (e.g., cells such as subpopulations of lymphocytes) or any other molecules to which the antibodies or their active fragments can be directed.

An additional feature of this aspect of the invention is the observation that the antibody/neutral polymer compounds greatly reduce the tendency to aggregate cells or particles, which would hinder specific cell separation, while at the same time providing concentration-dependent reduction in cell or particle electrophoretic mobility. The analytical useful immuno-affinity compounds of the present invention are a great improvement over previous compounds as reported above which only achieved up to about 40% reduction in electrophoretic mobility. In the present invention, reduction in charge and the mobility of up to 100% can be achieved, and this reduction is obtained in a concentration-dependent manner. In many cases the interaction can be reversed and in the case of living cells surface localized affinity compounds may be metabolized following cell separation.

In another embodiment of the present invention, the second component in the polymer-linked affinity compound comprises a hydrophobic compound. It is particularly preferred that the hydrophobic compound comprise a fatty acid alkyl derivative or acyl group with a hexadecane, octadecane, etc. tail including palmitic acid, stearic acid, oleic acid, etc. However, lipids and other hydrophobic compounds which can be covalently linked to the hydrophilic neutral polymer, such as 1-amino-octadecane may also be employed. The hydrophobic compounds can be linked to the hydrophilic neutral polymer by means of an ester linkage (see Figure 2), but linkage through an ether, amine or other functional group is possible. The hydrophobic-affinity compounds of the present invention show some specifity as to the cells and particles for which mobility is to be reduced, and these compounds have been preferably used in the separation of specific cells such as cancer cells or red blood cells. A particular advantage of the

hydrophobic-affinity compounds is that the binding with the cells treated is readily reversible. This feature is highly advantageous where it is desired to separate cells, then remove the affinity compound so that the separated cells can be examined. The compounds are not harmful to electrophoretic apparatii and are used in such low concentration that they can be easily and cheaply added to the buffer solutions normally used in electrophoretic techniques.

In still another aspect of the present invention, the second component of the polymer-linked affinity compound comprises a ligand. Ligands useful in the present invention may comprise a wide variety of small macromolecular substrates, such as hormones or antigens, or low molecular weight substances such as enzyme inhibitors and haptens. The present invention differs from the previous work of Muller et al, Nucleic Acid Research 9:95-115 (1981), in which dyes were covalently bound to a polyethylene glycol to specifically increase the frictional "surface" coefficient of DNA molecules gel electrophoresis, in that the Muller et al article did not disclose use of the dyes in binding cells or other particles. The ligand-affinity compounds of the present invention can be employed to reduce the gel and non-gel based electrophoretic mobility of cells and particles in addition to macromolecules. Additionally, the ligand-affinity compounds of the present invention will be of varying reversibility, depending upon the particular ligand chosen, and reduce the mobility of cells and particles in a concentration-dependent manner.

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In accordance with the present invention, electrophoretic separation can be accomplished in a variety of different manners. Depending on the affinity component utilized, separation can be achieved on the basis of specific/irreversible (e.g., PEG-derivatized antibody), specific/reversible (e.g., PEG-derivatized lectin), semi-specific, reversible (e.g., PEG-hydrophobic affinity ligand such as unsaturated PEG-fatty acid ester), relatively nonspecific reversible (e.g., saturated PEG-fatty acid ester or PEG-ionic interaction group (trimethylamine or sulphate)) or relatively nonspecific-irreversible (e.g., PEG-cyanuric chloride - amine group) interactions. 9 DA 7-8-89

In general, the affinity compounds appear to operate in reducing mobility of the macromolecules, cells, or particles treated by masking the "effective or apparent" surface charge. The neutral polymers apparently increase fluid viscous resistance to "surface" flow when anchored via the second component of the affinity compound to the particular cell, particle, or macromolecule separated. A general background as to the theory of electrophoresis and the effects of increasing local surface resistance to fluid flow can be found in Levine et al, Biophys. J. 42:127-135 (1983). In the present invention, the more neutral polymers which can be attached to the unit to be separated, the greater the effect in reducing mobility. Thus the reduction in electrophoretic mobility may be obtained in a manner directly proportional to the amount of affinity compound employed in the electrophoretic assay. The ability of the affinity compounds of the present invention to achieve concentration-dependent reduction in

electrophoretic mobility has been confirmed in experimental tests. Their ability to specifically interact with a variety of macromolecules and particles (particularly cells) in a concentration dependent manner is documented. The polymer-linked affinity compounds of the invention can thus be used in a variety of applications when it is desired to achieve enhanced reduction of electrophoretic mobility in a controlled, concentration-dependent manner.

The following examples are presented as illustrative only and are not intended to limit the scope of the invention in any way:

EXAMPLE I

Electrophoretic mobility profiles of standard model cells (human erythrocytes) were obtained in phosphate buffered saline in a Rank Bros. cylindrical chamber cytopherometer set at 40 V DC. Standard cell mobility is roughly 1.1 micrometer per second per volt per cm (i.e., velocity per field strength) expressed as $\mu m\ s^{-1}\ V^{-1}\ cm$. Five separate experiments with polyethylene glycol-immunoglobulin used to treat the cells were run, and the results are observed in the graph of Figure 1. Each different immunoglobulin is represented by a different symbol. The standard error associated with each measurement (point) was $\pm$ 10%, or less, of the mean. The results indicated that cell mobility was decreased for all PEG-immunoglobulin treated cells in a concentration dependent manner. The range of reduction ranged from roughly 1 ug to 1 mg, and at the higher concentration levels of PEG-IgG, cell mobility effectively approached zero or a 100% reduction in mobility. It was also noted in these tests that cell aggregation normally associated with treatment by immunoglobulins alone was not observed with the PEG-immunoglobulin affinity compounds of the present invention.

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EXAMPLE II

The effect of a PEG-fatty acid ester affinity compound on cell mobility was tested in the same manner as described in Example 1. The results of these tests can be observed in Figure 2. Tests were made on PEG alone (as a control), and on PEG-fatty acid ester combinations of varying fatty acyl tail length and saturation. The PEG employed in most of these trials had a molecular weight of roughly 8000, and in one of the tests, a PEG of molecular weight of about 1000 was studied. The tests involved 3-5 trials for each of the affinity compounds as indicated in Figure 2. Typical standard deviation for each point was ± 10%, or less, of the mean. The results again indicated that the PEG-fatty acid ester affinity compounds were successful in producing a concentration-dependent reduction in electrophoretic mobility of the treated cells. It was also determined that the reduction in mobility was dependent upon the PEG head group molecular weight and the fatty acyl tail length and saturation. The particularly effective affinity compounds were those indicated at C-F in Figure 2, wherein molecular weight of the PEG was around 8000, and the acyl tails of the compound had substantial tail length (hydrophobicity). The trials with lower molecular weight PEG and shorter tail length compounds both affected the mobility of the cells, but not as greatly as the effect observed with the compounds C-F.

The hydrophobic affinity compounds of the invention, although not as specific as the immuno-affinity compounds such as described in Example 1, are advantageous in that they can be produced at

low cost, and are easily reversible with regard to the cells treated. Most cells have similar native electrophoretic mobilities due to the nature of the surface charges involved. The affinity compounds of the present invention will therefore allow for the controlled electrophoretic separation of cells and other particles in a manner heretofore unachievable, using equipment already owned by many laboratories.

ABSTRACT OF THE DISCLOSURE

A method of reducing electrophoretic mobility of macromolecules, particles, cells and the like is provided which comprises interacting the particles or cells with a polymer-linked affinity compound comprised of: a hydrophilic neutral polymer such as polyethylene glycol, and an affinity component consisting of a hydrophobic compound such as a fatty acid ester, an immunocompound such as an antibody or active fragment thereof or similar macromolecule, or other ligands. The reduction of electrophoretic mobility achieved is directly proportional to the concentration of the polymer-linked affinity compound employed, and the mobility reduction obtainable is up to 100% for particular particles and cells. The present invention is advantageous in that analytical electrophoretic separation can not be achieved for macromolecules, particles and cells whose native surface charge structure had prevented them from being separated by normal electrophoretic means. Depending on the affinity component utilized, separation can be achieved on the basis of specific/irreversible, specific/reversible, semi-specific/reversible, relatively nonspecific/reversible, or relatively nonspecific/irreversible ligand-substance interactions. The present method is also advantageous in that it can be used in a variety of standard laboratory electrophoresis equipment.