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

(19) **World Intellectual Property Organization**
International Bureau

(43) **International Publication Date**
17 September 2015 (17.09.2015)

WIPO | PCT

(10) **International Publication Number**
WO 2015/137860 A1

(51) **International Patent Classification**:
B01J 20/32 (2006.01) ***B01D 15/38*** (2006.01)
B01D 15/32 (2006.01) ***B01J 20/281*** (2006.01)
B01D 15/34 (2006.01) ***C08J 7/00*** (2006.01)

(21) **International Application Number**:
PCT/SE2015/050186

(22) **International Filing Date**:
18 February 2015 (18.02.2015)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
1450290-0 14 March 2014 (14.03.2014) SE

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(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, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, 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, SA, 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, ST, 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, KM, ML, MR, NE, SN, TD, TG).

Published:

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

(54) Title: SEPARATION MATRICES FOR PURIFICATION OF BIOLOGICAL PARTICLES

(57) Abstract: The invention discloses a separation matrix for purification of biological particles, comprising a plurality of particles having a porous core entity and a porous shell entity covering the core entity, wherein the core entity comprises at least 50 micromole/ml primary amines present on covalently attached ligands displaying at least two primary amines per ligand and the shell entity comprises less than 20 micromole/ml primary amines. The invention further discloses a method of purifying biological particles and a method of manufacturing a separation matrix.

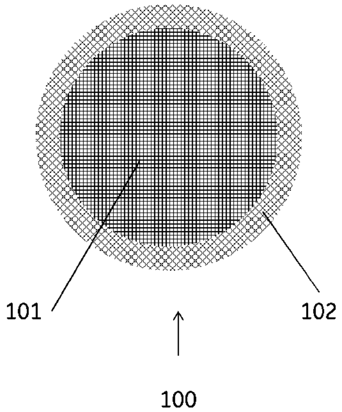

Fig. 1.

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SEPARATION MATRICES FOR PURIFICATION OF BIOLOGICAL PARTICLES

Technical field of the invention

The present invention relates to separation matrices for purification of biological particles, and more particularly to separation matrices for purification of vaccine antigens, e.g. virus particles. The invention also relates to use of separation matrices for purification of biological particles, to methods for purification of biological particles and to methods of manufacturing separation matrices.

Background of the invention

In many vaccines the antigen is composed of particles having a size considerably larger than protein molecules. This is the case for e.g. viruses, virus-like particles, bacteria and plasmid antigens. In the manufacturing process of these antigens they are produced together with proteins and other contaminating species of considerably smaller size than the antigens. These species can be e.g. egg proteins for egg-derived antigens and host cell proteins as well as culture media components for antigens produced in cell cultures. These proteins are present in high amounts and have to be removed during processing of the antigen, as they can e.g. give rise to allergic reactions.

Size exclusion chromatography has traditionally been used for size-based separation of antigens and contaminating proteins, but increasing demands on throughput and process economics have prompted the search for alternative methods. Recently, a class of chromatography media has been made available where an inner core of chromatography beads is functionalized with ligands having both a positive charge and a hydrophobic moiety, while an outer shell is inert and has a pore size smaller than a virus antigen. The contaminating proteins can penetrate through the shell into the core where they are strongly bound by the ligands and the virus particles will pass through the column without binding and can be collected in the flowthrough. Such media are commercially available from GE Healthcare Bio-Sciences AB under the name of Capto™ Core 700. Similar media have also been described in WO9839364, WO2009131526 and US7208093, which are hereby incorporated by reference in their entireties.

Most antigens are produced under conditions where the ionic strength is close to what is normal under physiological conditions, i.e. about 0.1-0.2 mol/l, corresponding to conductivities of about 10-20 mS/cm. Under these conditions the binding capacity of existing products, particularly those relying on charge interaction mechanisms, is limited and it would be desirable to increase the binding capacity at high salt conditions in order to avoid dilutions or buffer exchanges before application of the feed to the chromatography media.

Further, it is desirable to re-use the chromatography media for a large number of separation cycles. This necessitates a cleaning (also called regeneration) of the media between the cycles to remove the adsorbed proteins. With hydrophobic moieties present, this can be challenging and may require addition of solvents such as alcohols, which causes a fire and explosion hazard during operation. This problem becomes further accentuated in cases where the hydrophobicity of the ligands is increased in order to increase the binding capacity at high ionic strengths.

Accordingly there is a need for new matrices allowing the purification of vaccine antigens and other biological particles from contaminating proteins at high ionic strengths and at the same time allowing a safe and convenient cleaning of the matrices before re-use.

Summary of the invention

One aspect of the invention is to provide a regeneratable separation matrix capable of binding high amounts of proteins at high ionic strengths and allowing biological particles to be retrieved in a flowthrough fraction. This is achieved with a separation matrix as defined in claim 1.

One advantage is that the matrix still has a high binding capacity for common contaminating proteins at ionic strengths of 100-200 mM and higher. Further advantages are that the matrix does not bind virus particles or virus-like particles and that a wide range of tenaciously adsorbed contaminating proteins can be easily removed by the application of solvent-free regenerating solutions, allowing convenient reuse of the matrix.

A second aspect of the invention is to provide a method for separation of biological particles. This is achieved with a method as defined in the claims. Advantages of this method include the possibility to bind high amounts of contaminating proteins at high ionic strengths and the ease of regeneration of the matrix.

A third aspect of the invention is to provide a use of a separation matrix for purification of biological particles. This is achieved with a method as defined in the claims.

A fourth aspect of the invention is to provide a method of manufacturing a separation matrix suitable for purification of biological particles. This is achieved with a method as defined in the claims.

Further suitable embodiments of the invention are described in the dependent claims.

Figures

Fig. 1 shows a schematic structure of the matrix of the invention.

Fig. 2 shows examples of ligands: a) tris(2-aminoethyl)amine ligand precursor, b) tris(2-aminoethyl)amine coupled ligand, c) 1,3-diamino-2-propanol ligand precursor, d) 1,3-diamino-2-propanol coupled ligand, e) polyallylamine ligand precursor and f) polyallylamine coupled ligand.

Fig. 3 shows examples of synthesis routes of the invention, a) allylation, b) bromination, c) inactivation and d) coupling.

Fig. 4 shows chip electrophoresis data for extracts of E Coli-fouled Capto^{TM} Core 700 after regeneration with different regeneration solutions. Lanes: 1 – Mw markers; 2 – 1 M NaOH; 3 – 2 M NaOH; 4 – 2 M NaOH + 30% 2-propanol; 5 – 30% 2-propanol; 6 – 1 M NaOH + 20% 1-propanol; 7 - 1 M NaOH + 10% 1-propanol; 8 – 1 M NaOH + 5% 1-propanol; 9 – 20% 1-propanol; 10 – 0.5M NaOH/10 mM HCl/0.5M NaOH; 11 – 1M NaOH/10 mM HCl/1M NaOH; 12 – 20% propylene glycol; 13 – 40% propylene glycol; 14 – 1M NaOH + 20% propylene glycol; 15 – 1M NaOH + 40% propylene glycol; 16 – 8M urea/1MNaOH; 17 – 1Murea/1M NaOH; 18 – 8M urea; 19 - 8M urea + 0.1 M citric acid; 20 - 8M urea + 1M NaCl + 0.1 M citric acid.

Fig. 5 shows chip electrophoresis data for extracts of E Coli-fouled polyallylamine prototype after regeneration with different regeneration solutions. Lanes: 1 – 1 M NaOH; 2 – 2 M NaOH; 3 – 1 M NaOH + 30% 2-propanol; 4 – 1 M NaOH + 20% 1-propanol; 5 – 8 M urea; 6 – 8M urea +0.1 M citric acid; 7 - 6M guanidinium hydrochloride; 8 – 0.5 M NaOH + 1M NaCl; 9 – 1M

NaOH/10 mM HCl/0.5M NaOH; 10 – 1M urea/1M NaOH; 11 – No regeneration; 12 – Mw markers

Fig. 6 shows chip electrophoresis data for extracts of E Coli-fouled tris(2-aminoethyl)amine prototype after regeneration with different regeneration solutions. Lanes: 1 – 1 M NaOH; 2 – 2 M NaOH; 3 – 1 M NaOH + 30% 2-propanol; 4 – 1 M NaOH + 20% 1-propanol; 5 – 8 M urea; 6 – 8M urea +0.1 M citric acid; 7 - 6M guanidinium hydrochloride; 8 – 0.5 M NaOH + 1M NaCl; 9 – 1M NaOH/10 mM HCl/0.5M NaOH; 10 – 1M urea/1M NaOH; 11 – No regeneration; 12 – Mw markers

Detailed description of embodiments

In one aspect the present invention, as illustrated by Figs. 1 and 2, discloses a separation matrix for purification of biological particles, which comprises a plurality of particles **100** having a porous core entity **101** and a porous shell entity **102** covering the core entity. Suitably, each particle **100** within the plurality can have a porous core entity **101** covered by a porous shell entity **102**. The core entity comprises at least 50 micromole/ml primary amines present on covalently attached ligands displaying at least two primary amines per ligand and the shell entity comprises less than 20 micromole/ml primary amines, such as less than 10 or less than 1 micromole/ml. The term displaying means that the ligands have at least two primary amines per ligand when the ligand is in the attached state. The presence of a shell entity with a lower ligand content than the core entity can e.g. be detected by microscopy. The particles can be contacted with an acidic fluorescent dye (e.g. fluorescein), or a fluorescent dye reactive towards primary amines (e.g. 5-carboxyfluorescein succinimidyl ester), and the spatial distribution of the fluorescence can be measured in a confocal microscope. Alternatively the particles can be dyed, embedded in a resin, sectioned with a microtome and observed in a conventional microscope. With both methods, the relative ligand concentrations in the two regions can be assessed by densitometry and absolute concentrations calculated by multiplication with the total ligand content for the matrix as determined e.g. by titration methods or spectroscopic methods well known in the art.

In certain embodiments, the matrix is capable of binding at least 20 mg ovalbumin per ml matrix, such as at least 30 or at least 40 mg ovalbumin, in an aqueous buffer having a conductivity of at least 10 mS/cm, such as at least 20 mS/cm. 10 mS/cm corresponds to about

0.1 M NaCl and 20 mS/cm to about 0.2 M NaCl. As most physiological systems such as cell cultures, egg fluids, blood plasma, milk etc have conductivities in these ranges, it is advantageous to have a high capacity for protein binding, as exemplified by ovalbumin, under these conditions. The matrix can also be capable of binding at even higher conductivities such as e.g. 20-30 or 30-40 mS/cm, which is of interest e.g. when the liquid is an eluate from an ion exchange or a HIC matrix. The test for binding capacity can suitably be carried out in a Tris buffer at pH 7.5, using a static capacity test with 60 min contact time as described in the Examples.

In some embodiments, said ligands display at least two, such as at least three or at least four primary amines per ligand. It appears that the number of available primary amines per ligand is advantageous for the binding capacity at high ionic strengths and that it should be at least two. It can further be advantageous if the primary amines are bound to primary carbon atoms with only one bond to another carbon, such as in aminomethyl- or aminoethyl groups, H_2N-CH_2- and $H_2N-CH_2-CH_2-$ respectively. This provides better accessibility to proteins compared to primary amines bound to secondary or tertiary carbons. The ligands can be of different molecular weight, monomeric or polymeric and may form part of larger structures.

In certain embodiments, the ligands are attached to the core entity via secondary or tertiary amine links. Amine links are chemically stable and easy to achieve by coupling between an amine on a ligand precursor and an electrophilic group on a support material, such as e.g. an epoxide or a halohydrin. The ligand precursor can suitably be a species with at least three primary amines, such that one primary amine is consumed to make the attachment link and at least two remaining primary amines per ligand are displayed in the coupled matrix.

In some embodiments, the combined content of nitrogen and oxygen atoms in said ligands is at least 20 wt %. The content of primary amine nitrogen in the attached ligands can advantageously be at least 18 wt%. The ratio of displayed primary amines to the total amount of nitrogen atoms can be at least 0.5, such as at least 0.7 or at least 0.9. With these ratios the ligands are hydrophilic, i.e. they do not bind proteins irreversibly, and the primary amine content is high enough to give high binding at high ionic strengths.

In certain embodiments, the ligands are selected from the group consisting of tris(2-aminoethyl)amine and polyallylamine. These ligands are hydrophilic and display a plurality of primary amines even when attached via an amine. The ligands can e.g. comprise polyallylamine of molecular weight at least 1 kDa, such as at least 5 kDa or at least 10 kDa. The polyallylamine can suitably be non-crosslinked to provide mobility and access to proteins. The attachment can be in a single point or by multipoint attachment. Single point attachment can e.g. be achieved by graft polymerization or by coupling via a reactive end-group on the polyallylamine. Multipoint attachment can be achieved by coupling the polyallylamine via the amines to electrophilic groups or aldehydes on the core entities according to methods known in the art.

In some embodiments, the shell entity has an average thickness of 1 to 10 micrometers, such as 1 to 6 micrometers. A minimum shell thickness ensures that ligands are not available for binding to the biological particles on the outside of the separation matrix particles, while the shell thickness should suitably not be too high, in order to maximize the binding capacity for proteins in the core entity. The particles can be substantially spherical, e.g. with a volume-weighted average diameter of 15 – 400 micrometers, and in this case the shell entity can have an average thickness of 0.5 to 6 % of the volume-weighted average diameter of the particles. If the particles are non-spherical, the shell entity can have an average thickness of 0.5 to 6 % of the sphere-equivalent diameter of the particles. The average sphericity of the particles, defined as the ratio of the surface area of a sphere (having the same volume as a given particle) to the surface area of the particle, can be at least 0.9, such as at least 0.97. The shell thickness can be determined microscopically on dyed particles, as discussed above. The separation matrix particles can e.g. be manufactured according to the method disclosed below, in which case the thickness of the shell can be controlled by a partial deactivation process.

In certain embodiments, the shell entity has a molecular weight cutoff for globular proteins of 60 to 1000 kDa, such as 100-1000 or 400-800 kDa. This is advantageous to allow rapid mass transport of contaminating proteins into and out of the particles, while preventing the biological particles from reaching the core entity. The cutoff can e.g. be measured by comparing binding capacities for proteins of different molecular weight under conditions where they bind to the ligands.

In a second aspect the present invention discloses a method for purification of biological particles, comprising the steps of

a) providing a separation matrix according to any embodiment disclosed above;

b) contacting said matrix with a liquid comprising biological particles and at least one contaminant protein, such that said contaminant protein binds to said matrix;

c) separating said liquid from said matrix and recovering said liquid with purified biological particles.

The liquid can be e.g. an allantoic fluid from eggs, a cell culture supernatant, a cell lysate etc and it can e.g. have a conductivity of at least 10 mS/cm or even at least 15 mS/cm, which means that the liquid does not have to be extensively diluted. In addition to contaminant protein(s), also other contaminants such as e.g. DNA and/or endotoxins may be bound to the matrix and thus removed. The method may be a chromatographic method, where the matrix is packed into a column, but it may also be a batch adsorption method (e.g. using a magnetic matrix) or e.g. an expanded bed adsorption method (e.g. using matrix particles comprising high density filler particles). When the matrix is used as a packed bed in a column, the column may have a bed height of 1 mm – 1 m, such as 1 cm to 30 cm or 1 cm to 10 cm. The packed bed may comprise only the matrix of the invention but mixed beds with other matrices are also contemplated.

In certain embodiments the method further comprises the steps of
d) regenerating said matrix by contacting it with a regeneration solution, and;
e) repeating steps a) – c) at least once, such as at least five, at least 10 or at least 50 times. The regeneration solution can suitably be free from organic solvents or contain less than 15 wt%, such as less than 5 wt% or less than 1 wt%, flammable solvents, which is advantageous in that expensive explosion-proof equipment and localities does not have to be used in the process. In addition, it also provides a more environmentally friendly process.

In some embodiments the regeneration solution comprises alkali, such as NaOH, of e.g. 0.1-2 M concentration, such as 0.5-2 M. Alkali, and in particular NaOH, is a useful cleaning agent in that it hydrolyses proteins and in that after washing and neutralization it does not leave any potentially toxic residuals.

In certain embodiments the residual protein content in the matrix after step d) is less than 100 micrograms/ml matrix. The residual protein amount can be determined by a total extraction by boiling in a 0.5% SDS + 28 mM DTT extraction solution and detection of the extracted proteins by chip electrophoresis, as described in the Examples.

In some embodiments the biological particles are selected from the group consisting of viruses, virus-like particles, cells, organelles and plasmids.

In certain embodiments at least 40 mg/ml, such as at least 60 mg/ml, of said contaminant protein binds to the matrix. It is advantageous if the matrix can be loaded with a large amount of liquid, which is possible with the new matrix. As is well known, the loading should not exceed the binding capacity of the matrix as this would lead to breakthrough and consequently poor removal.

In some embodiments the liquid comprises a buffering substance. This buffering substance may e.g. comprise buffering cations, such as Tris, Bis-Tris, Tricine or piperazine, and/or monovalent buffering anions, such as acetate, lactate etc. It can be advantageous if the liquid does not comprise polyvalent anions, e.g. phosphate, as they may interact too strongly with the ligands under some conditions.

In certain embodiments the pH of the liquid is 6.0-8.5, such as 6.5-8.5 or 6.5-8.0. When live/viable biological particles are to be purified, they are highly sensitive to pH, which in these cases limits the usable range.

In some embodiments the contaminant protein is an albumin, such as ovalbumin or serum albumin. Albumins are common contaminant proteins both in egg-derived feeds and in cell cultures.

In certain embodiments the biological particles comprise an influenza virus. The liquid can e.g. comprise allantoic fluid derived from fertilized eggs or alternatively it can comprise a cell culture supernatant or a cell lysate.

In some embodiments the method can further comprise, before step b), a step a’) of conditioning said liquid by crossflow filtration, such as ultrafiltration and/or microfiltration. Microfiltration, e.g. in hollow fibers, can be used to remove particulates which may otherwise clog the chromatography column. If desired, the liquid may also be subjected to ultrafiltration to increase the concentration of biological particles before applying the liquid to the matrix.

In certain embodiments the method can further comprise, after step c), a step c’) of ion exchange chromatography, such as cation exchange chromatography. Any remaining positively charged contaminants can be removed with a subsequent cation exchange step. Subsequent steps may also comprise further flowthrough purification on matrices with functional core entitites and inert shell entitites.

In a third aspect the present invention discloses the use of a separation matrix as disclosed above for purification of biological particles. The biological particles can e.g. be selected from the group consisting of viruses, virus-like particles, cells, organelles and plasmids. The use can e.g. be in a method as disclosed above.

In a fourth aspect the invention discloses a method for manufacturing a separation matrix as disclosed above, comprising the steps of:
a) providing a plurality of porous polysaccharide support particles, such as crosslinked agarose gel particles;
b) reacting said particles with an allylation reagent to obtain at least 50, at least 100 or at least 200 micromole/ml allyl groups covalently attached to the particles;
c) reacting said particles with a halogen for a period of up to 30 minutes and then with an alkaline aqueous solution;
d) coupling a ligand precursor comprising a plurality of primary amines to the remaining allyl groups.
The allylation reagent can be e.g. allyl glycidyl ether or an allyl halide, such as allyl bromide. The halogen is suitably bromine and can be applied in an aqueous solution. In step b), allyl groups are introduced uniformly throughout the particles, while in step b), the short contact time with the bromine only allows reaction between the allyl groups in the outermost part of each particle and the bromine. The reaction with bromine causes the formation of bromohydrins, which in aqueous alkali are converted to hydrophilic, non-reactive diols, thus causing the formation of an inert shell entity. If the aqueous alkali solution comprises a polyol, the polyol may react with the bromohydrins and be immobilized as hydrophilic non-reactive moieties. Such methods can also be used to fine-tune the pore structure of the shell entity. The allyl groups in the interior are left unreacted and can then be used to couple ligands in what becomes the core entity. The thickness of the shell entity can be controlled primarily by the contact time with the halogen and by the amount of halogen used in step c).

In certain embodiments the support particles are substantially spherical, with a volume average diameter of 15-400 micrometers, such as 30-100 micrometers. The support particles can e.g. be made from dextran, agarose, agar, carrageenan, alginate, cellulose, konjac or other suitable polysaccharides. Agarose, agarose derivatives (e.g. hydroxyethyl agarose), agar and cellulose can be particularly suitable as they can conveniently be prepared with suitable porosity and rigidity. The support particles can also be crosslinked, either by straight forward crosslinking with a crosslinking agent such as epichlorohydrin or a diepoxide or by two-stage crosslinking such as described in US Pat. 6,602,990. The latter method provides an improved rigidity.

In some embodiments the ligand precursor has at least three primary amines per molecule and is coupled to the allyl groups by first reacting the allyl groups with an aqueous halogen, e.g. bromine, and then under alkaline conditions with the ligand precursor.

In certain embodiments the ligand precursor is selected from the group consisting of tris(2-aminoethyl)amine and polyallylamine, e.g. polyallylamine of Mw at least 5 kDa, such as at least 10 kDa.

Examples

Example 1. Synthesis of prototypes.

Support particles

The support particles used were highly crosslinked agarose beads, prepared according to the methods described in US Pat. 6,602,990, which is hereby incorporated by reference in its entirety. The beads had a volume-weighted average diameter (D50,v) of 88 micrometers and a pore size distribution such that 69 % of the pore volume was available to dextran molecules of Mw 110 kDa. This can also be expressed such that Kd for dextran 110 kDa on the beads was 0.69, when measured according to the methods described in "Handbook of Process Chromatography, A Guide to Optimization, Scale-Up and validation" (1997) Academic Press, San Diego. Gail Sofer & Lars Hagel eds.ISBN 0-12-654266-X, p. 368.

Allylations

Intermediate 5803

400 mL (g) of support particles were washed with six gel volumes distilled water and then three gel volumes with 50% NaOH. The gel was then sucked dry and transferred to a 2 L round bottom flask. 775 mL of 50% NaOH was added, mechanical propeller stirring was applied and

the flask was immersed into a water bath at 50°C. After 30 minutes 128 mL of allyl glycidyl ether (AGE) was added. The reaction progressed for 17 h. The gel was washed with one gel volume distilled water, five gel volumes of ethanol and then eight gel volumes of distilled water.

Intermediate 5840

120 mL (g) of support particles were washed with six gel volumes distilled water, was dried by vaccum and transferred (90.7 g) to a 250 mL round bottom flask. 149.3 mL of 50% NaOH was added (11.8 M), mechanical propeller stirring was applied and the flask was immersed into a water bath at 50°C. After 30 minutes 36 mL of AGE was added. The reaction progressed for 17 h. The gel was washed with one gel volume distilled water, three gel volumes of ethanol and then eight gel volumes distilled water.

Intermediate 6383

200 mL (g) allylated support particles 5803, in distilled water, was washed on a glass filter with 3 gel volumes of 50% aqueous NaOH. The gel was then sucked dry and transferred to a 1 L round bottom flask. 388 mL of 50% aqueous NaOH was added, mechanical propeller stirring was applied and the flask was immersed into a water bath at 50°C. After 30 minutes 64 mL of allyl glycidyl ether was added and the reaction progressed for 16 h. The gel was washed with one gel volume of distilled water, 5 gel volumes of ethanol and then 8 gel volumes of distilled water.

Intermediate 3266A

256 mL(g) support particles were washed with distilled water and with 50% aqueous NaOH on a glass filter. The gel was then sucked dry and transferred to a 1 L round bottom flask. 200 mL of 50% aqueous NaOH was added, mechanical propeller stirring was applied and the flask was immersed into a water bath at 50°C. After 30 minutes 200 mL of AGE was added and the reaction progressed for 17 h. The gel was washed with distilled water, ethanol and distilled water.

Partial bromination and shell inactivation

Intermediate 6478

192 g (mL) of allylated support particles, 6383, was transferred drained into a 3 L round bottomed flask together with 1728 mL of distilled water. Mechanical stirring was applied. A

solution of 1000 µL bromine in 200 mL of water was prepared. The bromine solution (equivalent to the amount of allyl groups in a 5 µm shell of the 88 µm bead) was added slowly during approximately 2 minutes at a stirring speed of 300 rpm. After 20 minutes the gel was washed with 10 gel volumes of distilled water.

196 g (mL) of the partially activated gel was transferred drained into a 1 L round bottomed flask. 175.3 g of distilled water and 20.7 mL of 50% NaOH (1 M) were added and mechanical stirring was applied. The flask was immersed into a water bath at 50 °C and the reaction progressed for 18.5h. The gel was then washed with 10 gel volumes of distilled water. 2x1 mL was titrated for remaining allyl content.

Intermediate 5860

115 g (mL) of allylated support particles, 5840, was transferred drained into a 2 L round bottomed flask together with 1035 mL of distilled water. Mechanical stirring was applied. A solution of 408 µL bromine in 100 mL of water was prepared. The bromine solution (equivalent to the allyls in a 5.5 µm shell of the 88 µm bead) was added slowly during approximately 2 minutes at a stirring speed of 250 rpm. Some of the bromine was lost during handling when pouring from the E-flask. Thereafter additions were made by using a plastic pipette. After 20 minutes the gel was washed with ten gel volumes distilled water.

116 g (mL) of the partially activated gel was transferred drained into a 500 mL round bottomed flask. 104 g of distilled water and 12.25 mL of 50% NaOH (1 M) were added and mechanical stirring was applied. The flask was immersed into a water bath at 50 °C and the reaction progressed for 16.5h. The gel was then washed with ten gel volumes distilled water. 2x1 mL was titrated for remaining allyl content.

Intermediate 3266B

260 g (mL) of allylated support particles, 3266A, was transferred drained into a 3 L round bottomed flask together with 2300 mL of distilled water. Mechanical stirring was applied. A solution of 3.55 g bromine in 250 mL of water was prepared. The bromine solution was added slowly during stirring. After 5 minutes the gel was washed with distilled water.

The partially activated gel was transferred drained into a 1 L round bottomed flask. 250 mL 2 M aqueous NaOH were added and mechanical stirring was applied. The flask was immersed into a water bath at 50 °C and the reaction progressed for 17 h. The gel was then washed with distilled water. 2x1 mL was titrated for remaining allyl content.

Core bromination

80 g (mL) of core allylated support particles, 6478, was transferred drained into a 500 mL E-flask with 80 mL of water and 3.2 g of sodium acetate. An aqueous solution of bromine was then added until a persistent yellow colour existed. Sodium formate (~2g) was added to quench the excess of bromine. The gel was washed with 10 gel volumes of distilled water.

Titration of allyl and remaining allyl (to determine inactivated shell thickness)

- The gel is washed with water.
- 1.0 mL gel is measured with a cube and transferred to a suction flask with 9 mL of distilled water.
- A saturated solution of bromine in water is added until a yellow color due to an excess of Br2 is persisting.
- The sample is left with magnetic stirring for 5 min.
- The sample is put under vacuum (water suction) with magnetic stirring to remove the excess of bromine.
- The sample is then transferred to a titration beaker by rinsing the flask with 10 mL of distilled water.
- 2-3 drops of conc. HNO3 are added and the titration with 0.1 M AgNO3 for indirect measurement of allyl content is started.
- The result is given as micromol/mL gel.
- The theoretical shell thickness is calculated from a model where the residual allyl content after partial bromination and inactivation is assumed to be entirely in a core surrounded by a shell with no allyl groups. With a bead of 88 micrometer diameter and a shell thickness of x micrometers, the ratio of the allyl contents before and after bromination/inactivation will be $44^3/(44-x)^3$, from which x can be calculated.

Table 1. Allyl group content of allylated and of brominated/deactivated intermediates.

Prototype	Allyl content µmol/mL	Theoretical shell thickness µm
5840	**210**	n/a
5860 After partial bromination and inactivation	**164**	3.5

6383	**336**	n/a
6478 After partial bromination and inactivation	**264**	3.5
3266 A	**255**	n/a
3266 B After partial bromination and inactivation	**190**	4.0

Ligand coupling

1,3-diamino-2-propanol coupling

24.8 g of 1,3-diamino-2-propanol (270 mmol, 34 eq) was transferred into a 100 mL round bottomed flask. The flask was immersed into a water bath at 60 °C to melt the ligand for 75 minutes.

30 mL (g) of core activated allylated support particles 6478 (7.92 mmol allyl, 1 eq) was transferred drained into the flask and mechanical propeller stirring (230 rpm) was applied. The reaction progressed for 17h. After cooling down the flask, pH was measured to ~12. The gel was washed with 12 gel volumes of distilled water.

Tris(2-aminoethyl)amine coupling

40 mL (g) of core activated allylated base matrix 6478 (10.56 mmol allyl, 1 eq) was transferred drained into a 250 mL round bottomed flask. 39.6 mL of tris(2-aminoethyl)amine (264.3 mmol, 25 eq) was added and mechanical propeller stirring (200 rpm) was applied. The reaction progressed for 17h. pH was measured to 8.8. The gel was washed with 10 gel volumes of distilled water.

Polyallylamine coupling

37 mL (g) of core activated allylated base matrix, 3266B, was transferred drained into a 250 mL round bottomed flask. 11.3 g polyallylamine Mw 15 kDa (Aldrich, 283215) was dissolved in 20 ml distilled water + 2 ml 50% aqueous NaOH and added to the flask. Mechanical propeller stirring (200 rpm) was applied and the reaction was allowed to proceed at 50 C for 17h. The gel was first washed with 0.5 M HCl, then with 1 mM HCl and then with 10 gel volumes of distilled water.

Titration of amine content

- The gel is washed with water.
- The gel is washed with 3x0.5 M HCl and then with 3x1 mM HCl.
- 1.0 mL gel is measured with a cube and transferred to a titration cup with 19 mL of distilled water.
- 2-3 drops of conc. HNO_3 are added and titration with 0.1 M $AgNO_3$ for indirect measurement of amine content is started.
- The result is given as micromol/mL gel.

Table 2. Amine content of core-shell prototypes.

Prototype	Amine content µmol/mL	Ligand density µmol/mL
1,3-diamino-2-propanol	**168**	**84**
Tris(2-aminoethyl)amine	**221**	**55**
Polyallylamine Mw 15 kDa	**210**	**0.8**

Example 2. Binding capacities for proteins on prototypes.

Empty 96-well filter plates were filled with 6 µl gel per well. The method described in Table 3 was used during the plate experiments. The number of washes after incubation varied between two and three dependent of what was needed to wash out any unbound protein.

Table 3. Method for plate experiments

Step	**Volume (µl)**	**Buffer**	**Execution**
Equilibration	3x200	Buffer	1 min shake + vacuum
Protein addition	100 + 100	Protein solution + Buffer	60 min shake + centrifugation
Wash	Xx200	Buffer	1 min shake + Centrifugation

Analysis

Unbound protein is analyzed by absorbance at 280 nm and the concentration calculated from a calibration curve.

The amount of bound protein is calculated from the difference between the added protein and the amount of unbound protein.

From the concentration the capacity for each well is calculated.

The maximum capacity is expressed as g/L, this is based on calculations preparing the protein solution for each experiment not through UV-measurements in cuvette.

For each series a blank experiment was conducted with no protein added, to exclude any interference from other UV-absorbing materials.

Binding capacity results for BSA and Ovalbumin on PAA

Prototype: Polyallylamine
Ion capacity: 210µmol/ml
Protein 1: BSA 2g/l, IP~4.7
Protein 1: Ovalbumin 2g/l, IP~4.7
Buffer system 1: 40mM Tris pH 7.2-8.2
Buffer system 2: 20mM Piperazine pH 6
Salt: NaCl

Table 4. Binding capacity (g/L) for BSA on Polyallylamine prototype.

NaCl (mM)	**0**	**250**	**500**	**750**	**1000**	**1250**
pH 7,2 40mM Tris	32	62	33	11	1	0
pH 7,7 40mM Tris	32	62	44		3	0
pH 8,2 40mM Tris	44	61	40	14	3	0

As shown in Table 4 the polyallylamine prototype had the highest binding capacity for BSA at 250mM NaCl. At 1250mM NaCl the polyalylamine prototype has no capacity for BSA which indicates good possibilities for salt elution of BSA.

Table 5. Binding capacity (g/L) for BSA and Ovalbumin on Polyallylamine prototype.

		BSA
A	pH6 20mM Piperazine	63
B	pH6 20mM Piperazine 250mM NaCl	49
C	pH6 20mM Piperazine 500mM NaCl	17
		Ovalbumin
E	pH6 20mM Piperazine	65
F	pH6 20mM Piperazine 250mM NaCl	41

G	pH6 20mM Piperazine 500mM NaCl	18

When using Piperazine as a buffer system polyallylamine has the best binding capacity for both BSA and Ovalbumin at no salt, rows A and E in Table 5. However the capacity is still good with 250 mM NaCl.

Prototype: Polyallylamine
Ion capacity: 210µmol/ml
Protein: Ovalbumin 2g/l, IP~4.7
Buffer system : 20-40mM Phosphate pH 7-7.5
Salt: NaCl

Table 6. Binding capacity (g/L) for Ovalbumin on the polyallylamine prototype.

	Ovalbumin
20mM Phosphate + 0mM NaCl, pH 7.0	20
20mM Phosphate + 0mM NaCl, pH 7.5	21
20mM Phosphate + 150mM NaCl, pH 7.0	13
20mM Phosphate + 150mM NaCl, pH 7.5	15
40mM Phosphate + 300mM NaCl, pH 7.0	5
40mM Phosphate + 300mM NaCl, pH 7.5	8
40mM Phosphate + 500mM NaCl, pH 7.0	7
40mM Phosphate + 500mM NaCl, pH 7.5	8

Prototype: Polyallylamine
Ion capacity: 210µmol/ml
Protein 1: Ovalbumin 4g/l, IP~4.7
Protein 2: BSA 4g/L, IP~4.7
Buffer system: 40mM Tris pH7.2-8.2
Salt: NaCl

Table 7. Binding capacity (g/L) for Ovalbumin and BSA on Polyallylamine prototype.

	Ovalbumin	**BSA**
40mM Tris pH 7.2 + 250 mM NaCl	73	73

40mM Tris pH 7.7 + 250 mM NaCl	65	70
40mM Tris pH 8.2 + 250 mM NaCl	52	63

In Table 6, polyallylamine shows the highest binding capacity for Ovalbumin when no salt is added, an influence by pH cannot be seen in the phosphate system.

Comparing Table 6 with Table 7 it is clear that polyallylamine favors capacity for Ovalbumin with a Tris buffer system more than a Phosphate buffer system.
In the Tris system of Table 7 the capacity on polyallylamine increases with decreasing pH. The binding capacity for Ovalbumin is more affected by pH than it is for BSA.

Prototype: Polyallyl amine
Ion capacity:210µmol/ml
Protein: Ovalbumin 2g/l, IP~4.7
Buffer system : 40mM Tris pH7.2-7.5
Salt: NaCl

Table 8. Binding capacity (g/L) for Ovalbumin on the polyallylamine prototype

	pH	7.2	7.5
A	40mM Tris + 0mM NaCl	66	66
B	40mM Tris + 150mM NaCl	57	57
C	40mM Tris + 300mM NaCl	45	44

Again it is shown that polyallylamine and Tris gives a high binding capacity for Ovalbumin, see Table . Even if the capacity decreases with salt addition it is still high.

Comparison of binding capacity for Ovalbumin on the polyallylamine prototype and Capto Core 700

Prototype: Polyallyl amine
Ion capacity:210µmol/ml
Reference media: Capto core 700
Ion capacity:40-85µmol/ml
Protein: Ovalbumin 3g/l, IP~4.7
Buffer system: 40mM Tris pH 7.2-7.5

Salt: NaCl

Table 9. Static binding capacity (g/L) for Ovalbumin on the polyallylamine prototype and Capto Core 700

		Polyallylamine			**Capto^{TM} Core 700**		
	NaCl (mM)	0	150	250	0	150	250
A	40mM Tris pH 7.2	101	83	69	29	22	22
B	40mM Tris pH 7,5	101	82	70	27	23	22

Capto^{TM} Core 700 (Ge Healthcare Bio-Sciences AB) is a commercially available product, based on highly crosslinked agarose beads of 85-90 micrometer volume-weighted average diameter, an inert shell with Mw cutoff 700 kDa and a core derivatised with octylamine ligands coupled via the amine nitrogen. The shell structure is very similar to the currently prepared prototypes and is known to give virtually zero interaction with e.g. virus particles.

In the Tris buffer systems the binding capacity for Ovalbumin is significantly better on polyallylamine.

Summary polyallylamine

When running polyallylamine the isoelectric point (IP) for the ligand is suitably considered so the pH of the buffer is not close to the IP of the ligand, making it uncharged. The IP of polyallylamine is ~8.7.

Polyallylamine works very well for ovalbumin and BSA. Ovalbumin has been tested the most and it is clear that a Tris buffer system increases the capacity compared to a Phosphate buffer. The binding capacity of BSA is zero at 1.25M NaCl, see Table 4, which indicates that it is possible to elute with salt. The characteristics of BSA and Ovalbumin are similar so it is likely that also Ovalbumin can be eluted from polyallylamine with salt.

Results 1.3-diamino-2-propanol and tris(2-aminoethyl)amine prototypes

Prototype: 1.3-diamino-2-propanol

Ligand density: 84µmol/ml

Prototype: tris(2-aminoethyl)amine

Ligand density: 55µmol/ml

Protein solution: Ovalbumin 2g/L, IP ~4.7

Buffer system 1: 50 mM Tris pH 7.4

Buffer system 2: 50 mM Phosphate pH 7.2

Salt: NaCl

Amount matrix/well: 6µl

Table 10. Binding capacity (g/L) for Ovalbumin on the 1,3-diamino-2-propanol and Tris(2-aminoethyl)amine prototypes

		1,3-diamino-2-propanol						**Tris(2-aminoethyl)amine**					
	NaCl (mM)	0	150	300	500	1000	1500	0	150	300	500	1000	1500
A	50mM Tris pH 7.4	23	12	7	4	2		32	20	12	6	2	1
B	50mM Phosphate pH 7.2	1	1	1				4	2				

The 1,3-diamino-2-propanol and Tris(2-aminoethyl)amine prototypes bind at high salt concentrations, as can be seen in row A. Tris(2-aminoethyl)amine has a higher binding capacity for Ovalbumin and both prototypes favor Tris for binding of Ovalbumin.

Example 3. Cleaning of fouled prototypes.

This study was performed in 96-well filter plates with 800 microliters well size. The plate wells were filled with20 microliter matrix and fouled with an *E.coli* homogenate. Cleaning was performed with different regeneration liquids and the contaminants still left in the beads were forced into solution by boiling with 24 mM dithiotreitol (DTT) and 0.5% sodium dodecyl sulfate (SDS) to then be analysed with chip electrophoresis in a Caliper HT Protein LabChip system.

E.coli lysate

To foul the media *E.coli* homogenate was used. Frozen *E.coli* homogenate was thawed and clarified through a glass fiber filter, followed by a 0.45 µm membrane filter.

Plate experiments

Empty filter plates were filled with 20 µl gel per well. The method described in Table was used during the plate experiments. One plate was run with every solution in triplicate. The incubation time for the CIP was 15 minutes.

Table 11. Method for plate experiments

Step	**Volume (µl)**	**Buffer**	**Execution**
Equilibration	3x200	PBS	Quick shake + vacuum
Fouling	300	*E.coli*	30 min shake + vacuum
Wash	3x200	PBS	Quick shake + vacuum
CIP	300	See Appendix 1	15min shake + vacuum
Wash	3x200	PBS	Quick shake + vacuum
Centrifugation	-	-	5 min - 972xg, to remove all liquid

After the end centrifugation of the beads the media plugs were transferred to a collection plate by connecting the collection plate on top of the filter plate and securing them together with rubber bands. The tower of plates was turned to have the filter plate on top and centrifuged for 5 min (972xg). 200 µl Caliper sample buffer was then added to all wells in the collection plate. The wells were covered with micro foil seal and put on a microplate shaker for 2 minutes. The plate was put in a heat chamber (104°C) for 5 minutes. After heating the samples were additionally shaken for 2 minutes and centrifuged for 2 minutes (500xg) to sediment the gel particles. 40 µl of the supernatant was carefully transferred to a PCR plate (conical bottom). The order of samples were reversed during the transfer of gel plugs from the filter plate to the collection plate, here it was changed back by pipetting the samples in row H to row A and so on. The PCR plate was centrifuged for 1 minute (500xg) to spin down gel particles that might have been transferred. The plate was then ready to be put in the Caliper chip electrophoresis equipment (Caliper Life Sciences Inc., USA)

Caliper LabChip electrophoresis

The chip, buffer strip and the molecular weight ladder was prepared according to the HT Protein LabChip^{TM} Kit user guide (Caliper Life Sciences Inc.,Nov. 2010). The HT Protein Express 200 High Sensitivity program was run.

The results are shown in Figs 4-6. On Capto Core 700, only the solutions containing isopropanol were really effective in removing all the contaminants. In contrast, on the polyallylamine prototype all the solutions with the exception of 8 M urea showed an efficient impurity removal. Also the diaminopropanol and trisaminoethylamine prototypes could be efficiently cleaned with all solutions except 8M urea and 8M urea + citric acid.

This written description uses examples to disclose the invention, including the best mode, and also to enable any person skilled in the art to practice the invention, including making and using any devices or systems and performing any incorporated methods. The patentable scope of the invention is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal languages of the claims.

CLAIMS

1. A separation matrix for purification of biological particles, comprising a plurality of particles (100) having a porous core entity (101) and a porous shell entity (102) covering said core entity, wherein
a) said core entity comprises at least 50 micromole/ml primary amines present on covalently attached ligands displaying at least two primary amines per ligand and
b) said shell entity comprises less than 20 micromole/ml primary amines.

2. The separation matrix of claim 1, wherein said matrix is capable of binding at least 20 mg ovalbumin per ml matrix, such as at least 40 mg ovalbumin, in an aqueous buffer having a conductivity of at least 10 mS/cm, such as at least 20 mS/cm.

3. The separation matrix of claim 1, wherein said ligands display at least two, such as at least three or at least four primary amines per ligand.

4. The separation matrix of any preceding claim, wherein said ligands are attached to said core entity via secondary or tertiary amine links.

5. The separation matrix of any preceding claim, wherein the content of nitrogen and oxygen atoms in said ligands is at least 20 wt %.

6. The separation matrix of any preceding claim, wherein the content of primary amine nitrogen is at least 18 wt %.

7. The separation matrix of any preceding claim, wherein the ratio of displayed primary amines to the total amount of nitrogen atoms is at least 0.5, such as at least 0.7 or at least 0.9.

8. The separation matrix of any preceding claim, wherein said ligands are selected from the group consisting of tris(2-aminoethyl)amine and polyallylamine, attached via an amine.

9. The separation matrix of any preceding claim, wherein said ligands comprise polyallylamine of molecular weight at least 1 kDa, such as at least 10 kDa.

10. The separation matrix of claim 9, wherein the polyallylamine is non-crosslinked.

11. The separation matrix of any preceding claim, wherein said shell entity has an average thickness of 1 to 10 micrometers.

12. The separation matrix of any preceding claim, wherein said particles are substantially spherical.

13. The separation matrix of any preceding claim, wherein said shell entity has an average thickness of 0.5 to 6 % of the diameter or the sphere-equivalent diameter of said particles.

14. The separation matrix of any preceding claim, wherein said shell entity has a molecular weight cutoff for globular proteins of 60 to 1000 kDa, such as 100-1000 or 400-800 kDa.

15. A method for purification of biological particles, comprising the steps of
a) providing a separation matrix according to any one of claims 1 – 14;
b) contacting said matrix with a liquid comprising biological particles and at least one contaminant protein, such that said contaminant protein binds to said matrix;
c) separating said liquid from said matrix and recovering said liquid with purified biological particles.

16. The method of claim 15, wherein said liquid has a conductivity of at least 10 mS/cm.

17. The method of claim 15 or 16, further comprising the steps of
d) regenerating said matrix by contacting it with a regeneration solution comprising less than 15% organic solvents, such as less than 5%, and;
e) repeating steps a) – c) at least once, such as at least five, at least 10 or at least 50 times.

18. The method of claim 17, wherein the regeneration solution comprises alkali.

19. The method of claim 17 or 18, wherein the residual protein content in the matrix after step d) is less than 100 micrograms/ml.

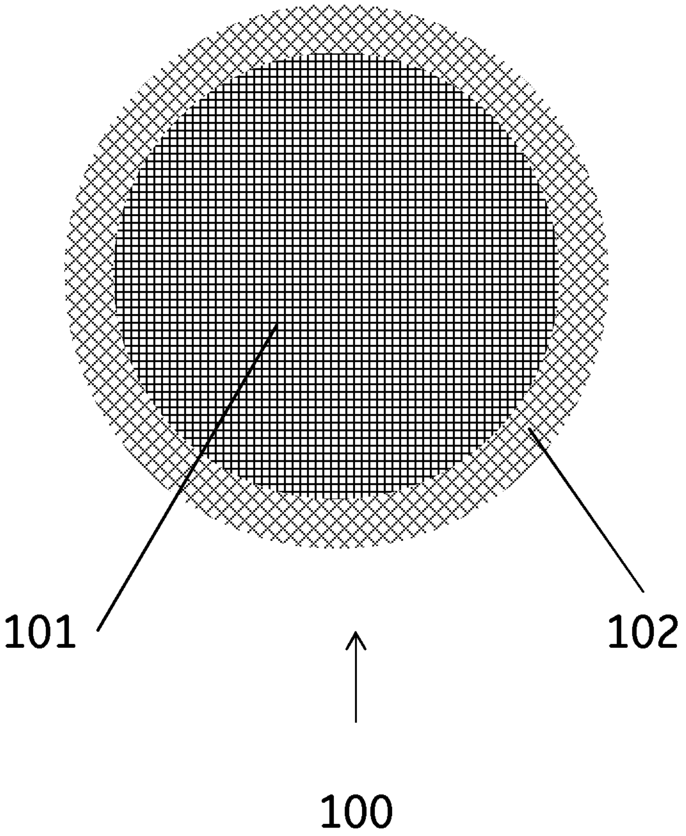
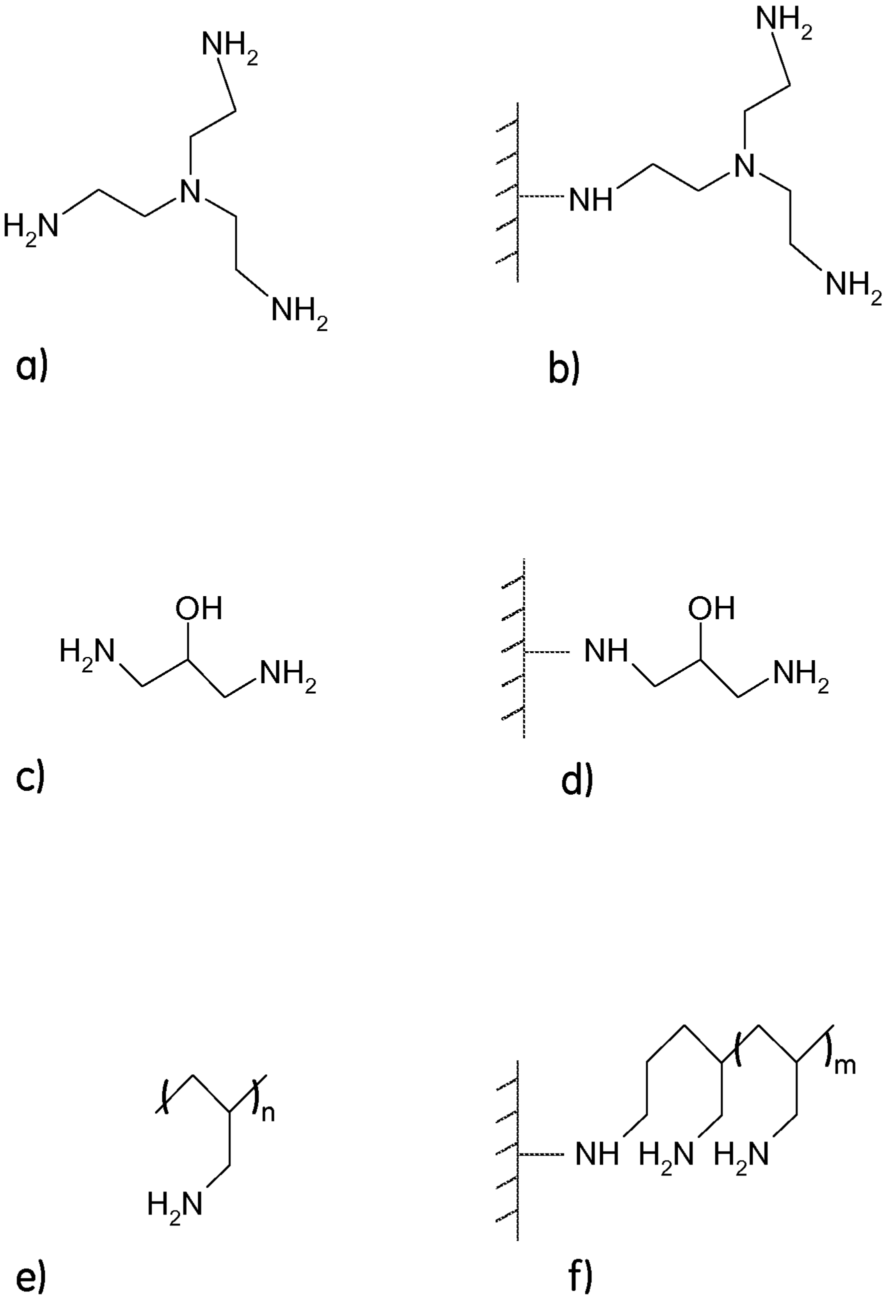


Fig. 1.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NH2
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f)

Fig. 2.

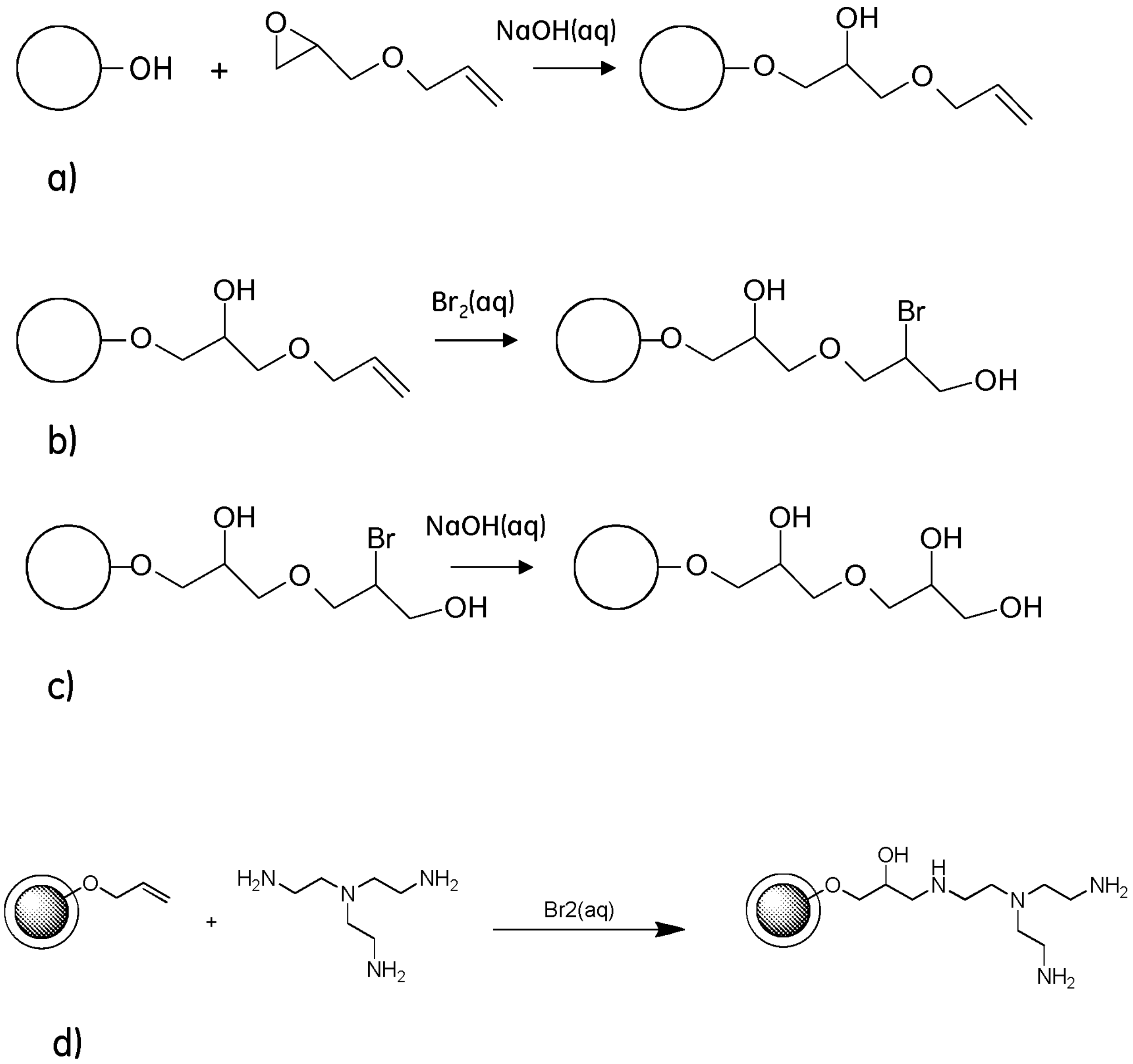

Fig. 3.

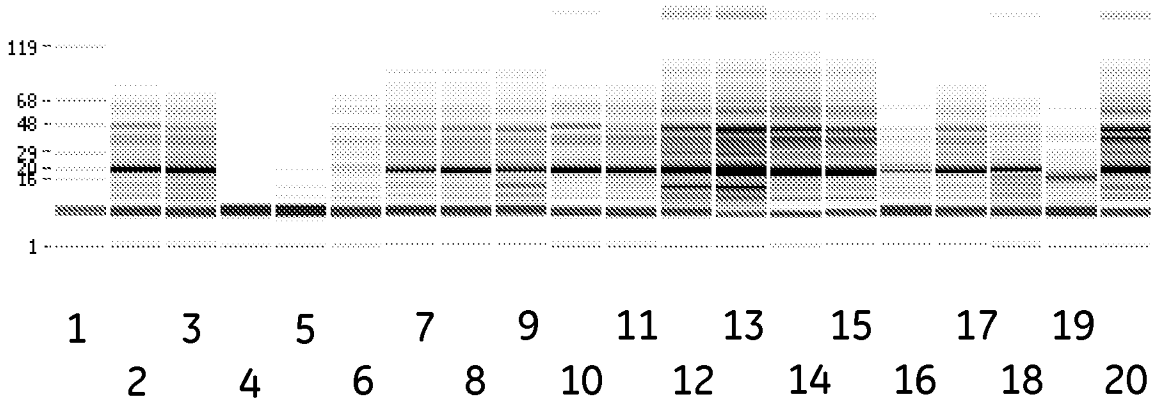

Fig. 4.

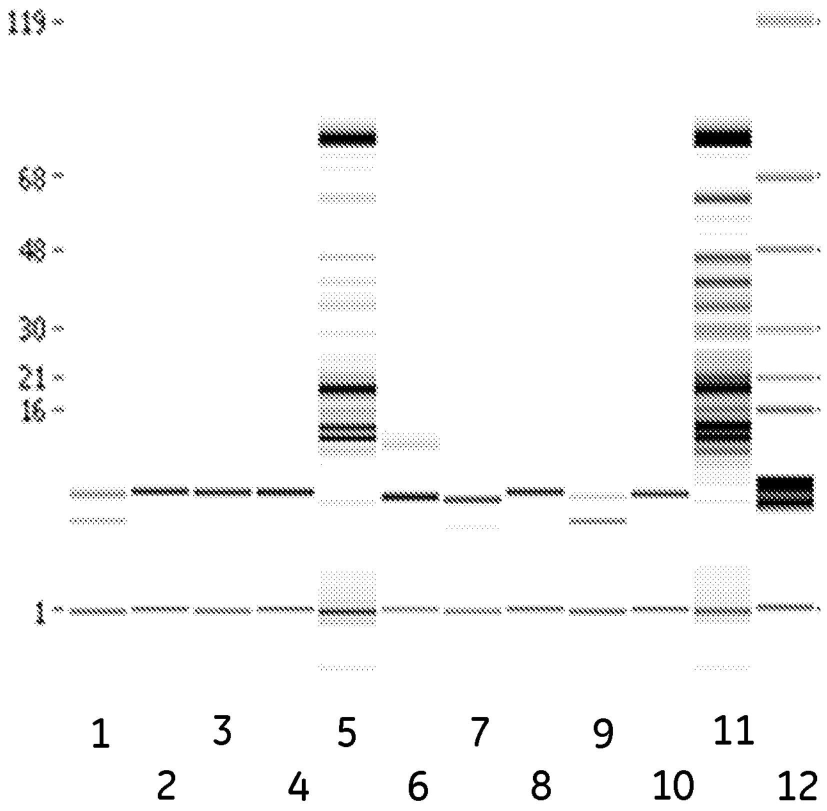

Fig. 5.

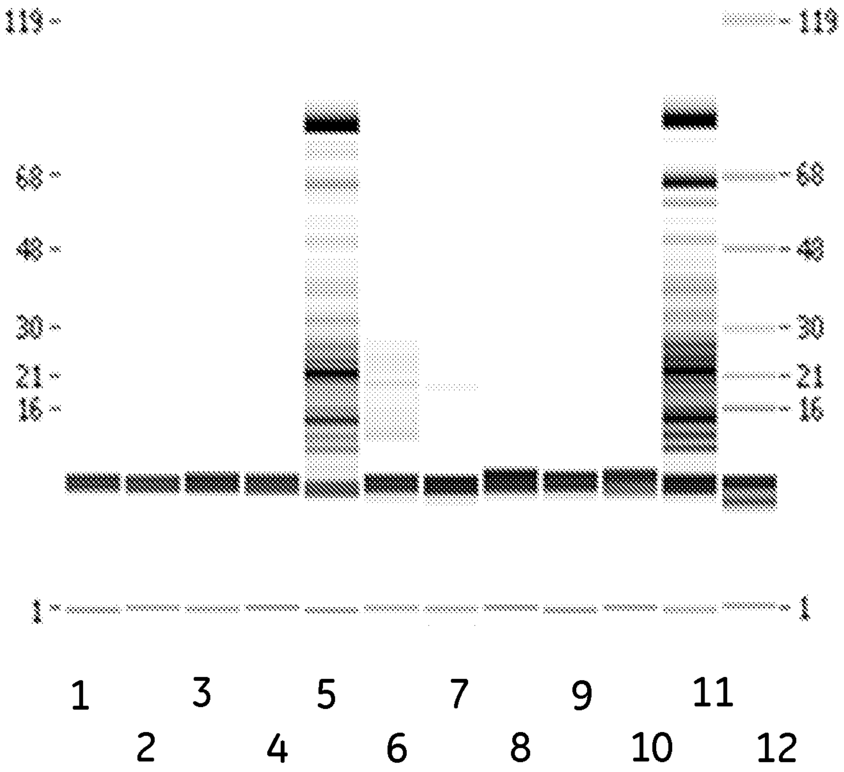

Fig. 6.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2015/050186

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

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: B01D, B01J, C08J

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

SE, DK, FI, NO classes as above

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

EPO-Internal, PAJ, WPI data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009145722 A1 (GE HEALTHCARE BIO SCIENCES AB ET AL), 3 December 2009 (2009-12-03); page 9, line 25 - page 10, line 18; claim 1 --	1-36
A	WO 2013028330 A2 (EMD MILLIPORE CORP ET AL), 28 February 2013 (2013-02-28); abstract; claims 30, 31 --	1-36
A	WO 2008028218 A (INNOVATIVE PURIFICATION TECHNO ET AL), 13 March 2008 (2008-03-13); abstract; page 10, line 4 - line 21 --	1-36

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* 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
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"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 23-06-2015	Date of mailing of the international search report 23-06-2015
Name and mailing address of the ISA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Moa Emling Telephone No. + 46 8 782 28 00

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 20120193278 A1 (KOZLOV MIKHAIL ET AL), 2 August 2012 (2012-08-02); paragraph [0040] --	1-36
A	US 20120077249 A1 (RAMASWAMY SENTHIL ET AL), 29 March 2012 (2012-03-29); claims 1, 11 --	1-36
A	WO 2011140406 A1 (MILLIPORE CORP ET AL), 10 November 2011 (2011-11-10); claims 1-5 --	1-36
A	GB 2445579 A (SECR DEFENCE), 16 July 2008 (2008-07-16); abstract --	1-36
A	WO 2009131526 A1 (GE HEALTHCARE BIO SCIENCES AB ET AL), 29 October 2009 (2009-10-29); abstract; figure 1 -- --------	1-36

Continuation of: second sheet

International Patent Classification (IPC)

B01J 20/32 (2006.01)
B01D 15/32 (2006.01)
B01D 15/34 (2006.01)
B01D 15/38 (2006.01)
B01J 20/281 (2006.01)
C08J 7/00 (2006.01)

WO	2009145722	A1	03/12/2009	US	20110065900	A1	17/03/2011
WO	2013028330	A2	28/02/2013	CN	102977182	A	20/03/2013
				EP	2574618	A1	03/04/2013
				JP	2013044748	A	04/03/2013
				JP	2015071629	A	16/04/2015
				KR	101484659	B1	21/01/2015
				KR	20130020950	A	04/03/2013
				TW	201313735	A	01/04/2013
				US	20130197200	A1	01/08/2013
WO	2008028218	A	13/03/2008	AU	2007294455	A1	13/03/2008
				GB	2454153	A	29/04/2009
				US	20100129889	A1	27/05/2010
US	20120193278	A1	02/08/2012	EP	2027921	A2	25/02/2009
				EP	2319622	A3	02/05/2012
				JP	5031695	B2	19/09/2012
				JP	2012018171	A	26/01/2012
				JP	2011247903	A	08/12/2011
				JP	2009053191	A	12/03/2009
				SG	150458	A1	30/03/2009
				SG	183660	A1	27/09/2012
				SG	183083	A1	30/08/2012
				US	8435406	B2	07/05/2013
				US	8137561	B2	20/03/2012
				US	20120121819	A1	17/05/2012
				US	20110288277	A1	24/11/2011
				US	20110284446	A1	24/11/2011
				US	20100200507	A1	12/08/2010
				US	20090050566	A1	26/02/2009
US	20120077249	A1	29/03/2012	CN	102234311	A	09/11/2011
				EP	2386628	A1	16/11/2011
WO	2011140406	A1	10/11/2011	US	20120168382	A1	05/07/2012
				US	20120006752	A1	12/01/2012
GB	2445579	A	16/07/2008	WO	2008084247	A1	17/07/2008
WO	2009131526	A1	29/10/2009	AU	2009238686	A1	29/10/2009
				CN	102015094	A	13/04/2011
				EP	2268393	A4	14/08/2013
				JP	2011518337	A	23/06/2011
				JP	5643747	B2	17/12/2014
				US	20110045574	A1	24/02/2011