(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 April 2013 (25.04.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/059690 A1

(51) **International Patent Classification**:
C07K 1/16 (2006.01) ***B01D 15/10*** (2006.01)
C07K 1/22 (2006.01) ***B01D 15/20*** (2006.01)
C07K 1/36 (2006.01)

(21) **International Application Number**:
PCT/US2012/061154

(22) **International Filing Date**:
19 October 2012 (19.10.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/549,146 19 October 2011 (19.10.2011) US

(71) **Applicant**: **BIO-RAD LABORATORIES, INC.** [US/US]; 1000 Alfred Nobel Drive, Hercules, CA 94547 (US).

(72) **Inventors**: **LIAO, Jiali**; 225 Gale Ridge Court, San Ramon, CA 94582 (US). **OLECH, Lee**; 542 Tennent Avenue, Pinole, CA 94564 (US). **CHEN, Hong**; 2244 Britannia Drive, San Ramon, CA 94582 (US). **HE, Xuemei**; 3606 Winchester Place, Walnut Creek, CA 94598 (US). **FROST, Russell**; 991 Rolling Woods Way, Concord, CA 94521 (US).

(74) **Agents**: **HEINES, M., Henry** et al.; Kilpatrick Townsend & Stockton LLP, Two Embarcadero Center, Eighth Floor, San Francisco, CA 94111-3834 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2013/059690 A1

(54) **Title**: SOLID PHASE FOR MIXED-MODE CHROMATOGRAPHIC PURIFICATION OF PROTEINS

(57) **Abstract**: Proteins are purified by a mixed-mode chromatography system formed by attaching a ligand with cation exchange and hydrophobic functionalities to a large-pore support matrix, the only linkage between the ligand and the support matrix being a chain having a backbone of no more than three atoms between the hydrophobic group and the support matrix.

SOLID PHASE FOR MIXED-MODE CHROMATOGRAPHIC PURIFICATION OF PROTEINS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of United States Provisional Patent Application No. 61/549,146, filed October 19, 2011, the contents of which are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0002] Technologies for extracting immunoglobulins or other proteins from source liquids for purposes of purification or isolation, with particular emphasis on chromatographic separation techniques and materials are provided.

2. Description of the Prior Art

[0003] The extraction of immunoglobulins and other proteins from source liquids, which are primarily mammalian bodily fluids or cell culture harvest, is of value in obtaining the proteins in a sufficiently concentrated or purified form for diagnostic and therapeutic uses as well as laboratory studies in general. Purifications of proteins, and particularly immunoglobulins, often suffer however from such factors as low yield, the use of costly separation media, the leaching of separation media into the product, and concerns for the safe disposal of extraneous materials used in the extraction process. The present invention seeks to address at least some of these issues.

SUMMARY OF THE INVENTION

[0004] It has now been discovered that an unusually efficient extraction (i.e. purification) of immunoglobulins and other proteins can be achieved by use of a mixed-mode chromatography system that combines cationic exchange and hydrophobic functionalities with a large-pore support matrix. The cationic exchange and hydrophobic functionalities are incorporated in a ligand that is bound to a solid matrix that has pores whose median diameter is 0.5 micron or greater, with substantially no pores of less than 0.1 micron in diameter, and the ligand is coupled to the support matrix at the hydrophobic group on the ligand through a linkage of a chain of one to three atoms. Binding of proteins to the matrix-supported ligand is achieved at low pH, and elution of the bound proteins is achieved at a higher pH, using conventional binding and elution conditions. Highly purified immunoglobulin in high yield, for example, is achieved with a single pass of the source liquid through the separation medium. The separation medium, i.e., the matrix-supported ligand, is itself novel, as is the method of attachment of the ligand to the matrix.

[0005] In some embodiments, a method for purifying a protein from a source solution is provided, as follows:

(a) contacting the source solution with a mixed-mode chromatography medium comprising a ligand coupled to a solid support, the ligand comprising a hydrophobic group and either a carboxyl group or a sulfo group, in which the hydrophobic group is joined to the carboxyl or sulfo group by a peptide-containing linkage, and in which the solid support has pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter, and in which the ligand is coupled to the solid support at the hydrophobic group through a chain of one to three atoms, to bind the protein in the source solution to the solid support through the ligand; and

(b) eluting the bound protein from the solid support.

[0006] In some embodiments, the protein is an antibody.

[0007] In some embodiments, the contacting step (step (a)) is performed at a pH of 4.0 to about 6.0 and the eluting step (step (b)) is performed at a pH of from about 6.1 to about 8.5.

[0008] Certain methods within the scope of the invention involve the following steps:

(a) at a pH of 4.0 to about 6.0, contacting a source solution containing antibodies with a mixed-mode chromatography medium comprising a ligand coupled to a solid support, the ligand comprising a hydrophobic group and either a carboxyl group or a sulfo group, and the solid support having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter, the ligand being coupled to the solid support at the hydrophobic group through a chain of one to three atoms, to bind at least a portion of the antibodies in the source solution to the solid support through the ligand; and

(b) eluting bound antibodies from the solid support at a pH of from about 6.1 to about 8.5.

[0009] In some embodiments, the hydrophobic group is a phenyl group, and in some embodiments, the phenyl group is joined to the acid moiety by a peptide-containing linkage.

[0010] In some embodiments, the ligand is benzamidoacetic acid. In some embodiments, the linkage is an alkylamino group at a para-position on the phenyl ring of the benzamidoacetic acid whereby the ligand and linkage together constitute a para-aminobenzamidoacetic acid group.

[0011] In some embodiments, the solid support consists of particles having a median particle size of from about 25 microns to about 150 microns.

[0012] In some embodiments, the solid support is a membrane. In some embodiments, the solid support is a monolith.

[0013] In some embodiments, a mixed-mode chromatography medium is provided. In some embodiments, the mixed-mode medium comprises a ligand coupled to a solid support, the ligand comprising a hydrophobic group and either a carboxyl group or a sulfo group, the solid support comprising particles having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter, and the ligand coupled to the solid support at the hydrophobic group by a chain of one to three atoms.

[0014] In some of the media, the particles have a median particle size of from about 25 microns to about 150 microns. In some of the media, the hydrophobic group is a phenyl group, and in some cases, the phenyl group is joined to the acid moiety by a peptide-containing linkage. In some of the media, the ligand is benzamidoacetic acid. In some of these media, the ligand is coupled to the solid support at the hydrophobic group by an alkylamino group at a para-position

on the phenyl ring of the benzamidoacetic acid whereby the ligand and the alkylamino group together constitute a 4-aminobenzamidoacetic acid group.

[0015] Also provided herein is a method for manufacturing a mixed-mode chromatography medium, including the following steps:

(a) oxidizing diol groups on diol-functionalized solid particles having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter and having a diol density of from about 200 to about 300 μmol/mL to aldehyde groups, thereby converting the diol-functionalized solid particles to aldehyde-functionalized solid particles; and

(b) coupling amine-functionalized ligands to the aldehyde-functionalized particles, the amine-functionalized ligands comprising an amine-substituted hydrophobic group joined to either a carboxyl group or a sulfo group.

[0016] In some embodiments of this method, the hydrophobic group is a phenyl group, and in some of these embodiments, the phenyl group is joined to the carboxyl group by a peptide-containing linkage. In some embodiments, the ligand is benzamidoacetic acid.

[0017] The term "sulfo group" throughout this specification means the group having the formula

$$—SO_3H$$

[0018] These and other objects, aspects, features, and advantages of the invention will be better understood by the explanations that follow.

BRIEF DESCRIPTION OF THE FIGURES

[0019] FIG. **1** is an absorbance profile of a purification of an IgG1 monoclonal antibody on a *p*-aminohippuric acid-functionalized resin in accordance with the present invention.

[0020] FIG. **2** is an electropherogram of the flow-through fraction from the initial column wash and two pooled fractions from the elution of FIG. **1**.

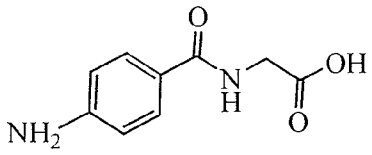
[0021] FIG. **3** is an absorbance profile of an HPLC-size exclusion chromatography analysis of the two pooled fractions from the elution of FIG. **1**.

DETAILED DESCRIPTION OF THE INVENTION AND ILLUSTRATIVE EMBODIMENTS

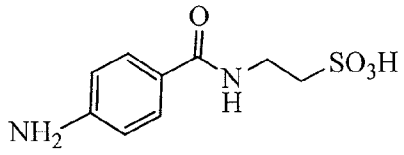
[0022] Structural groups that are useful as hydrophobic functionalities in the ligands described herein include aromatic and substituted aromatic groups. Phenyl and biphenyl groups, particularly phenyl groups, are common examples of aromatic groups and are used in certain embodiments herein. Suitable substituents are those that retain the hydrophobic character of the aromatic group; examples include certain alkyl groups such as hexyl. Substituents that create steric hindrance to the immunoglobulins are less preferred. Structural groups that are useful as cationic exchange functionalities include carboxylic acids, carboxylates, and sulfo groups, including both the sulfo group itself and sulfates. The cationic exchange and hydrophobic moieties can be joined by a chain, preferably a chain that contains no more than five atoms, excluding hydrogen atoms and substituents. Examples of such chains are peptide–containing chains, such as —R^1—C(O)—NH—R^2— where R^1 and R^2 are alkyl groups and one or both of R^1 and R^2 can be absent. A specific example is —C(O)—NH—CH_2—. A ligand containing the latter linkage between a carboxylic functionality as the weak cation exchange group and a phenyl functionality as the hydrophobic group is benzoylamino acetic acid.

[0023] The linkage joining the ligand to the support matrix (also referred to herein as a solid support) is a chain whose one end is directly coupled to the hydrophobic functionality of the ligand and whose other end is directly coupled to the matrix, the chain thereby including any group pendant from the matrix as a result of activation of the matrix for the coupling reaction. No spacer in addition to this linkage is included. As noted above, the chain is an amine-containing chain of one to three atoms. Examples of such a chain are those having the formula —R^3—NH—R^4— where R^3 and R^4 are methyl or ethyl groups and one or both of R^3 and R^4 can be absent. A specific example of such a group is —CH_2—NH—. In embodiments in which the ligand is a benzamidoacetic acid or a 2-benzamidoethanesulfonic acid, a particularly convenient linkage between the phenyl ring of the ligand and the matrix is one in which the amine group of the linkage is bonded to the phenyl ring at a *para*-position relative to the carbonylamino acetic acid group in the case of the benzamidoacetic acid or to the carbonylaminoethylsulfonic acid group in the case of the 2-benzamidoethanesulfonic acid.

[0024] An example of a compound that can form both the ligand and at least part of the linkage is 4-aminobenzamidoacetic acid, also known as *para*-aminohippuric acid, whose formula is as follows:



and another example of a compound that can form both the ligand and at least part of the linkage is 2-(4-aminobenzamido)ethanesulfonic acid, whose formula is as follows:



[0025] The term "chain" is used herein to denote a series of atoms joined together in a linear arrangement, preferably by single bonds, such as -A-B-C- ... etc. where the atoms are either all the same or contain one or more that differ from the other(s). The term includes both substituted and unsubstituted chains, "substituted" referring to atoms or groups other than hydrogen atoms, such as for example –OH, $-NH_2$, and =O, but in all cases, the number of atoms indicated as constituting the chain, such as in the expression "chain of one to three atoms," refers to the atoms in the linear array, excluding any hydrogen atoms and any substituent groups. The linearly arranged atoms are also referred to as the backbone of the chain.

[0026] The support matrix, as noted above, is one with pores of a median diameter of 0.5 micron or greater, with substantially no pores of less than 0.1 micron in diameter. In certain embodiments of the invention, the median pore diameter ranges from about 0.5 micron to about 2.0 microns. The pore volume can vary, although in many embodiments, the pore volume will range from about 0.5 to about 2.0 cc/g. The matrix can be particles, a membrane or a monolith, and by "monolith" is meant a single block, pellet, or slab of material. Particles when used as matrices can be spheres or beads, either smooth-surfaced or with a rough or textured surface. Many, and in some cases all, of the pores are through-pores, extending through the particles to serve as channels large enough to permit hydrodynamic flow or fast diffusion through the pores. When in the form of spheres or beads, the median particle diameter, where the term "diameter" refers to the longest exterior dimension of the particle, is preferably within the range of about 25 microns to about 150 microns. Disclosures of matrices meeting the descriptions in this paragraph and the processes by which they are made are found in Hjertén et al., United States Patent No. 5,645,717, Liao et al., United States Patent No. 5,647,979, Liao et al., United States

Patent No. 5,935,429, and Liao et al., United States Patent No. 6,423,666. Examples of monomers that can be polymerized to achieve useful matrices are vinyl acetate, vinyl propylamine, acrylic acid, methacrylate, butyl acrylate, acrylamide, methacrylamide, vinyl pyrrolidone (vinyl pyrrolidinone), with functional groups in some cases. Crosslinking agents are also of use in many embodiments, and when present will generally constitute a mole ratio of from about 0.1 to about 0.7 relative to total monomer. Examples of crosslinking agents are dihydroxyethylenebisacrylamide, diallyltartardiamide, triallyl citric triamide, ethylene diacrylate, bisacrylylcystamine, N,N'-methylenebisacrylamide, and piperazine diacrylamide.

[0027] For purposes of the formation of a linkage with the ligand, and particularly ligands with amine groups, the inclusion of monomers with vicinal diols is often useful. One example is allyloxy propandiol (3-allyloxy-1,2-propanediol). Vicinal diol monomers can be used with other monomers to prepare copolymers. The diol group density in the polymers produced from diol-containing monomers can vary widely, such as for example densities within a range of from about 100 to 1,000 μmol/mL (i.e., micromoles of diol per milliliter of packed beads), and in many cases a range of from about 200 to 300 μmol/mL. An example of a matrix that meets this description and is commercially available is UNOsphere™ Diol (Bio-Rad Laboratories, Inc., Hercules, California, USA). To couple a pendant amine-containing ligand to a matrix with exposed vicinal diols, the diols can be oxidized to aldehyde groups, and the aldehyde groups can then be coupled to amine groups to form secondary amino linkages, all by conventional chemistry techniques well known in the art.

[0028] Protein purification utilizing a resin (i.e., separation medium) in accordance with the present invention can be achieved by conventional means known to those of skill in the art. Examples of proteins include but are not limited to antibodies, enzymes, growth regulators, clotting factors, transcription factors and phosphoproteins. In many such conventional procedures, the resin prior to use is equilibrated with a buffer at the pH that will be used for the binding of the target protein (e.g., antibody or non-antibody protein). Equilibration can be done with respect to all features that will affect the binding environment, including ionic strength and conductivity when appropriate.

[0029] In some embodiments, the resins described herein can be used in "bind-elute" mode to purify a target protein from a biological sample. In some embodiments, following binding of the target protein to the resin, a change in pH can be used to elute the target protein.

[0030] In some embodiments, once the resin is equilibrated, the source liquid is loaded onto the resin while maintaining the source liquid, and any additional carrier liquid when used, to a pH below 6.0 with an appropriate buffer, allowing the target protein to bind to the resin. Notably, it has been found that the mixed mode resins described herein function with solutions having salt concentrations in the range of salt concentrations of cell cultures (e.g., 50-300 mM, or about 100-150 mM). Thus, in some embodiments, the protein is loaded to the resin under such salt concentrations.

[0031] In some embodiments, the resin is then washed with a wash liquid, optionally at the same pH as that of the loading step, to remove any unbound biological species that may have been present in the source liquid.

[0032] The bound protein (e.g., antibody or non-antibody protein, as desired) can be subsequently eluted. In some embodiments, the protein is then eluted with an elution liquid at a pH above 6.0. Illustrative pH ranges, as cited above, are pH 4.0-6.0 for the binding and washing steps, and pH 6.1-8.5 for the elution step. In certain embodiments, the binding and washing steps are performed with the inclusion of a salt in the sample and wash liquids. Examples of salts that can be used for this purpose are alkali metal and alkaline earth metal halides, notably sodium and potassium halides, and as a specific example sodium chloride. The concentration of the salt can vary; in most cases, an appropriate concentration will be one within the range of about 10mM to about 1M. As will be seen in the working examples below, optimal elution conditions for some proteins will involve a buffer with a higher salt concentration than that of the binding buffer, and in other cases by a buffer with a lower salt concentration than that of the binding buffer. The optimal choice in any particular case is readily determined by routine experimentation.

[0033] The resin can be utilized in any conventional configuration, including packed columns and fluidized or expanded-bed columns, and by any conventional method, including batchwise modes for loading, washes, and elution, as well as continuous or flow-through modes. The use of a packed flow-through column is particularly convenient, both for preparative-scale extractions and analytical-scale extractions. A column may thus range in diameter from 1cm to 1m, and in height from 1cm to 30cm or more.

[0034] "Antibody" refers to an immunoglobulin, composite (e.g., fusion), or fragmentary form thereof. The term may include but is not limited to polyclonal or monoclonal antibodies of the

classes IgA, IgD, IgE, IgG, and IgM, derived from human or other mammalian cell lines, including natural or genetically modified forms such as humanized, human, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies. "Antibody" may also include composite forms including but not limited to fusion proteins containing an immunoglobulin moiety. "Antibody" may also include antibody fragments such as Fab, $F(ab')_2$, Fv, scFv, Fd, dAb, Fc and other compositions, whether or not they retain antigen-binding function.

[0035] Any antibody preparation can be used in the present invention, including unpurified or partially purified antibodies from natural, synthetic, or recombinant sources. Unpurified antibody preparations can come from various sources including, but not limited to, plasma, serum, ascites, milk, plant extracts, bacterial lysates, yeast lysates, or conditioned cell culture media. Partially purified preparations can come from unpurified preparations that have been processed by at least one chromatography, precipitation, other fractionation step, or any combination of the foregoing. In some embodiments, the antibodies have not been purified by protein A affinity prior to purification.

[0036] As noted above, it is believed that the resins are also useful for purification of non-antibody proteins. Examples of therapeutic proteins include, but are not limited to, Factor VIII von Willebrand Factor enzymes, growth regulators, clotting factors, transcription factors and phosphoproteins.

EXAMPLE 1. Preparation of *p*-Aminohippuric Acid-Functionalized Resin

[0037] UNOsphere™ Diol (10mL), a copolymer of 3-allyloxy-1,2-propanediol and vinyl pyrrolidinone, crosslinked with N,N'-methylenebisacrylamide and with a diol density of 200-300μmol/mL, was used in the form of spherical beads. The beads were suspended in 10mL of either 0.1M sodium acetate or water. Sodium periodate was added to a concentration within the range of 50 to 100mM, and the resulting mixture was incubated at room temperature (approximately 70°F (21°C)) for 3-24 hours. The reaction resulted in conversion of the diol groups to aldehyde groups in the range of 150-250μmol/mL. The resulting aldehyde-functionalized resin was transferred to a 20-mL column where it was washed with 100mL of water.

[0038] The washed resin was then suspended in 10mL of 0.05M sodium phosphate buffer at pH 7.0 and mixed with *p*-aminohippuric acid, and the resulting mixture was incubated in a shaker at 200rpm for thirty minutes at 37°C. To the mixture was then added $NaBH_3CN$ (100mg), and the concentration of *p*-aminohippuric acid in the reaction mixture was in the range of 25 to 100mM. After three hours of reaction time, the resulting *p*-aminohippuric acid-functionalized resin was transferred to a 20-mL column where it was washed with three column volumes of water followed by one to two column volumes of 0.1N aqueous NaOH, then washed with water again until the pH of the eluent was below 10. The *p*-aminohippuric acid ligand density in the resulting product was in the range of 25-100 μmol/mL.

EXAMPLE 2. Binding of Immunoglobulin to *p*-Aminohippuric Acid-Functionalized Resin

[0039] A column measuring 7mm in inner diameter and 5.5cm in length was packed with the *p*-aminohippuric acid-functionalized resin prepared in Example 1 and equilibrated with 20mM sodium acetate buffer containing 150mM NaCl at pH 4.5. A 1.0 mg/mL solution of human immunoglobulin G in this buffer was then applied to the column at a flow rate of 1mL/min. When the column effluent absorbance at 280nm reached a value equal to 10% of the value corresponding to the 1.0 mg/mL human IgG solution, thereby indicating 10% breakthrough, the column was washed with equilibration buffer. Binding capacity was determined by multiplying the retention time to 10% breakthrough by the flow rate and the immunoglobulin concentration. The dynamic binding capacity of the immunoglobulin was 40mg/mL (i.e., 40mg of immunoglobulin per mL of column packing). Bound immunoglobulin was eluted using 100mM sodium phosphate buffer, pH 7.0.

EXAMPLE 3. Purification of Immunoglobulin G on *p*-Aminohippuric Acid-Functionalized Resin from Mammalian Culture Filtrate

[0040] A 0.57cm × 4cm column of *p*-aminohippuric acid-functionalized resin prepared as in Example 1 (60 μmoles *p*-aminohippuric acid/mL beads), was equilibrated with 50mM sodium acetate, 125mM NaCl, pH 5.0. Ten milliliters of Chinese hamster ovary (CHO) cell culture harvest, containing 12 mg of mAb 1, an IgG 1 monoclonal antibody, were applied to the column at a linear flow rate of 300 cm/h. The column was then washed with the equilibration buffer until the absorbance at 280nm reached baseline. At approximately 36 minutes, an elution buffer containing 50mM sodium phosphate, 50mM NaCl, pH 6.2, was passed through the column to

elute bound antibody, and the pooled eluate obtained with this buffer was collected and designated Pool 1. At approximately 44 minutes, the column was further eluted with 20mM sodium phosphate, 1M NaCl, pH 7.5, and the eluate obtained with this buffer until the absorbance returned to baseline was pooled and designated Pool 2. The column was then cleaned by washing with 1M NaOH. Optical densities were measured at 260nm and 280nm, and the detector output is shown in FIG. **1**, which is a plot of optical density expressed in absorbance units vs. time minutes and shows the detector signal for the flow-through fraction ("Flow-through") during the wash with equilibration buffer in addition to Pools 1 and 2.

[0041] Polyacrylamide gel electrophoresis of the Flow-through fraction and Pools 1 and 2 (FIG. **1**) produced the electropherogram shown in FIG. **2**, which shows that no antibody was present in the flow-through fraction while Pool 1 was highly enriched in antibody. The host cell protein level in Pool 1 was reduced from 3.5×10^4 ng/mg in the CHO cell harvest, to 391 ng/mg, as determined using host cell protein assay kit CM015 from Cygnus Technologies (Southport, North Carolina, USA). The DNA level in Pool 1 was decreased from $>5.0 \times 10^3$ ng/mg in the CHO cell harvest to 458 ng/mg, as determined by PicoGreen assay. Size exclusion chromatography using a Bio-Sil 250 column (Bio-Rad Laboratories, Inc., Hercules, California, USA) was performed on Pools 1 and 2 with results shown in FIG. **3**. The results show that Pool 1 contained monomeric antibody while Pool 2 contained a small amount of monomeric antibody and a significant amount of aggregated antibody.

EXAMPLE 4. Binding of Immunoglobulin G to *2-(4-*Aminobenzamido)ethanesulfonic Acid-Functionalized Resin

[0042] A 0.7cm × 5.5cm column of *2-(4-*aminobenzamido)ethanesulfonic acid-functionalized resin prepared in a manner analogous to that of Example 1 on the same solid support was equilibrated with 20mM sodium acetate, 150mM NaCl, pH 4.5. A 1.0 mg/mL solution of human immunoglobulin G in this buffer was then applied to the column at a flow rate of 1 mL/min. When the column effluent absorbance at 280nm reached a value equal to 10% of the value corresponding to the 1.0 mg/mL human IgG solution, thereby indicating 10% breakthrough, the column was washed with equilibration buffer. Binding capacity was determined by multiplying the retention time to 10% breakthrough by the flow rate and the immunoglobulin concentration. The dynamic binding capacity of the immunoglobulin was 36mg/mL. Bound immunoglobulin was eluted using 100mM sodium phosphate buffer, pH 7.0. The recovery was measured at 87%.

EXAMPLE 5. Binding and Elution Studies of Various Proteins on p-Aminohippuric Acid-Functionalized Resin

[0043] Example 2 was repeated with series of proteins to determine optimal conditions for both binding and elution. The results are shown in the table below.

TABLE: Binding and Elution Conditions for Proteins on p-Aminohippuric Acid-Functionalized Resin

Test protein	pI	Molecular Mass	Optimal Conditions	
			Binding	Elution
Bovine serum albumin	4.8 – 5.5	56 kDa	100mM NaCl, pH 4.0	1000mM NaCl, pH 8.0
Bovine carbonic anhydrase	5.9	29 kDa	10mM NaCl, pH 4.6	800mM NaCl, pH 7.6
Lysozyme	9.3	15 kDa	10mM NaCl, pH 4.0	1000mM NaCl, pH 8.0
Conalbumin	6.9	78 kDa	10mM NaCl, pH 4.0	505mM NaCl, pH 6.0
Lactoferrin	9.2	78 kDa	205mM NaCl, pH 4.0	1000mM NaCl, pH 8.0
mAbX	9.2 – 9.5	150 kDa	300mM NaCl, pH 4.6	800mM NaCl, pH 8.0

[0044] The table shows that the binding of bovine serum albumin, bovine carbonic anhydrase, and lysozyme bound to the resin occurred in a primarily cation-exchange mode, as indicated by the great increase in salt concentration in the optimal elution buffer as compared to the optimal binding buffer. Conversely, the binding of lactoferrin and mAbX occurred in a mixed mode (cation exchange and hydrophobic interaction), as indicated by the need for 200-300 mM NaCl to achieve sufficient binding of the target proteins and the need for increases in both salt concentration and pH to achieve complete elution. The binding of conalbumin occurred primarily in the cation exchange mode, while the concentration of NaCl in the optimal elution buffer for this protein was modest at 505 mM at pH 6. Salt concentrations higher than 505 mM produced stronger binding of conalbumin to the column and therefore made elution more difficult, which indicates a transition to a hydrophobic interaction mode as buffer conductivity increases.

EXAMPLE 6. Purification of IgM BF on p-Aminohippuric Acid-Functionalized Resin

[0045] This example illustrates the conditions for purifying IgM BF (pI = 5.3-5.5) from a sample whose main contaminant is bovine serum albumin (pI = 5) on a p-aminohippuric acid-functionalized resin. Using the resin of Example 1 and the procedure of Example 2, a 30-mL sample was applied to the resin, washed with 20 mL of a binding buffer containing 20 mM sodium phosphate and 125 mM NaCl at pH 6.5 at 200 cm/h, and eluted with 10 mL of an elution buffer containing 20 mM sodium phosphate and 400 mM NaCl at pH 7.0 at 200 cm/h, followed by column regeneration with 19 mL of 1N NaOH. The BSA did not bind to the column, but instead appeared in a broad peak extending from 14 mL to 56 mL of column fraction collection; IgM eluted in a sharp peak at 80 mL, and other impurities eluted in a sharp peak at 94 mL. The three peaks were fully resolved with no overlap.

EXAMPLE 7. Polishing of mAb1 on p-Aminohippuric Acid-Functionalized Resin

[0046] This example illustrates the conditions for purifying mAb1 on a p-aminohippuric acid-functionalized resin. Using the resin of Example 1 and the procedure of Example 2, the sample was applied to the resin with a binding buffer containing 50 mM sodium acetate and 125 mM NaCl at pH 5.0 for 35 minutes, followed by a gradient elution to 100% elution buffer at 35-45 minutes (fifteen column volumes), followed by 100% elution buffer the elution buffer at 45-55 minutes (five column volumes), the elution buffer containing 50 mM sodium phosphate and 50 mM NaCl at pH 6.2. The elution buffer was followed by a stripping buffer at 55-65 minutes, the stripping buffer containing 200 mM sodium phosphate and 700 mM NaCl at pH 7.5, and a final 1N NaOH stripping solution at 70 minutes. The mAb1 eluted in a sharp peak at 48 minutes, followed by smaller but still sharp peaks at the starts of the stripping buffer and the NaOH solution, respectively. All peaks were fully resolved with no overlap. This example shows that elution can take place at a lower salt concentration than that present in the binding buffer.

EXAMPLE 8. Removal of mAb2 Aggregates From mAb2 Using p-Aminohippuric Acid-Functionalized Resin

[0047] This example illustrates the conditions for the removal of mAb2 Aggregates from mAb2 on a p-aminohippuric acid-functionalized resin. Using the resin of Example 1 in a column 0.56 cm in diameter and 4cm in length and a flow rate of 300 cm/h, an mAb2 sample was applied to the resin with a binding buffer (Buffer A) containing 20 mM sodium acetate and 300

mM NaCl at pH 4.5 for 33 minutes, then washed with 20 mM MES and 20 mM NaCl at pH 6.0 (Buffer B), followed by a gradient elution of Buffer B to Buffer C (20 mM sodium phosphate and 1 M NaCl at pH 7.5) over 33-52 minutes (25 column volumes), then held at 100% Buffer C at 52-57 minutes, and finally regenerated with 1N NaOH. A first peak eluted at 42 minutes and a second peak at 60 minutes, both peaks fully resolved. The first peak was analyzed by size exclusion chromatography (HPLC) which indicated that the peak contained monomer with less than 0.2% aggregate, as compared to 11% aggregate in the sample. Monomer recovery was greater than 80%.

[0048] In the claims appended hereto, the term "a" or "an" is intended to mean "one or more." The term "comprise" and variations thereof such as "comprises" and "comprising," when preceding the recitation of a step or an element, are intended to mean that the addition of further steps or elements is optional and not excluded. All patents, patent applications, and other published reference materials cited in this specification are hereby incorporated herein by reference in their entirety. Any discrepancy between any reference material cited herein or any prior art in general and an explicit teaching of this specification is intended to be resolved in favor of the teaching in this specification. This includes any discrepancy between an art-understood definition of a word or phrase and a definition explicitly provided in this specification of the same word or phrase.

WHAT IS CLAIMED IS:

1. A method for purifying a protein from a source solution, said method comprising:

(a) contacting said source solution with a mixed-mode chromatography medium comprising a ligand coupled to a solid support, said ligand comprising a hydrophobic group and an acid moiety selected from the group consisting of a carboxyl group and a sulfo group wherein said hydrophobic group is joined to said acid moiety by a peptide-containing linkage, said solid support having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter, and said ligand coupled to said solid support at said hydrophobic group through a chain of one to three atoms, to bind said protein in said source solution to said solid support through said ligand; and

(b) eluting said protein so bound from said solid support.

2. The method of claim **1** wherein said protein is an antibody.

3. The method of claims **1** or **2** wherein step (a) is performed at a pH of about 4.0 to about 6.0 and step (b) is performed at a pH of from about 6.1 to about 8.5.

4. The method of claim **1** wherein said hydrophobic group is a phenyl group.

5. The method of claim **1** wherein said ligand is benzamidoacetic acid.

6. The method of claim **1** wherein said ligand is 2-benzamidoethanesulfonic acid.

7. The method of claim **5** wherein said linkage is an alkylamino group at a *para*-position on the phenyl ring of said benzamidoacetic acid whereby said ligand and chain together constitute a 2-(4-aminobenzamido)acetic acid group.

8. The method of claim **6** wherein said linkage is an alkylamino group at a *para*-position on the phenyl ring of said 2-benzamidoethanesulfonic acid whereby said ligand and chain together constitute a 2-(4-aminobenzamido)ethanesulfonic acid group.

9. The method of claim **1** wherein said solid support consists of particles having a median particle size of from about 25 microns to about 150 microns.

10. The method of claim **1** wherein said solid support is a membrane.

11. The method of claim **1** wherein said solid support is a monolith.

12. The method of claim **1** wherein said source solution contains a salt selected from alkali metal and alkaline earth metal halides at a concentration of from about 50mM to about 300mM.

13. The method of claim **1** wherein said source solution contains a salt selected from alkali metal and alkaline earth metal halides at a concentration of from about 100mM to about 150mM.

14. A mixed-mode chromatography medium comprising a ligand coupled to a solid support, said ligand comprising a hydrophobic group and an acid moiety selected from the group consisting of a carboxyl group and a sulfo group, wherein said hydrophobic group is joined to said acid moiety by a peptide-containing linkage, said solid support comprising particles having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter, and said ligand coupled to said solid support at said hydrophobic group through a chain of one to three atoms.

15. The mixed-mode chromatography medium of claim **14** wherein said particles have a median particle size of from about 25 microns to about 150 microns.

16. The mixed-mode chromatography medium of claim **14** wherein said hydrophobic group is a phenyl group.

17. The mixed-mode chromatography medium of claim **16** wherein said acid moiety is a carboxyl group.

18. The mixed-mode chromatography medium of claim **16** wherein said acid moiety is a sulfo group.

19. The mixed-mode chromatography medium of claim **14** wherein said ligand is benzamidoacetic acid.

20. The mixed-mode chromatography medium of claim **14** wherein said ligand is 2-benzamidoethanesulfonic acid.

21. The mixed-mode chromatography medium of claim **19** wherein said linkage is an alkylamino group at a *para*-position on the phenyl ring of said benzamidoacetic acid whereby said ligand and chain together constitute a 2-(4-aminobenzamido)acetic acid group.

22. The mixed-mode chromatography medium of claim **20** wherein said linkage is an alkylamino group at a *para*-position on the phenyl ring of said 2-benzamido-ethanesulfonic acid whereby said ligand and chain together constitute a 2-(4-aminobenzamido)-ethanesulfonic acid group.

23. A method for manufacturing a mixed-mode chromatography medium, said method comprising:

(a) oxidizing diol groups on diol-functionalized solid particles having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter and having a diol density of from about 200 to about 300 μmol/mL to aldehyde groups, thereby converting said diol-functionalized solid particles to aldehyde-functionalized solid particles; and

(b) coupling amine-functionalized ligands to said aldehyde-functionalized solid particles, said amine-functionalized ligands comprising an amine-substituted hydrophobic group joined to an acid moiety selected from the group consisting of a carboxyl group and a sulfo group.

24. The method of claim **23** wherein said hydrophobic group is a phenyl group.

25. The method of claim **24** wherein said phenyl group is joined to said carboxyl group by a peptide-containing linkage.

26. The method of claim **23** wherein said ligands are benzamidoacetic acid.

27. The method of claim 23 wherein said ligands are 2-benzamido-ethanesulfonic acid.

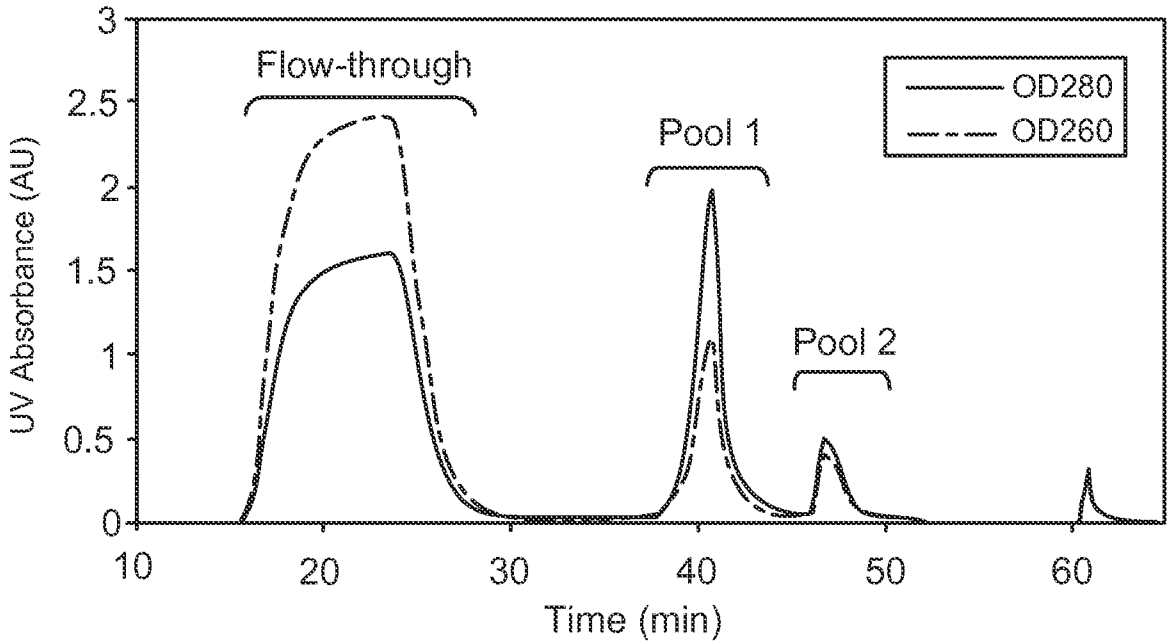

Flow-through
Pool 1
Pool 2
OD280
OD260
UV Absorbance (AU)
Time (min)
0
0.5
1
1.5
2
2.5
3
10
20
30
40
50
60

FIG. 1

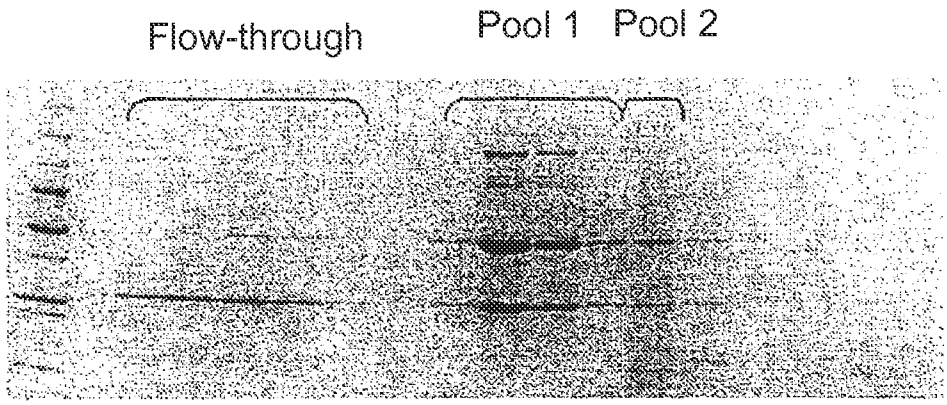

Flow-through
Pool 1
Pool 2

FIG. 2

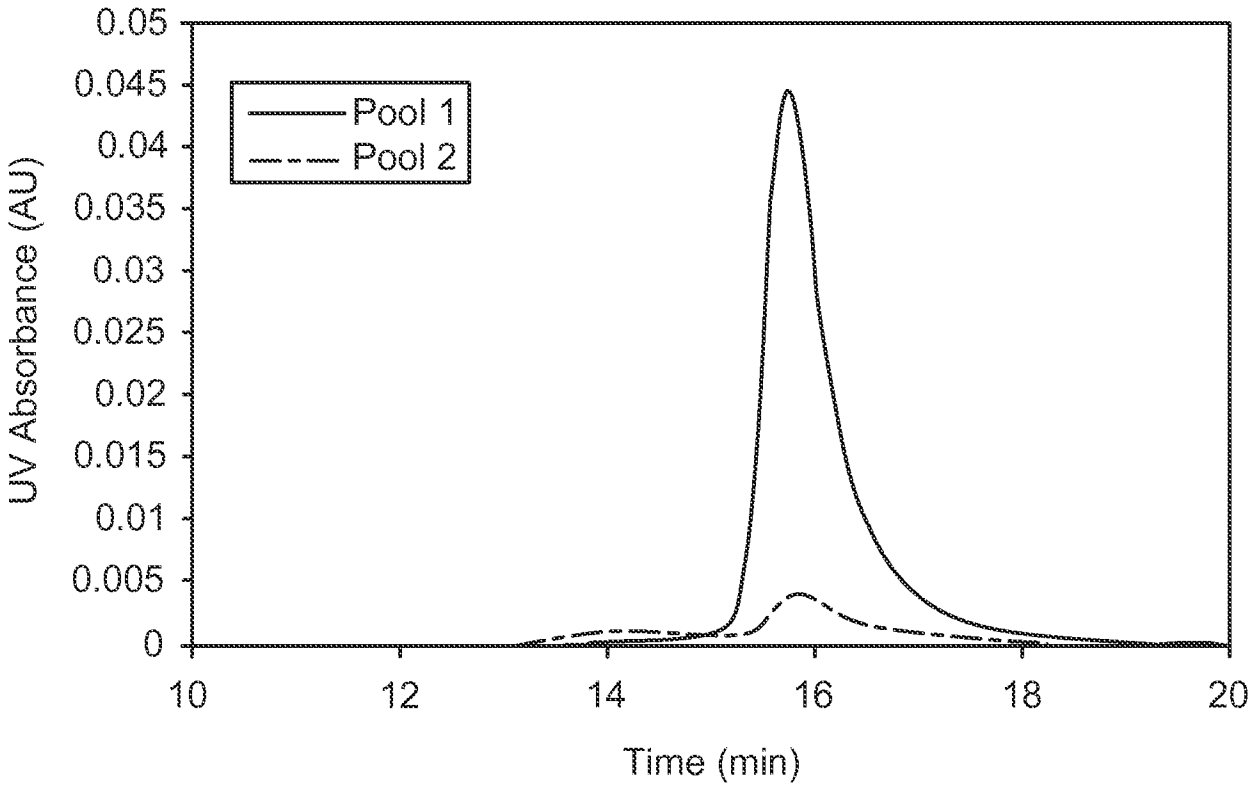

0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
UV Absorbance (AU)
Pool 1
Pool 2
10
12
14
16
18
20
Time (min)

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/61154

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 1/16, C07K 1/22, C07K 1/36, B01D 15/10, B01D 15/20 (2013.01)
USPC - 530/413; 210/198.2
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)
IPC (8)- C07K 1/16, C07K 1/22, C07K 1/36, B01D 15/10, B01D 15/2 (2013.01)
USPC - 530/413; 210/198.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 530/413; 210/198.2, 530/412, 413, 414, 416, 417; 210/198.2

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase (PGPB, USPT, USOC, EPAB, JPAB, DWPI, TDBD), FreePatentsOnline (US Pat, PgPub, EPO, JPO, WIPO, NPL), GoogleScholar (PL, NPL); search terms: mixed-mode chromatography medium ligand coupled solid support carboxy sulfo aminohippurate aminohippuric acid p-aminohippuric acid paraaminohippuric acid ethanesulfonic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2011/049798 A1 (Wenger et al.) 28 April 2011 (28.04.2011) pg 1, ln 6-9, pg 3, ln 16-22, pg 15, ln 12-15, pg 12, ln 19-26, with pg 13, Table 1, pg 14, ln 24 to pg 15, ln 11, pg 21, ln 16-18, pg 24, ln 5-8	1-5, 9-13 ---------- 6-8
Y	WO 2011/012722 A1 (Rau et al.) 03 February 2011 (03.02.2011) pg 18, ln 4-16, 24-27, pg 19, ln 10, pg 13, ln 6-12 , pg 14, ln 15-16	6-8
A	US 4,452,931 A (Okamoto et al.) 05 June 1984 (05.06.1984) col 1, ln 7-13, col 4, ln 49	1-13
A	US 2007/0244307 A1 (Engstrand et al.) 18 October 2007 (18.10.2007) Abstract	1-13
A	US 5,945,520 A (Burton et al) 31 August 1999 (31.08.1999) Abstract	1-13
A	US 2005/0020812 A1 (Angus) 27 January 2005 (27.01.2005) Abstract	1-13

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"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
"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
"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
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 2013 (12.02.2013)	05 MAR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/61154

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search 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 No. III Observations where unity of invention is lacking (Continuation of item 3 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 additional fees, this Authority did not invite payment of additional fees.

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.:
Group I: Claims 1-13

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/61154

Box III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-13 relate to methods for purifying a protein from a source solution comprising: (a) contacting a source solution with mixed-mode chromatography medium; and (b) eluting the protein bound to said solid support.

Group II: Claims 14-22 relate to a mixed-mode chromatography medium.

Group III: Claims 23-27 relate to a method for manufacturing a mixed-mode chromatography medium.

The inventions listed as Groups I-III do not relate to a single general 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:

Groups II and III do not include the intentive concept of methods for purifying a protein from a source solution comprising: (a) contacting a source solution with mixed-mode chromatography medium; and (b) eluting the protein bound to said solid support, as required by Group I.

Groups I and II do not include the intentive concept of a method for manufacturing a mixed-mode chromatography medium by (a) oxidizing diol groups on diol-functionalized solid particles to aldehyde groups, thereby converting said diol-functionalized solid particles to aldehyde-functionalized solid particles; and (b) coupling amine-functionalized ligands to said aldehyde-functionalized solid particles, said amine-functionalized ligands comprising an amine-substituted hydrophobic group joined to an acid moiety selected from the group consisting of a carboxyl group and a sulfo group; as required by Group III.

Groups I, II and III share the technical features of being related to a mixed-mode chromatography medium as set forth in claims 1 and 14. However, the shared technical features fail to make a contribution over the prior art WO/2011/049798 A1 to Wenger et al. (hereinafter 'Wenger') (publication date 28 April 2011), which discloses a method for purifying a protein (pg 1, ln 7-9) from a source solution (pg 14, ln 24 to pg 15, ln 11), said method comprising: (a) contacting said source solution with a mixed-mode chromatography medium (pg 3, ln 18 and pg 15, ln 12-15) comprising a ligand coupled to a solid support (pg 15, ln 14-15 and pg 14, ln 5-12), said ligand comprising a hydrophobic group and an acid moiety selected from the group consisting of a carboxyl group and a sulfo group (pg 12, ln 19-26, with pg 13, Table 1), wherein said hydrophobic group (phenyl group, Table 1) is joined to said acid moiety (acid moiety, Table 1) by a peptide containing linkage (-CH-NH-CO-; Table 1, as defined in para [0022] of applicants specification), said solid support having pores of a median diameter of 0.5 micron or greater with substantially no pores of 0.1 micron or less in diameter (pg 15, ln 18-20), and said ligand coupled to said solid support at said hydrophobic group through a chain of one to three atoms (pg 12, ln 19-26, with pg 13, Table 1), to bind said protein in said source solution to said solid support through said ligand (pg 3, ln 16-22); and (b) eluting said protein so bound from said solid support (pg 3, ln 19-22 and pg 16, ln 25-31). As mixed-mode chromatography mediums as set forth in claims 1 and 14 were known, as evidenced by the disclosure of Wenger, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I-III therefore lack unity under PCT Rule 13.1 because they do not share a same or corresponding special technical feature.