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(21) International Application Number: PCT/US00/11474 (22) International Filing Date: 28 April 2000 (28.04.00) (30) Priority Data: 60/131,751 30 April 1999 (30.04.99) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 60/131,751 (CIP) Filed on 30 April 1999 (30.04.99) (71) Applicant (for all designated States except US): APPLIED HYDROGEL TECHNOLOGY CORPORATION [US/US]; 6865 Flanders Drive, Suite G, San Diego, CA 92121 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SHIH, Lih-Bin [US/US]; 11042 Caminito Vista Pacifica, San Diego, CA 92131 (US). VILALTA, Patricia [MX/US]; 10727 Caminito Alvarez, San Diego, CA 92126 (US). WILLIAMS, Mark [US/US]; 5049 Sea Shell Place, San Diego, CA 92130 (US).		(74) Agent: STEWART, Ramsey, R.; Gray Cary Ware & Freidenrich LLP, Suite 1600, 4365 Executive Drive, San Diego, CA 92121 (US). (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: MIXED BUFFERS IN GEL ELECTROPHORESIS SYSTEMS (57) Abstract <p>In accordance with the present invention, there are provided methods for the separation of biological materials, based on the discovery that by including a small amount of organic agent to electrophoresis buffer, the rate of separation of biological materials is increased. Thus, according to the present invention, biological materials can be separated and analyzed by gel electrophoresis more rapidly than by prior methods.</p>		

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MIXED BUFFERS IN GEL ELECTROPHORESIS SYSTEMS

FIELD OF THE INVENTION

5 The present invention relates to gel electrophoresis, and particularly to improved methods for the separation of biological materials using gel electrophoresis.

BACKGROUND OF THE INVENTION

10 Gel electrophoresis is a technique that is often carried out for the analysis and purification of biological molecules such as peptides, proteins, nucleic acids, carbohydrates, and others. Electrophoresis is based on the principle that charged molecules will migrate when placed in an electric field, with the rate of migration dependent upon, among other things, the molecule's charge density (charge per unit of
15 mass). Gel electrophoresis uses a gel, made of natural or synthetic polymers, typically manufactured in a slab orientation or alternatively in a tube shape, to provide a support for the molecules so that they can be analyzed at the completion of separation, and to provide additional sieving characteristics. Gels for electrophoresis can be considered to consist of random networks of filaments, cross-linked by covalent, ionic, hydrogen or
20 other molecular bonds, to form a three-dimensional "sponge". The pores form a molecular sieve that retards the mobility of molecules migrating in the imposed electric field. The size of the pores is determined by the concentration of filaments (normally described as the percentage of solid in the gel) and the degree of cross-linking. For two molecules with equal charge density, their migration through the gel will be determined
25 by their size. For a given gel porosity, the rate of migration is inversely proportional to the log of the protein molecular weight over a limited range of molecular weights, so that smaller molecules migrate at a faster rate than those of larger size.

 Many support media for electrophoresis are in current use. The most popular are
30 sheets of paper or cellulose acetate, silica gels, agarose, starch and polyacrylamide. Paper, cellulose acetate, and thin layer silica materials are relatively inert and serve mainly for support and to minimize convection. Separation of proteins using these materials is based largely upon the charge density of the proteins at the pH selected. Most typically, electrophoresis is performed in an aqueous solution or gel across which a

voltage is applied. It is the voltage gradient that causes the migration of the species being separated. Gradients typically range from 10 volts/cm to many times higher, the magnitude depending on the nature of the separation being performed. Gel electrophoresis is typically performed by placing a complex mixture of molecules to be separated and analyzed or purified at one position in the gel by means of a well formed in the gel or by means of layering at one end of a tube. The mixture usually contains a visible dye that migrates at a known rate in the system. The gel is brought into contact with an electrolyte buffer solution that is in electrical contact with a power source, and a defined electrical field is applied to the gel. The molecules in the sample migrate toward the appropriate electrode, each at an individual rate until the marker dye reaches a predetermined position in the gel. The electrical field is then removed and the separated molecules are visualized by one or more methods well known in the field as bands corresponding in width to the width of the sample well. The size, relative concentration and other information on the molecules in the sample can then be determined.

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Investigators often use gel electrophoresis to separate peptides, proteins and nucleic acids by size rather than by charge density. For nucleic acids, the charge density of each molecule is approximately equal, since each individual nucleotide has one charged moiety. Care is taken to remove any possible tertiary structure from RNA and DNA, and any secondary structure from RNA and DNA for determinations based on specific sequence characteristics, typically by using a combination of heat and chemical denaturation. As a result, nucleic acids are typically separated based on their primary sequence length.

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To separate proteins based on their molecular weight, the effects of secondary structure and heterogeneous charge density are eliminated during sample preparation. Complexing proteins with an excess of sodium dodecyl sulfate (SDS) at high temperatures eliminates most secondary structure and provides an approximately equal charge density to all proteins. Inclusion of a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME) serves to remove intra- and intermolecular disulfide bonds between cysteine residues.

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Alternatively, biological molecules are sometimes separated using gel electrophoresis based on their charge differences or upon structural differences that cause molecules of similar molecular weight to assume different three-dimensional shapes and thus migrate at different rates in gels. The extent of molecular sieving is thought to depend on how closely the gel pore size approximates the size of the migrating particle. The pore size of agarose gels is sufficiently large that molecular sieving of most protein molecules is minimal and separation of proteins is based mainly on charge density. In contrast, polyacrylamide gels can have pores that more closely approximate the size of protein molecules and so contribute to the molecular sieving effect. Polyacrylamide has the further advantage of being a synthetic polymer which can be prepared in highly purified form. Polymeric materials consisting of block copolymers of partially hydrolyzed acrylonitrile have also been demonstrated to be of utility in the separations of biological materials (US Patent 5,888,365). The ability to produce gels having a wide range of polymer concentrations resulting in a wide range of controlled average pore size as well as to form pore size gradients within the gels by virtue of polymer concentration gradients, are additional advantages of synthetic polymers as electrophoresis gel media. Control over pore size enables a wide range of mixtures of biological materials to be sieved on the basis of molecular size and enables molecular weight determinations to be performed.

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Gels are typically saturated with an electrolyte solution that provides for efficient electrical conductivity. The rate of biomolecule migration in gel electrophoresis is also affected by the concentration and type of ions present in the electrolyte solution. The concentration of ions in the electrolyte solution must be high enough that the counter-ions keeping the biological molecule in solution are not separated from the biomolecule (which is itself a large ionic species), in which case the force attracting the large ion to the small counter-ions can become stronger than the electromotive force of the applied electric field. Electrolyte concentrations that are too great lead to degradation of molecule band resolution. The pH of the electrolyte also plays a role in maintaining electrophoretic separation. During electrophoresis, water is electrolyzed, which generates protons at the anode and hydroxyl ions at the cathode. The cathodal end of the electrophoresis chamber then becomes basic and the anodal end acidic. To prevent this,

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the electrolyte solution also contains a buffering system, typically based upon tris(hydroxy-methyl)aminomethane (Tris).

There are generally up to three distinct buffer systems employed in performing gel electrophoresis of biological materials. First, a “sample buffer” is used to prepare the biological materials for separation under a given set of electrophoretic conditions. Second, the “electrode buffer” is used as a conducting medium, among other functions, to conduct the electrical potential from the electrode to the gel, which causes the charged biological molecules to migrate and separate in the gel. The third is the “gel buffer” which impregnates the gel media so as to cause the biological molecules to migrate and resolve themselves according to their size and charge under electrophoretic conditions.

Gel buffers are often chosen so that the media has a suitable pH value for biological molecules to carry desired charges under a given set of electrophoretic conditions. The buffer type and ionic strength are also factors of consideration for achieving the optimal results of (a) controlling the pore size of the gel for sieving and (b) controlling and balancing the heat generation and the speed of separation.

To separate SDS-denatured protein molecules in gel electrophoresis applications, a variety of gel buffers are used based on the specific biomolecule separation required, including Tris-HCl, Tris-Borate, Tris-acetate, and many others known to those of skill in the art. The electrode buffer often consists of a Tris-glycine system, or the like. There may also be other electrolyte components such as SDS in the buffer system, but it has been very uncommon in the past for organic agents such as ethanol, methanol, acetonitrile, and the like to be added to gel buffer systems for use in electrophoretic applications. Part of the reason for this is that the material typically used for the preparation of gel matrix, polyacrylamide, is a very hydrophilic material whose polymerization is adversely affected by some agents that interfere with the free-radical initiated polymerization of polyacrylamide, resulting in lack of gelation, opaque gels, gels with reduced separation capacity, and other problems.

In a small number of cases, some organic agents have been used in combination with polyacrylamide and other polymer gels, most notably sulfolane, dimethyl formamide (DMF), urea, and phenol. These agents have been added to polyacrylamide gels with the intent of denaturing or solubilizing otherwise insoluble, hydrophobic biomolecules to make gel electrophoresis possible.

For example, Analytical Biochemistry 1984, 137:410-419 describes the use of the detergent sulfolane (tetrahydro-1,1-dioxide) as an agent for the electrophoretic separation of hydrophobic proteins and indicates as common knowledge that alcohols are inappropriate as additives to PAGE gels due to their inhibition of gel polymerization. No data on relative rates of electrophoresis plus or minus sulfolane were reported.

As another example of the use of organic agents with polyacrylamide gels, Analytical Biochemistry 1984, 137:420-428 describes use of organic agents formamide and phenol in certain nucleic acid separations to effect strand denaturation. Chloral hydrate, DMF, tetramethylurea (TMU), and DMSO were used for particular protein applications, typically for sample solubilization purposes. In order to utilize electrophoretic methods for highly hydrophobic molecules, the authors tested the compatibility of N-acryloyl morpholine/acrylamide copolymer gels with several organic agents, including DMSO, DMF, methanol, ethanol, isopropanol, benzaldehyde, acetophenone, m-cresol, TMU and dioxane. This reference focuses on the effects of certain organic agents on the physical properties of the molecules undergoing electrophoresis, and does not suggest that the speed at which a gel can be run can be increased.

As yet another example of the use of organic agents with polyacrylamide-based gels, Electrophoresis 1992, 13:824-831 reports the use of TMU, DMF, formamide, methanol, ethanol, DMSO, sulfolane, n-methylacetamide, N-methylpyrrolidinone and tetrahydrofuran to reswell dried polyethyleneglycol methacrylate/polyacrylamide gels, and suggests that the agents are compatible with this copolymer system. This paper describes experiments designed to allow for

separation of extremely hydrophobic proteins and notes the gels studied “as a whole have less rapid protein migration and poorer resolution” (p 829).

As still another example of the use of organic agents with electrophoresis gels,
5 Electrophoresis 1994 15:195-199 describes poly(ethylene glycol) methacrylate-
acrylamide copolymer media developed to enable electrophoresis of hydrophobic
proteins. The authors describe increasing the speed of electrophoresis in this media
by addition of 25% methanol or 30% DMF, which act as anti-freeze and allow for
running gels at temperatures beginning at -20°C . The authors claim their system
10 provides optimal results when the gel temperature is held at 12°C or below. Using
gels containing antifreeze and starting runs at -20°C , the authors were able to apply
much higher current while staying below the 12°C limit, resulting in 2 to 6-fold faster
run times. See also US Patents 5,397,449 and 5,290,411. This reference does not
describe increasing the speed at which electrophoresis can be conducted without
15 temperature control. Nor does the reference provide a method to increase the speed at
which a gel can be run, regardless of the temperature at which the gel is run. Organic
agents were specifically added to electrophoresis electrolyte buffers only to serve as
antifreeze so that gels could be run at higher than normal voltages without undue
heating, not to directly affect the sieving characteristics of the gel buffer system.

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As yet another example of separation systems designed for use with organic
agents, non-gel materials (sponges) have been described that are compatible with
organic agents (DMSO) and which are appropriate for electrophoresis of highly
hydrophobic proteins [Electrophoresis 1994 15:187-194]. See also US Patent
25 5,637,202.

As still another example of separation systems designed for use with organic
agents, the Journal of Biochemical and Biophysical Methods 19:37-50 (1989),
describes a proprietary matrix that is compatible with DMSO, TMU, acetonitrile and
30 THF, but the benefit considered is a theoretical opportunity to perform electrophoresis
of hydrophobic particles. See also US Patents 5,219,923; 5,153,166 and 5,055,517.

Thus, there remains a need in the art for a method to increase the speed of gel electrophoresis, without requiring extraordinary measures to keep the gel at a suitable temperature, e.g., by refrigeration or the like.

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BRIEF DESCRIPTION OF THE INVENTION

In the development of an appropriate buffer system for use in gel electrophoresis using polymeric materials consisting of block copolymers of partially hydrolyzed polyacrylonitrile, the inventors obtained an unexpected result. Addition of organic agent(s) to the electrophoresis buffer resulted in increased rate of migration of biomolecules in gels when an electric field is applied as compared to migration rates in gels containing only aqueous electrolyte buffer systems. A similar increase in migration rate was also observed in polyacrylamide gels. Accordingly, there is a clear benefit in using organic agent-containing electrophoresis buffers to speed the migration of molecules under electrophoresis and facilitate the more rapid completion of experiments.

It is believed that the conductivity of the gel media and of the gel buffer with its small electrolytes is modified by the addition of organic agents because the electrophoresis system conductivity is dependent on the viscosity and the dielectric constant of the buffer media. Due to the modification of the viscosity and conductivity of the buffers by addition of organic agent, the heat generation is reduced and the separation speed is enhanced.

Thus, in accordance with the present invention, there are provided methods and media for the separation of biomolecules that rely on the inclusion of organic agent(s) of a number of species and at a variety of concentrations into gels and their running buffers. Accordingly, invention methods and materials can serve to increase the rate of migration of biologic molecules in gel electrophoresis applications. As a result, the length of time required to complete a given electrophoresis run is decreased, allowing for higher data throughput in a given time period. The present invention has positive implications for increasing the efficiency of high-throughput biological molecule screening and characterization.

Electrophoresis gels contemplated for use in the practice of the present invention provide advantages over gels currently employed in the art. Decreases in separation times allow for additional gels to be run in a given time period, increasing efficiency in life science laboratories. Inclusion of organic agents allows for efficient separation of proteins with strong hydrophobic character compared to gels without added organic buffer.

DETAILED DESCRIPTION OF THE INVENTION

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In accordance with the present invention, there are provided methods for the separation of biological materials. Invention methods comprise:

- a) applying a mixture of biomolecules to a separation medium comprising a gellable polymeric material and a mixed medium, wherein said mixed medium comprises an aqueous component and one or more organic agents,
- b) applying an electric field to said separation medium, for a time sufficient to cause the electrophoretic separation of said biomolecules, according to the size and relative charge of said biomolecules.

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As will be understood by those of skill in the art, the aqueous component of the mixed medium contemplated for use in the practice of the present invention will comprise electrolytes in sufficient concentration, and combination, and having a suitable pH for conducting electrophoresis. Appropriate electrolytes will depend on the gellable polymeric material being used as well as the nature of the biomolecules being separated. Those of skill in the art can readily determine the appropriate type and amount of electrolytes to be employed. The aqueous electrolyte-containing component contemplated for use in the practice of the present invention comprises buffered media having a pH in the range of about 2 up to 12, such aqueous components include aqueous surfactant-containing mixtures, aqueous solutions containing salt or other electrolytes, and the like.

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The addition of organic agent to the separation media allows for higher running voltages at a given temperature without damage to the gel from heating. Accordingly, invention methods may be carried out at any temperature provided that the electric field applied to the gel does not damage the separation medium to an extent that prevents accurate electrophoresis. As will be understood by those of skill in the art, the accuracy of electrophoresis can be gauged by running appropriate molecular weight standards, or the like. In one embodiment of the present invention, application of electric field to the separation medium is conducted at or below room temperature. In another embodiment of the present invention, application of electric field to the separation medium is conducted at or above room temperature. In a presently preferred embodiment of the present invention, application of electric field to the separation medium is at room temperature. In a presently preferred embodiment, electrophoresis is conducted at a temperature range from about -20°C to room temperature (i.e., about 22°C).

Electrophoresis according to invention methods can be conducted with the application of any suitable electric field. Accordingly, in one embodiment of the present invention, the applied electric field is in the range of about 125V to 5000V. In another embodiment of the present invention, the applied electric field is in the range of about 1000V to 2000V. In still another embodiment of the present invention, the applied electric field is in the range of about 2000V to 5000V. In still another embodiment of the present invention, the applied electric field is in the range of about 250V to 1000V. In a presently preferred embodiment of the present invention, the applied electric field is in the range of about 350V to 450V.

As used herein, "organic agent" means any organic compound which, when added to the separation medium and mixed medium described herein, will provide for faster electrophoresis. Without wanting to be bound by theory, this effect is believed to be a result of a change in conductivity in the electrophoresis system due to changed viscosity and dielectric constant of a buffer containing organic agents. Molecular species are able to migrate faster in a given electrical field under the viscosity and dielectric constant conditions formed by the inclusion of organic agents in electrophoresis buffers. Examples of suitable organic agents contemplated for use in the practice of the present invention include those that are at least 5% soluble in aqueous

medium, such as select alcohols, ketones, nitriles, polar aprotic solvents, ethers, carbamides, and the like.

Alcohols contemplated for use in the practice of the present invention include
5 those that are at least 5% soluble in aqueous medium, such as methanol, ethanol, isopropanol, phenol, m-cresol, and the like.

Ketones contemplated for use in the practice of the present invention include
those that are at least 5% soluble in aqueous medium, such as acetone, methyl ethyl
10 ketone, ethyl ethyl ketone, acetophenone, and the like.

Nitriles contemplated for use in the practice of the present invention include
those that are at least 5% soluble in aqueous medium, such as acetonitrile,
glutaronitrile, propionitrile, or the like.
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Ethers contemplated for use in the practice of the present invention include
those that are at least 5% soluble in aqueous medium, such as tetrahydrofuran, dioxane,
dimethyl ether, or the like.

20 Carbamides contemplated for use in the practice of the present invention
include those that are at least 5% soluble in aqueous medium, such as urea, tetramethyl
urea (TMU), or the like.

Polar aprotic solvents contemplated for use in the practice of the present
25 invention include dimethyl formamide, dimethyl sulfoxide, chloral hydrate,
formamide, benzaldehyde, ethyl acetate, N-methylacetamide, N-methylpyrrolidinone,
and the like.

The relative amounts of aqueous component and organic agent(s)
30 contemplated for use in the practice of the present invention will vary as appropriate
for the intended application. Thus, in accordance with one embodiment of the present
invention, the mixed medium comprises in the range of about 20 to about 99.5 wt% of
aqueous component. In another embodiment of the present invention the mixed

medium comprises in the range of about 60 to about 99 wt% of aqueous component. In yet another embodiment of the present invention, the mixed medium comprises in the range of about 80 to about 99 wt% of aqueous component. In a presently preferred embodiment, the mixed medium comprises about 95 wt% of aqueous component.

The amount of organic agent in the mixed medium contemplated for use in the practice of the present invention will also vary according to the particular organic agent and gellable polymeric material employed. In one embodiment of the present invention, the mixed medium contains about 1-30 wt% of organic agent(s). In another embodiment of the present invention the mixed medium comprises in the range of about 3 to about 20 wt% of organic agent(s). In yet another embodiment, the mixed medium comprises in the range of about 4 to about 10 wt% of organic agent(s). In a presently preferred embodiment, the mixed medium comprises about 5 wt% of organic agent(s).

In accordance with another embodiment of the present invention, there are provided methods for the rapid separation of biomolecules by size and relative charge, said methods comprising:

- a) applying a mixture of biomolecules to a modified separation medium comprising a gellable polymeric material and a suitable electrophoresis buffer,
 - b) applying an electric field to said modified separation medium, for a time sufficient to cause the electrophoretic separation of said biomolecules, according to the size and relative charge of said biomolecules,
- wherein the viscosity and/or dielectric constant of said modified separation medium is selected such that biomolecules separate during electrophoresis therein at a more rapid rate than in unmodified separation medium.

Suitable dielectric constants for separation media which provide more rapid separation of biomolecules typically fall in the range of about 5-75, and preferably in the range of about 10-40.

Suitable viscosities for separation media which provide more rapid separation of biomolecules typically fall in the range of about 0.2 to 2.0 mPa s at 25°C.

5 In one embodiment of the present invention, the separation medium further comprises a denaturing agent. Any appropriate denaturing agent can be employed, including sodium dodecyl sulfate, urea, formamide, sulfolane, β -mercaptoethanol, dithiothreitol (DTT), methyl mercury, or the like.

10 Biological materials contemplated for treatment in accordance with invention separation methods include proteins, polypeptides, nucleic acids, oligonucleotides, carbohydrates, oligosaccharides, lipids, glycolipids, as well as other charged polymine materials (both natural and synthetic), and the like.

15 As employed herein, "polypeptides" refer to compounds containing two or more amino acids linked covalently through peptide bonds. Polypeptides of three or more amino acids are sometimes referred to as oligopeptides. As employed herein, "proteins" refer to macromolecules made up of one or more chains of amino acids covalently joined through peptide bonds. Proteins can vary greatly in molecular weight, from a few thousand to several million daltons.

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As employed herein, "nucleic acids" refer to either of two major types of macromolecule (i.e., DNA or RNA) formed by polymerization of nucleotides. As will be appreciated by those of skill in the art, DNA and RNA exist in many forms, all of which are contemplated for use in the practice of the present invention. As employed
25 herein, "oligonucleotides" refer to relatively short chain nucleic acids, i.e., from 2 to about 1000 bases in length.

As employed herein, "carbohydrates" refer to sugar-based compounds containing carbon, hydrogen and oxygen with the general formula $C_x(H_2O)_y$.
30 Carbohydrates can be divided into various sub-groups, i.e., monosaccharides, disaccharides, oligosaccharides or polysaccharides, depending on the degree of polymerization of the basic sugar units. As employed herein, "oligosaccharides" refer to carbohydrates containing a plurality of monosaccharides.

As employed herein, "lipids" refer to those compounds found in living organisms which are not carbohydrates, proteins or polynucleic acids. Lipids tend to be soluble in organic agents and insoluble in water, and include fats, waxes, phospholipids, glycolipids, steroids, terpenes and a number of different types of pigments. The major group of lipids contains those compounds whose structure is characterized by the presence of fatty acid moieties (acyl lipids). These include neutral lipids (glycerides and waxes) and polar lipids (phospholipids and glycolipids). As employed herein, "glycolipids" refer to lipids that contain one or more carbohydrate moieties. These lipids include the cerebrosides and gangliosides in animals and the galactosyl diglycerides and sulpholipids in plants. The lipid portion is usually glycerol phosphate, glycerol or sphingosine, and the carbohydrate is D-galactose, inositol or D-glucose.

As will be understood by those of skill in the art, the gellable polymeric material contemplated for use in the practice of the present invention may comprise thermally crosslinked gels of different monomer and crosslinker combinations selected for their suitability in separation of particular molecular species. Exemplary thermally crosslinked gellable polymeric materials contemplated for use in the practice of the present invention include polyacrylamide, substituted acrylamide (including, for example, N-substituted polyacrylamide, and the like), poly-N-acryloyl-tris, acrylomorpholine, HYDROLINK™, acrylic and methacrylic esters (e.g., poly(ethylene glycol) methacrylate-acrylamide copolymer, polyhydroxy methacrylate, poly(ethylene glycol) methacrylate, and the like), poly(N-acylalkylenimine), N-vinyl pyrrolidone-based polymers, methacrylic acid-based polymers (e.g., glycerol methacrylate-based polymers, 2-hydroxyethylmethacrylate-based polymers, and the like), acrylic acid-based polymers, and the like, wherein said polymers have one or more hydrophilic groups (e.g., one or more of hydroxy, amine, or the like), or combinations of two or more thereof.

Alternatively, the gellable polymeric material contemplated for use in the practice of the present invention may comprise gels of natural or synthetic polymers which form gels through chain-entanglement processes. Such gellable polymeric materials include agarose (natural material) and the synthetic hydrogel materials

described in United States Patent No. 5,888,365 (incorporated herein by reference in its entirety; referred to hereinafter as "AHT hydrogel"), where the gel network formation is based on chain entanglement of the hydrophobic domains of nitrile groups in a polyacrylonitrile-polyacrylamide copolymer, greater hydrophobic character is present in gels, by virtue of the presence of the nitrile groups, than that of the conventional polyacrylamide gel material used in electrophoresis. Because of the hydrophobicity of the AHT hydrogel material, the electrophoretic process can take place at a far higher voltage (and thus speed) while the heat generated by the process can be dissipated to tolerable levels by air cooling or a simple water bath.

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In addition, a number of electrophoresis matrices comprising chemically modified or differentially crosslinked acrylamide and other polymers have been developed for separation of biomolecules and are reviewed in Electrophoresis 1995 16:1815-1829, incorporated herein by reference in its entirety. Many of these are compatible with organic agents and are contemplated for use in the practice of the present invention.

Additional gellable polymeric materials contemplated for use in the practice of the present invention include electrophoresis sponges such as those comprising polyethylene, polypropylene, polytetrafluoroethylene, polyvinylidene difluoride, polynitrile, polystyrene, or the like, or two or more thereof.

One key property of the foregoing gellable polymeric materials is the ability to uptake a large weight percentage of aqueous medium. Thus suitable materials will be sufficiently hydrophilic so as to allow the desired uptake level of aqueous medium. In spite of the ability to uptake large weight percentages of aqueous medium, gellable polymeric materials suitable for use in the practice of the present invention are those having sufficient strength, in the presence of aqueous media, to retain the structural integrity thereof. Such structural integrity can be imparted by chemical linkage (e.g., covalent crosslinking, or ionic crosslinking), physical interaction (e.g., hydrogen bonding, hydrophobic interactions (such as the presence of crystalline domains), physical entanglement of polymer chains, dipolar forces, etc.), and the like. Where dipolar forces make a significant contribution to the structural integrity of the gellable

polymeric material, the pore size of the gel can be varied by appropriate modification of the chemical structure of the polymer, as well as manipulation of the electrolyte conditions (i.e., ionic strength, buffer type and pH).

5 Gellable polymeric materials suitable for use in the practice of the present invention can be either ionizable or non-ionizable, so long as they meet the criteria set forth herein.

 Crosslinking agents, when employed, are bifunctional compounds which serve to
10 bridge two different polymer chains. Commonly used crosslinking agents are α,ω -diolefins, which are incorporated into the forming polymer by free radical polymerization. The degree of crosslinking imparted to the resin impacts the pore size achievable by the resulting resin. When chemical crosslinking agents are not used for the preparation of gellable polymeric material, gel pore size can be controlled by
15 controlling the extent the gellable polymeric material is capable of chain entanglement and cohesive dipolar forces, and by controlling the electrolyte conditions (e.g., ionic strength, pH and buffer type) employed for the separation process. Thus, the longer the chain length of the polymer backbone between chemical crosslinks and/or chain entanglement points, the longer the potential pore size obtainable by the resulting gel.
20 Where the gellable polymeric material employed in the practice of the present invention forms a hydrogel, based at least in part, upon cohesive dipolar forces, the gel pore size can be varied by appropriate manipulation of the electrolyte conditions (e.g., ionic strength, buffer type and pH).

25 In accordance with a further aspect of the present invention, the separation medium is comprised of gellable polymeric materials characterized as thermally crosslinked gels (e.g., polyacrylamide), said gels containing in the range of about 4% to about 30% total monomer (generally expressed as %T), said gels formed by crosslinking polyacrylamide strands with crosslinking agent (e.g., bis-acrylamide) in the range of
30 about 2.5% to about 10% (generally known as %C). Addition of an organic agent in the gel buffer of the gels to achieve the benefits of enhanced separation speed has not previously been practiced. However, it has surprisingly been found that addition of about 1-30%, and more preferably 5-10% of organic agent to traditional

polyacrylamide gel media is quite beneficial, presumably as a result of decreased viscosity and dielectric constant of the modified media.

5 In another aspect of the invention, thermally crosslinked gels are equilibrated in gel buffer containing about 1-30% and more preferably about 5-10% of organic agent rather than carrying out polymerization in the presence of organic agent, in order to speed the polymerization step while retaining the beneficial aspects of inclusion of organic agents in the gel buffer.

10 As will be understood by those of skill in the art, the ratio of gellable polymeric material to mixed medium can vary widely depending on the intended use, the size and nature of biomolecules being separated, and the like.

15 In another embodiment of the present invention there are provided separation media for use in an electrophoresis system, wherein said separation media comprises a gellable polymeric material and a mixed medium, and wherein said mixed medium comprises an aqueous component and one or more organic agents. All components in the amounts specified herein are suitable for use in the practice of this embodiment of the invention.

20 In accordance with another embodiment of the present invention, there are provided articles useful for the separation of biological materials. Invention articles comprise a support having deposited thereon a layer of about 0.01-5 mm of a separation medium comprising a gellable polymeric material containing aqueous medium with
25 added organic buffer absorbed therein, wherein said gellable polymeric material is characterized as described herein.

30 In accordance with yet another embodiment of the present invention, there are provided articles comprising a support structure containing therein separation medium comprising gellable polymeric material containing aqueous medium with added organic buffer absorbed therein, wherein said gellable polymeric material is characterized as described herein.

Support materials contemplated for use in preparation of invention articles include glass plates, plastic sheets, and the like.

Alternatively, gellable polymeric material can be incorporated into support structures such as columns, glass tubing, capillary tubing, glass cells, and the like. Suitable support structures can be constructed of a variety of materials, as can be readily determined by those of skill in the art (e.g., glass, plastic, and the like).

The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1

Voltage-current relationship in gels with and without added organic agent.

The relationship between voltage and current, and thus resistance present in the gel electrophoresis system was investigated using random block copolymers of polyacrylonitrile and polyacrylamide, as described in US Patent 5,888,365, run in buffers with and without added organic agent. Typically, the gel buffer for AHT hydrogels comprises Tris (tris (hydroxy methyl) aminomethane)-borate buffer and 0.1% SDS with or without an organic agent such as 10% acetonitrile. The running buffers contain Tris-glycine buffer and 0.1% SDS. For comparison, polyacrylamide (Laemmli, 1970 *Nature*, 227, 680-686) gel buffer typically consists of 0.375 M Tris-HCl, pH 8.8, in the separating gel. The stacking gel consists of Tris-HCl, pH 6.8. The anode and cathode polyacrylamide running buffers Tris-glycine buffer and 0.1% SDS.

An example of the running conditions utilizing AHT HYDROGEL™ material for SDS-protein application is shown below.

	<u>Condition</u>	<u>AHT HYDROGEL™</u>	<u>Polyacrylamide gel</u>
30	Voltage	400-450 V	100-125 V
	Current	40-80 mA	30-66 mA

The following is the typical voltage – current relationship of the gel buffer containing 5-20% of organic agent at approximately 10 minutes after the onset of electrophoretic potential.

Organic Agent	Current and Voltage
No organic agent	62mA@350V
Glutaronitrile	45mA@350V
Acetonitrile	45mA@350V
Ethanol	60mA@338V
Methanol	60mA@327V

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The viscosity and the dielectric constants of water and some organic agents are as follows:

	Water	Methanol	Ethanol	Acetonitrile
10 Dielectric	77.9	30.3	24	36
Viscosity (mPa s at 25°C)	0.89	0.554	1.074	0.369

These data indicate that addition of organic agent to the electrophoresis buffer changes the resistivity of the system. The altered resistivity, derived from changes in the buffer and gel viscosity and dielectric constant, allow for more rapid mobility of biological molecules through the electrophoresis matrix.

Example 2.

20 Electrophoresis in copolymer hydrogels.

A series of AHT hydrogels that contained agents of decreasing dielectric constants were cast. Each gel was equilibrated in a buffer of appropriate composition for electrophoresis that included 5-20% of added organic agent such as acetonitrile, ethanol, acetone, or ethyl acetate. The gels were run without a stacking component. Gels were loaded with molecular weight standards (SIGMAMARKER™) prepared according to the manufacturer's instructions in 2x Laemmli loading buffer and

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electrophoresis was performed in an electrophoresis cell made to accommodate the hydrogels. Electrophoresis was performed by applying a electric charge limited to 450 volts and 80 milliamperes to the apparatus. The electrophoresis was terminated when the leading front of bromophenol blue marker dye had migrated to a position 55 mm from the sample well.

The time required to complete electrophoresis was recorded, and the gel was processed for staining with Coomassie Brilliant Blue stain. The distance (in mm) traveled by the protein markers from the sample well was measured following completion of the Coomassie Brilliant Blue staining procedure. The rate of electrophoretic separation (mm/min) was determined for each gel condition. In each case, gels with higher concentration of organic agent had a faster rate of separation than those with lower concentrations. For all gels with concentrations of organic agent at 10% or higher, the rate of separation was increased by 5 to 10% .

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Example 3.

Electrophoresis in polyacrylamide gels.

A series of polyacrylamide gels that contained agents of decreasing dielectric constants were cast. Each 5 ml gel contained 2.5 ml of 30% monomer acrylamide/bisacrylamide (29:1; 3.3% crosslinking), 0.375 M TrisHCl, pH 8.8, 0.1% SDS and 5-10% of an organic agent such as acetonitrile, ethanol, acetone, or ethyl acetate. The gels were run without a stacking component. Gels were loaded with molecular weight standards (SIGMAMARKER™) prepared according to the manufacturer's instructions in 2x Laemmli loading buffer and electrophoresis was performed in a BioRad Mini-PROTEAN II™ apparatus. The electrode buffer used was 0.025 M Tris, 0.192 M glycine, 0.1% SDS, with 125 ml in the cathodic end of the apparatus and 250 ml in the anodic end. The electrophoresis was performed by applying a constant 125 volts to the apparatus, and was continued until the leading front of bromophenol blue marker dye reached a point 5 mm from the bottom of the gel. The time required to complete electrophoresis was recorded, and the gel was processed for staining with Coomassie Brilliant Blue stain according to well-known protocols. The distance (in mm) traveled by the protein markers from the sample well

was measured following completion of the Coomassie Brilliant Blue staining procedure. The rate of electrophoretic separation (mm/min) was determined for each gel condition. Gels made with acetonitrile, acetone or ethyl acetate demonstrated increased rate of separation when compared with gels run in the absence of organic agent.

Example 4.

Electrophoresis in polyacrylamide gels.

The rate of electrophoresis in a polyacrylamide gel that contained 5% acetonitrile was compared to a standard polyacrylamide gel with no added organic agent. Each 5 ml gel contained 2.5 ml of 30% monomer acrylamide/bisacrylamide (29:1; 3.3% crosslinking), 0.375 M TrisHCl, pH 8.8, and 0.1% SDS. The gels were run without a stacking component. Gels were loaded with molecular weight standards (SIGMAMARKER™) prepared according to the manufacturer's instructions in 2x Laemmli loading buffer and electrophoresis was performed in a BioRad Mini-PROTEAN II™ apparatus. The electrode buffer used was 0.025 M Tris, 0.192 M glycine, 0.1% SDS, with 125 ml in the cathodic end of the apparatus and 250 ml in the anodic. The electrophoresis was performed by applying a constant 125 volts to the apparatus for 60 minutes, at which time the gel was processed for staining with Coomassie Brilliant Blue stain according to well-known protocols. The distance (in mm) traveled by the protein markers from the sample well was measured following completion of the Coomassie Brilliant Blue staining procedure. The rate of electrophoretic separation (mm/min) was determined for each gel condition. The gel made with acetonitrile demonstrated an increased rate of separation as compared to a standard polyacrylamide gel with no added organic agent.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

That which is claimed is:

1. A method for the rapid separation of biomolecules by size and relative charge, said method comprising:
 - a) applying a mixture of biomolecules to a separation medium comprising a gellable polymeric material and a mixed medium, wherein said mixed medium comprises an aqueous component and one or more organic agents,
 - b) applying an electric field to said separation medium, for a time sufficient to cause the electrophoretic separation of said biomolecules, according to the size and relative charge of said biomolecules.
2. A method according to claim 1, wherein said application of electric field is conducted at or above room temperature.
3. A method according to claim 1, wherein said application of electric field is conducted at or below room temperature.
4. A method according to claim 1, wherein said mixed medium comprises in the range of about 20 to about 99.5 wt% of said aqueous component.
5. A method according to claim 1, wherein said mixed medium comprises in the range of about 60 to about 99 wt% of said aqueous component.
6. A method according to claim 1, wherein said mixed medium comprises in the range of about 80 to about 99 wt% of said aqueous component.
7. A method according to claim 1, wherein said mixed medium comprises about 95 wt% of said aqueous component.
8. A method according to claim 1, wherein said mixed medium comprises in the range of about 1 to about 30 wt% of said organic agent.
9. A method according to claim 1, wherein said mixed medium comprises in the range of about 3 to about 20 wt% of said organic agent.
10. A method according to claim 1, wherein said mixed medium comprises in the range of about 4 to about 10 wt% of said organic agent.

11. A method according to claim 1, wherein said mixed medium comprises about 5 wt% of said organic agent.
12. A method according to claim 1, wherein said organic agent has a solubility of at least about 5% in water.
13. A method according to claim 1, wherein said organic agent is an alcohol, a ketone, a nitrile, a polar aprotic solvent, an ether, or a carbamide.
14. A method according to claim 13, wherein said alcohol is methanol, ethanol, isopropanol, phenol, or m-cresol.
15. A method according to claim 13, wherein said alcohol has a solubility of at least about 5% in water.
16. A method according to claim 13, wherein said ketone is acetone, methyl ethyl ketone, ethyl ethyl ketone or acetophenone.
17. A method according to claim 13, wherein said ketone has a solubility of at least about 5% in water.
18. A method according to claim 13, wherein said nitrile is acetonitrile, glutaronitrile, or propionitrile.
19. A method according to claim 13, wherein said nitrile has a solubility of at least about 5% in water.
20. A method according to claim 13, wherein said polar aprotic solvent is dimethyl formamide, dimethyl sulfoxide, chloral hydrate, formamide, benzaldehyde, ethyl acetate, N-methylacetamide, or N-methylpyrrolidinone.
21. A method according to claim 13, wherein said polar aprotic solvent has a solubility of at least about 5% in water.
22. A method according to claim 13, wherein said ether is tetrahydrofuran, dioxane, or dimethyl ether.
23. A method according to claim 13, wherein said ether has a solubility of at least about 5% in water.
24. A method according to claim 1, wherein said separation medium has a pH in the range of about 2 to 12.

25. A method according to claim 1, wherein said separation medium further comprises a denaturing agent.
26. A method according to claim 25, wherein said denaturing agent is sodium dodecyl sulfate, formamide, methyl mercury, β -mercaptoethanol, dithiothreitol or urea.
27. A method according to claim 1, wherein said gellable polymeric material is ionizable.
28. A method according to claim 1, wherein said gellable polymeric material is non-ionizable.
29. A method according to claim 1, wherein said gellable polymeric material is thermally crosslinked.
30. A method according to claim 29, wherein said thermally crosslinked gellable polymeric material is polyacrylamide, N-substituted polyacrylamide, poly-N-acryloyl-tris, acrylomorpholine, HYDROLINK™, acrylic and methacrylic esters, or poly(N-acylalkylenimine), an N-vinyl pyrrolidone-based polymer, a methacrylic acid-based polymer, or an acrylic acid-based polymer.
31. A method according to claim 29, wherein said thermally crosslinked gellable polymeric material is polyacrylamide.
32. A method according to claim 30, wherein said methacrylic acid-based polymer is a glycerol methacrylate-based polymer, or a 2-hydroxyethylmethacrylate-based polymer.
33. A method according to claim 30, wherein said acrylic or methacrylic ester is poly(ethylene glycol) methacrylate-acrylamide copolymer, polyhydroxy methacrylate, or poly(ethylene glycol) methacrylate.
34. A method according to claim 30, wherein said polymeric materials have one or more hydrophilic groups.
35. A method according to claim 34, wherein said hydrophilic group is hydroxy, amine, or a combination of two or more thereof.

36. A method according to claim 1, wherein said gellable polymeric material is an electrophoresis sponge.
37. A method according to claim 36, wherein said electrophoresis sponge comprises polyethylene, polypropylene, polytetrafluoroethylene, polyvinylidene difluoride, polynitrile, polystyrene, or combinations of two or more thereof.
38. A method according to claim 1, wherein said gellable polymeric material is a chain entangled polymer.
39. A method according to claim 1, wherein said gellable polymeric material chain entangled polymer comprises agarose.
40. A method according to claim 1, wherein said gellable polymeric material chain entangled polymer comprises AHT hydrogel.
41. A method according to claim 1, wherein said electric field is in the range of about 125V to 5000V.
42. A method according to claim 1, wherein said electric field is in the range of about 2000V to 5000V.
43. A method according to claim 1, wherein said electric field is in the range of about 1000V to 2000V.
44. A method according to claim 1, wherein said electric field is in the range of about 350V to 450V.
45. A separation medium for use in an electrophoresis system, wherein said separation medium comprises a gellable polymeric material and a mixed medium, and wherein said mixed medium comprises an aqueous component and one or more organic agents.
46. An article useful for the separation of biomolecules, said article comprising a support having deposited thereon a layer of separation medium according to claim 45.
47. An article according to claim 46, wherein said layer of separation material is in the range of about 0.01mm to 5mm in thickness.

48. A method for the rapid separation of biomolecules by size and relative charge, said method comprising:

- a) applying a mixture of biomolecules to a modified separation medium comprising a gellable polymeric material and a suitable electrophoresis buffer,
- b) applying an electric field to said modified separation medium, for a time sufficient to cause the electrophoretic separation of said biomolecules, according to the size and relative charge of said biomolecules,

wherein the viscosity and/or dielectric constant of said modified separation medium is selected such that biomolecules separate during electrophoresis therein at a more rapid rate than in unmodified separation medium.

49. A method according to claim 48 wherein said dielectric constant is in the range of about 10-40.

50. A method according to claim 48 wherein said viscosity is in the range of about 0.2 to 2.0 mPa s at 25°C.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11474

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 27/26, 27/447

US CL :204/456, 468, 469, 606

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 204/456, 468, 469, 606

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT

search terms: electrophor\$4, gel, buffer, organic additives recited in claims,e.g. methanol, ethanol, etc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,324,401 A (YEUNG et al) 28 June 1994, see col. 11, line 56 - col. 12, line 15, col. 14, lines 40-44, and col.15, line 65- 67.	1 - 3, 12 - 24, 27 - 29,31, 45 ----- 4-11
X --- Y	US 5,560,811 A (BRIGGS et al) 01 October 1996, see col. 9, line 24-42.	1-3,12-24, 26-31,38,45 ----- 4-11,41-44,46,47



Further documents are listed in the continuation of Box C.



See patent family annex.

"	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

21 JULY 2000

Date of mailing of the international search report

14 AUG 2000

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/11474

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 5,567,292 A (MADABHUSHI et al) 22 October 19996, see col. 2, lines 49-54, col. 5, lines 33-58, col. 8, line 51-col. 9, line 7, col.13, line 54-61, col. 14, lines 10 & 11.	1-5,8-28, 30,34,35,38,45 ----- 6,7,29,41- 44
Y	US 5,637,202 A (HARRINGTON et al) 10 June 1997, see abstract, col. 3, line 44-51 & lines 55-60, col. 9, lines 4-14.	36,37
X ---- Y	US 5,874,212 A (PROCKOP et al) 23 February 1999, see col. 4, line 63-col. 5, line 8, col.8, lines 26-54, col. 13, lines 28,29,33, & 34.	1-5,8-25, 27- 31,41,44,45 ----- 6,7,26,32- 40,42,43,46,47
X ---- Y	US 5,885,432 A (HOOPER et al) 23 March 1999, see col.4, lines 9-23 & 31-36.	1-5,8-10, 12-25,27- 31,34,35,39,45 ----- 6,7,11,46, 47
X ---- Y	US 5,888,365 A (SHIH et al) 30 March 1999, see col. 5, lines 16-22, col. 10, line 33.	1-3,12-24, 30,32,34, 35,40,45 ----- 4-11,46,47

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/11474

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-47

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/11474

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claim(s) 1-47, drawn to a separation medium and the use of the separation medium wherein the special technical feature is one or more organic additives.

Group II, claim(s) 48-50, drawn to a separation method wherein the special technical feature is the viscosity and/or dielectric constant of the separation medium.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical of the two groups is clearly different. In Group I the special technical feature is the organic additive(s) and in Group II the special technical feature is the viscosity and/or the dielectric constant of the separation medium.