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(54) Title: CHROMATOGRAPHY MEDIUM

(57) Abstract: The present invention provides a process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non- woven sheets, each comprising one or more cellulose acetate nanofibres, (ii) pressing the one or more non-woven sheets, (iii) heating the one or more non- woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose, and (v) contacting the thus-obtained product in a batchwise fashion between two and four times with a reagent which functionalises the product of step (iv) as a chromatography medium. The present invention further provides a process for the isolation of biological molecules i a mobile phase with the said functionalised cellulose chromatography medium.

CHROMATOGRAPHY MEDIUM

Field of the Invention

The invention relates to functionalised chromatography media which are suitable for isolating biological molecules from mobile phases.

Background to the Invention

The biotechnology market is the fastest growing sector within the world pharmaceutical market, accounting for 20% ($153bn) of all market sales in 2012. This growth from 10% of the market share in 2002 is set to grow by 41% between 2012 and 2018 from $153bn to $215bn. There are currently around 200 monoclonal antibody (MAb) products on the market and with over 1000 in clinical trials the need for technological advancement in this area is clear. Over the last decade typical fermentation titres of biomolecules have grown from 0.5g/L - 50g/L, and while the downstream purification processes have also received some research and development, improvements in this area have not matched those in the upstream. The heavily relied on bind/elute chromatography unit operations are, in economic terms, the key to advancements in the downstream processing of biomolecules, such as MAbs. Chromatography accounts for a very significant part of the downstream processing costs of biomolecules, which in turn impacts on the overall costs of the biomolecules themselves.

Historically, conventional packed bed chromatography has been an extremely powerful separation tool. However, it is becoming ever more apparent that radically new systems must be employed to allow biomolecules to be recovered efficiently and economically after preparation.

One area which has seen development is the synthesis of new ligands to replace

current expensive affinity ligands.

Another route which has been explored is modification of conventional support structures such as porous beaded packed bed adsorbents. This is typically to address drawbacks associated with such adsorbents, in particular problems with pressure drop and residence times. These drawbacks typically result in inefficient separations. The development of new adsorbent structures that allow for flowrate independent operation offers the advantage of increased throughput, but has generally only proven useful at small scale. Issues with adsorbent fouling are common, and this often limits chromatographic separation techniques to late stage polishing operations. A trade-off must be made between fouling and capture capacity with regard to adsorbent pore sizes. Small pore sizes are required for good separation with sharp breakthrough curves but result in increased fouling. Conversely, larger pore size adsorbents (10μm - 150+μm) may offer better handling of foulants but small target biomolecules may pass through such an adsorbent without binding.

More recently, membrane chromatography has been reported as a potentially viable alternative in contaminant capture mode.

Another focus of research has been the development of monolith structures which have proved to offer good separation for large biomolecules such as plasmids and viruses due to the relatively large pores present on the surface. The current industry trend to move towards single-use systems favours membrane chromatography as the economics of single-use membranes are more favourable than single-use packed bed columns.

Another route for development is a move into continuous processing. The drive towards continuous processing may allow efficiencies to be achieved in many systems. Thus, continuous operation presents opportunities for real-time process monitoring and automated control with potential benefits including predictable

product specification, reduced labour costs, and integration with other continuous processes. However, little in the way of truly continuous chromatography operation has been developed thus far.

It will therefore be appreciated that there are many different avenues of research being employed to provide improved processes for recovering biomolecules.

Electrospun polymeric nanofibres have properties which offer one potential solution to the issues observed with conventional support matrices used in downstream bioprocessing. Their properties readily lend themselves to ligand support surfaces with the potential for high capacity and high mass transfer rate operations, thus yielding flowrate independent binding with a high porosity and relatively small surface pore size system.

Adsorbent cartridges containing electrospun polymeric nanofibres with diethylaminoethyl (DEAE) functionality have been reported with binding capacities around 10% of a typical packed bed system but with flowrates around fifty times that of a typical packed bed system. Such nanofibre systems present a surface area to volume ratio similar to that of a porous beaded system. However, such existing nanofibre systems have somewhat lower binding capacities than typical packed bed systems. This represents a limit on their utility in recovering biomolecules.

Thus, it has not previously been possible to prepare nanofibre adsorbent systems with binding capacities greater than around 10% of a typical packed bed system whilst retaining the porosity and robust reproducible operations associated with such nanofibre systems.

The thickness of nanofibre adsorbent systems produced by electrospinning is limited during fabrication as the deposition of nanofibres onto an earthed collector surface yields a less earthed surface as deposition increases. The residual charge in the

deposited fibres therefore makes that area less attractive to continued deposition resulting in the fibres spreading further over the collector surface. This has the effect of limiting the thickness of nanofibre mats produced by electrospinning to about 100-200μm. The limited thickness of these nanofibre mats brings with it an inherent limit in physical strength of the overall mat which limits the materials' usefulness for process applications such as chromatography.

Summary of the Invention

In its broadest sense, the present invention provides processes for preparing functionalised chromatography media, which process involves treating one or more polymer nanofibres with a combination of physical and chemical processing steps to yield a functionalised product that is suitable for use as a chromatography medium in a chromatography method.

It has now been found that a specific series of physical and chemical processing steps greatly increases the binding capacity of nanofibre adsorbent systems, typically increasing said binding capacity by over 250% under typical operation conditions.

Accordingly, the present invention provides a process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non-woven sheets, each comprising one or more cellulose acetate nanofibres, (ii) pressing the one or more non-woven sheets, (iii) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose, and (v) contacting the thus-obtained product in a batchwise fashion between two and four times with a reagent which functionalises the product of step (iv) as a chromatography medium.

The present invention also provides:

- A process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non-woven sheets, each comprising one or more cellulose acetate nanofibres, (ii) pressing the one or more non-woven sheets, (iii) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose, (v) placing the thus-obtained product in a holder, and (vi) causing a reagent to flow through the holder so that the reagent flows in contact with the product obtained in step (iv) which functionalises the product of step (iv) as a chromatography medium.

- A process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres, (II) pressing the one or more polymer nanofibres, (III) heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres, and (IV) contacting the pressed and heated product with a reagent which functionalises the product of step (III) as a chromatography medium.

- A process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres, (II) optionally pressing the one or more polymer nanofibres, (III) optionally heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres, and (IV) contacting the product of step (I), (II) or (III) in a batchwise fashion at least two times with a reagent which functionalises the product of step (I), (II) or (III) as a chromatography medium.

- A process for preparing a functionalised polymeric chromatography medium,

which process comprises (I) providing one or more polymer nanofibres, (II) optionally pressing the one or more polymer nanofibres, (III) optionally heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres, (IV) placing the product of step (I), (II) or (III) in a holder, and (V) causing a reagent to flow through the holder so that the reagent flows in contact with the product of step (I), (II) or (III) which functionalises the product of step (I), (II) or (III) as a chromatography medium.

- A functionalised chromatography medium obtainable by the process of the present invention.

- A process for preparing a chromatography cartridge, which process comprises carrying out the process of the present invention and incorporating the thus-obtained product into a cartridge.

- A chromatography cartridge which (a) is obtainable by said process, or (b) which comprises one or more functionalised chromatography media of the invention.

- Use of a functionalised chromatography medium of the invention or a chromatography cartridge of the invention in chromatography.

- A process for isolating one or more biological molecules from a mobile phase, which process comprises contacting one or more biological molecules in a mobile phase with a functionalised chromatography medium of the invention or a chromatography cartridge of the invention.

Brief Description of the Figures

Figure 1 shows the performance of a functionalised chromatography medium of the invention in anion exchange chromatography.

Detailed Description of the Invention

Polymer nanofibres

The functionalised chromatography media of the present invention are formed from one or more polymer nanofibres. The polymer nanofibres are typically electrospun polymer nanofibres. Such electrospun polymer nanofibres are well known to the person skilled in the art and optimised conditions for their production can be found in, for example, O. Hardick, et al, J.Mater. Sci. 46 (2011) 3890, the entirety of which is incorporated herein by reference. The processes of the present invention typically comprise an initial step of electrospinning a polymer to produce one or more polymer nanofibres. This may involve electrospinning a polymer to produce one or more non-woven sheets, each comprising one or more polymer nanofibres

Polymer nanofibres for use in the present invention typically have mean diameters from 10nm to 1000nm. For some applications, polymer nanofibres having mean diameters from 200nm to 800nm are appropriate. Polymer nanofibres having mean diameters from 200nm to 400nm may be appropriate for certain applications.

The length of polymer nanofibres for use in the present invention is not particularly limited. Thus, conventional electrospinning processes can produce polymer nanofibres many hundreds of metres or even kilometres in length. Typically, though, the one or more polymer nanofibres have a length up to 10km, preferably from 10m to 10km.

Typically, the one or more polymer nanofibres are provided in the form of one or more non-woven sheets, each comprising one or more polymer nanofibres. A non-woven sheet comprising one or more polymer nanofibres is a mat of said one or more polymer nanofibres with each nanofibre oriented essentially randomly, i.e. it has not been fabricated so that the nanofibre or nanofibres adopts a particular pattern. Non-woven sheets comprising polymer nanofibres are typically provided by known methods, such as that disclosed in O. Hardick, et al, J.Mater. Sci. 46 (2011) 3890. Non-woven sheets may, in certain circumstances, consist of a single polymer nanofibre. Alternatively, non-woven sheets may comprise two or more polymer nanofibres, for example 2, 3, 4, 5, 6, 7, 8, 9 or 10 polymer nanofibres.

Non-woven sheets typically have area densities from 1 to 20g/m_2, preferably from 5 to 15g/m_2.

Non-woven sheets typically have a thickness from 5 to 40μm, preferably from 10 to 30μm, in some circumstances from 15 to 25μm.

The polymer used to produce the nanofibres used in the processes of the present invention is not particularly limited, provided the polymer is suitable for use in chromatography applications. Thus, typically, the polymer is a polymer suitable for use as a chromatography medium, i.e. an adsorbent, in a chromatography method. Suitable polymers include polamides such as nylon, polyacrylic acid, polymethacrylic acid, polyacrylonitrile, polystyrene, polysulfones, polycaprolactone, collagen, chitosan, polyethylene oxide, agarose, agarose acetate, cellulose, cellulose acetate, and combinations thereof. Cellulose and cellulose acetate are preferred.

Typically, the process of the present invention is for preparing a functionalised cellulose chromatography medium, and the process comprises providing one or more cellulose acetate nanofibres. Preferably, the process comprises providing one or more non-woven sheets, each comprising one or more cellulose acetate nanofibres.

Cellulose acetate is readily electrospun and can readily be transformed into cellulose after electrospinning. Thus, preferably the process comprises providing one or more non-woven sheets, each comprising one or more electrospun cellulose acetate nanofibres.

Physical modification of nanofibres

The processes of the present invention typically involve physical modification of the one or more polymer nanofibres or one or more non-woven sheets prior to chemical modification.

Thus, typically, the process of the present invention comprises providing one or more polymer nanofibres, pressing the one or more polymer nanofibres and heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres. These steps improve the structural stability of the material. The pressing and heating conditions may also be varied to alter the thickness and/or porosity of the resultant material.

Preferably, the process of the present invention comprises providing one or more non-woven sheets, each comprising one or more polymer nanofibres, pressing the one or more non-woven sheets and heating the one or more non-woven sheets to fuse points of contact between sections of the one or more polymer nanofibres. For the avoidance of doubt, fusion of points of contact between sections of the one or more polymer nanofibres refers to the one or more polymer nanofibres contained in the one or more non-woven sheets.

Use of multiple non-woven sheets of polymer nanofibres enables a thicker material to be prepared which has a greater capacity for adsorbence (once functionalised). Thus, preferably, the process of the present invention comprises providing two or more non-woven sheets, each comprising one or more polymer nanofibres, pressing the two or

more non-woven sheets and heating the two or more non-woven sheets to fuse points of contact between sections of the one or more polymer nanofibres. Fusion of points of contact between sections of the one or more polymer nanofibres refers here to the one or more polymer nanofibres contained in the two or more non-woven sheets.

Typically, from two to thirty non-woven sheets are provided. In certain circumstances, between five and twenty five non-woven sheets may be provided. In certain circumstances, between 10 and 20 non-woven sheets may be provided. The number of non-woven sheets employed will affect the thickness of the eventual chromatography medium, and its permeability to liquids. Thus, a thicker medium will generally have a lower permeability than a thinner medium. Thus, where a high permeability is required, typically a lower number of sheets are employed. Where a lower permeability medium is required a higher number of sheets may be employed

For the avoidance of doubt, the one or more non-woven sheets are pressed in a direction parallel to their thinnest dimension. Non-woven sheets will typically have two dimensions which are much larger than the third dimension, and the one or more sheets are pressed parallel to this third dimension. Where two or more non-woven sheets are provided and pressed, the two or more non-woven sheets are typically stacked one on top of the other so that they substantially overlap and the smallest dimension of each non-woven sheet is aligned.

Typically, pressing the one or more polymer nanofibres or one or more non-woven sheets involves subjecting them to a pressure of from 0.01 to 5MPa, more typically from 0.05 to 3MPa, for instance from 0.1 to 1MPa. Pressure may be applied by any suitable means. For instance, pressure may be applied using a manual press or hydraulic press. The pressure applied may be varied to alter the physical properties of the media. Generally, a higher pressure will result in a more robust medium, having a lower porosity and lower thickness. A lower pressure tends to yield a comparatively less robust medium, with a higher porosity and higher thickness. Thicker

chromatography media may be preferred when it is desirable to maximise the binding properties.

The length of time for which the one or more polymer nanofibres or one or more non-woven sheets are pressed is not particularly restricted, and typical pressing times may be determined by one of skill in the art.

Heating the one or more polymer nanofibres or one or more non-woven sheets may be effected by conventional means, for example using an oven.

The one or more polymer nanofibres or one or more non-woven sheets are heated to fuse points of contact between sections of the one or more polymer nanofibres (contained in the one or more non-woven sheets). For the avoidance of doubt, sections of a polymer nanofibre may be in contact with sections of the same nanofibre, and/or with sections of other nanofibres. Thus, heating may fuse points of contact between sections of a nanofibre with other sections of the same nanofibre, and/or between sections of a nanofibre with sections of a different nanofibre. Preferably, heating fuses points of contact between sections of a nanofibre with other sections of the same nanofibre, and between sections of a nanofibre with sections of a different nanofibre. Thus, in a simple scenario where first and second nanofibres are provided, heating the first and second nanofibres preferably fuses points of contact between sections of the first nanofibre with other sections of the first nanofibre, and between sections of the first nanofibre with sections of the second nanofibre.

In embodiments where two or more non-woven sheets, each comprising one or more nanofibres, are subjected to conditions of heat, sections of a polymer nanofibre may be in contact with sections of the same nanofibre, and/or with sections of other nanofibres in the same non-woven sheet, and/or with sections of nanofibres in adjacent non-woven sheets. Sections of nanofibres in non-woven sheets are not typically in contact with sections of nanofibres in other non-woven sheets which are

not adjacent. Thus, heating two or more non-woven sheets may fuse points of contact between sections of a nanofibre with other sections of the same nanofibre, and/or between sections of a nanofibre with sections of another nanofibre in the same non-woven sheet, and/or or between sections of a nanofibre with sections of a nanofibre in an adjacent non-woven sheet. Preferably, heating two or more non-woven sheets fuses points of contact between sections of a nanofibre with other sections of the same nanofibre, and between sections of a nanofibre with sections of another nanofibre in the same non-woven sheet, and or between sections of a nanofibre with sections of a nanofibre in an adjacent non-woven sheet. Thus, in a simple example where two non-woven sheets are provided, each non-woven sheet containing a single polymer nanofibre, heating the first and second non-woven sheets preferably fuses points of contact between sections of the polymer nanofibre in the first non-woven sheet with other sections of the polymer nanofibre in the first non-woven sheet, and between sections of the polymer nanofibre in the first non-woven sheet with sections of the polymer nanofibre in the second non-woven sheet.

Typically, the one or more polymer nanofibres or one or more non-woven sheets are heated to a temperature below the melting point of the polymer. Use of a higher temperature could result in destruction of the nanofibre structure. In some circumstances it is advantageous to use a temperature which is below the glass transition temperature of the polymer. In other circumstances a temperature above the glass transition temperature of the polymer may be used. The melting points and glass transition temperatures of polymers suitable for use in the claimed processes are well known to the skilled person.

Typically, the one or more polymer nanofibres or one or more non-woven sheets are heated to a temperature between a temperature at which points of contact between sections of the one or more polymer nanofibres begin to fuse and the melting point of the polymer. Preferably, the one or more polymer nanofibres or one or more non-woven sheets are heated to a temperature between a temperature at which points of

contact between sections of the one or more polymer nanofibres begin to fuse and the glass transition temperature of the polymer.

In the case where the polymer is cellulose acetate, the one or more cellulose acetate nanofibres or one or more non-woven sheets, each comprising one or more cellulose acetate nanofibres, are heated to a temperature between 200 to 220°C, preferably from 205 to 218°C, for instance from 210 to 215°C.

The one or more polymer nanofibres or one or more non-woven sheets are typically heated for from 1 to 120 minutes, for instance from 5 to 60 minutes.

Typically, the one or more polymer nanofibres or one or more non-woven sheets have an average pore size after pressing and heating at of from 0.1 to 1.0μm, preferably from 0.3 to 0.9 μm, more preferably from 0.4 to 0.8 μm, even more preferably from 0.5 to 0.7 μm, yet more preferably from 0.6 to 0.7 μm, for example from 0.6 to 0.65μm.

Typically, the one or more polymer nanofibres or one or more non-woven sheets have an average density after pressing and heating of from 250 to 750 kg/m^3, preferably from 350 to 650 kg/m^3, in some circumstances from 450 to 550 kg/m^3.

Typically, the one or more polymer nanofibres or one or more non-woven sheets are pressed then subsequently heated. Alternatively, the one or more polymer nanofibres or one or more non-woven sheets may be pressed and heated simultaneously.

Typically, the pressed and heated one or more polymer nanofibres or one or more non-woven sheets have a thickness of 0.05 to 10mm, for instance 0.1 to 5mm.

Chemical modification of nanofibres

The processes of the present invention typically involve chemical modification of the one or more polymer nanofibres or one or more non-woven sheets to functionalise them for use in chromatography. In its simplest form this involves contacting the one or more polymer nanofibres or one or more non-woven sheets (which may have been pressed and heated) with a reagent to functionalise the product as a chromatography medium.

Optionally, prior to this step of contacting with a reagent, the one or more polymer nanofibres or one or more non-woven sheets (which may have been pressed and heated) may be treated to deprotect or activate any functional groups on the polymer.

Deprotection of the functional groups is typically effected so that the functional groups can react with the reagent. For instance, when the polymer is cellulose, typically one or more cellulose acetate nanofibres or non-woven sheets, each comprising one or more cellulose acetate nanofibres, is provided and, prior to contacting with a reagent, the cellulose acetate is treated to convert it to cellulose. This involves the deprotection of acetylated hydroxyl groups to give hydroxyl groups. Conversion of cellulose acetate to cellulose is typically effected using aqueous alkali, preferably NaOH in water:ethanol, more preferably water:ethanol 2:1, for a period of greater than 12hrs, for example from 12 to 36 hours. This step typically takes place after the one or more cellulose acetate nanofibres or non-woven sheets, each comprising one or more cellulose acetate nanofibres, has been pressed and heated. Alternatively, this step may be carried out before the one or more cellulose acetate nanofibres or non-woven sheets, each comprising one or more cellulose acetate nanofibres, has been pressed and heated. Activation of functional groups is discussed further below.

The reagent typically functionalises the chromatography medium by introducing one

or more moieties which render the functionalised product comprising the one or more moieties suitable for use as a chromatography medium. The one or more moieties introduced will depend on the particular chromatography technique for which the medium is to be used. Suitable moieties and reagents are discussed further below. Typically, the reagent reacts with one or more functional groups present on the one or more polymer nanofibres, typically contained within the one or more non-woven sheets, to create the one or more moieties. Typical functional groups include hydroxyl, amino and carboxy groups. Thus, typically one or more hydroxyl, amino and/or carboxy groups are functionalised in the process of the present invention.

Although the present invention envisages processes involving only a single treatment with a reagent, processes involving multiple functionalising steps are preferred. Such processes lead to products with improved binding properties.

Thus, typically, functionalisation by contacting with a reagent is effected by contacting in a batchwise fashion two or more times with a reagent. Batchwise functionalisation means that the polymer nanofibre material (which has been optionally pressed, heated, deprotected and/or activated) is reacted with a reagent to functionalise it, that reaction is then stopped and the resultant (partially) functionalised material reacted with a separate batch of reagent. Reacting in a batchwise fashion does not simply refer to adding more portions of reagent to a reaction vessel, for instance.

Batchwise functionalisation is typically carried out from two to ten times, i.e. 2, 3, 4, 5, 6, 7, 8, 9 or 10 times. Preferably batchwise funtionalisation is carried out between two and four times.

Each step of contacting with a reagent in a batchwise fashion typically comprises (a) contacting with the reagent, (b) isolating the product of step (a) from the reagent, (c) optionally treating the product of step (b) with aqueous alkali, and (d) optionally

washing the product of step (b)/(c) with water. Preferably, each step of contacting with a reagent in a batchwise fashion comprises (a) contacting with the reagent, (b) isolating the product of step (a) from the reagent, (c) treating the product of step (b) with aqueous alkali, and (d) optionally washing the product of step (b)/(c) with water. More preferably, each step of contacting with a reagent in a batchwise fashion comprises (a) contacting with the reagent, (b) isolating the product of step (a) from the reagent, (c) treating the product of step (b) with aqueous alkali, and (d) washing the product of step (b)/(c) with water.

The steps of treating with aqueous alkali typically employ hot aqueous alkali, i.e. between 70 and 90°C.

The reagent used in each step of contacting with a reagent in a batchwise fashion may be the same or different, but is preferably the same.

In circumstances where between two and four steps of contacting with a reagent in a batchwise fashion are employed, the one or more polymer nanofibres or one or more non-woven sheets, each comprising one or more polymer nanofibres, which may have been pressed, heated and/or deprotected, are typically treated by

- (1) (a1) contacting with the reagent, (b1) isolating the product of step (a1) from the reagent, (c1) optionally treating the product of step (b1) with aqueous alkali, and (d1) optionally washing the product of step (b1)/(c1) with water, and
  (2) (a2) contacting the product of step (b1)/(c1)/(d1) with the reagent, (b2) isolating the product of step (a2) from the reagent, (c2) optionally treating the product of step (b2) with aqueous alkali, and (d2) optionally washing the product of step (b2)/(c2) with water; or
- (1) (a1) contacting with the reagent, (b1) isolating the product of step (a1) from the reagent, (c1) optionally treating the product of step (b1) with aqueous alkali, and (d1) optionally washing the product of step (b1)/(c1) with water,
  (2) (a2) contacting the product of step (b1)/(c1)/(d1) with the reagent, (b2)

isolating the product of step (a2) from the reagent, (c2) optionally treating the product of step (b2) with aqueous alkali, and (d2) optionally washing the product of step (b2)/(c2) with water, and

(3) (a3) contacting the product of step (b2)/(c2)/(d2) with the reagent, (b3) isolating the product of step (a3) from the reagent, (c3) optionally treating the product of step (b3) with aqueous alkali, and (d3) optionally washing the product of step (b3)/(c3) with water; or

- (1) (a1) contacting with the reagent, (b1) isolating the product of step (a1) from the reagent, (c1) optionally treating the product of step (b1) with aqueous alkali, and (d1) optionally washing the product of step (b1)/(c1) with water,

(2) (a2) contacting the product of step (b1)/(c1)/(d1) with the reagent, (b2) isolating the product of step (a2) from the reagent, (c2) optionally treating the product of step (b2) with aqueous alkali, and (d2) optionally washing the product of step (b2)/(c2) with water,

(3) (a3) contacting the product of step (b2)/(c2)/(d2) with the reagent, (b3) isolating the product of step (a3) from the reagent, (c3) optionally treating the product of step (b3) with aqueous alkali, and (d3) optionally washing the product of step (b3)/(c3) with water, and

(4) (a4) contacting the product of step (b3)/(c3)/0(d3) with the reagent, (b4) isolating the product of step (a4) from the reagent, (c4) optionally treating the product of step (b4) with aqueous alkali, and (d4) optionally washing the product of step (b4)/(c4) with water.

Typically, each step of contacting with a reagent in a batchwise fashion comprises treating with the reagent for between 1 and 20 minutes.

In certain circumstances, contacting with a reagent may comprise placing one or more polymer nanofibres or one or more non-woven sheets, each comprising one or more polymer nanofibres, which may have been pressed, heated, deprotected and/or activated (i.e. the polymer material) in a holder, and causing a reagent to flow through

the holder so that the reagent flows in contact with the polymer material which functionalises the polymer material as a chromatography medium. Functionalising polymer material in this manner may in certain circumstances be more efficient than simply contacting the polymer material with the reagent, in a flask or beaker for example.

Typically, the holder is a filter holder adapted to hold the polymer material. Typically, the filter holder holds the polymer material such that an aqueous or liquid substance which is passed through the filter holder flows in contact with the polymer material. Thus, in the context of the present invention, the filter holder preferably holds the polymer material such that a reagent which is caused to flow through the filter holder flows in contact with the polymer material.

Typically, the reagent is caused to flow through the holder under pressure.

Typically, the reagent is caused to flow through the holder using a pump, preferably an HPLC pump.

Typically, the reagent is caused to flow through the holder in a cyclical manner. Thus, any reagent exiting the holder is recycled and passed through the holder one of more further times.

Typically, the reagent is caused to flow through the holder for a period of time from 1 to 20 minutes.

Typically, the reagent is caused to flow through the holder at a rate of 10 to 100mL/min.

Typically, after the reagent has been caused to flow through the holder the resultant product is treated with aqueous alkali, and optionally washed with water. Preferably,

after the reagent has been caused to flow through the holder the resultant product is treated with aqueous alkali, and washed with water. Treatment with aqueous alkali is preferably treatment with hot aqueous alkali as defined above. Typically, after the reagent has been caused to flow through the holder the resultant product is removed from the holder prior to any further treatment steps. Thus, preferably, after the reagent has been caused to flow through the holder the resultant product is removed from the holder, treated with aqueous alkali, and optionally washed with water. More preferably, after the reagent has been caused to flow through the holder the resultant product is removed from the holder, treated with aqueous alkali, and washed with water.

The reagent functionalises the product of the preceding physical and chemical processing steps to yield a chromatography medium, specifically a functionalised chromatography medium. Typically, the reagent functionalises the product of the preceding steps so that it is suitable for use in an ion exchange, affinity capture or hydrophobic chromatography method. Thus, contacting with the reagent typically yields a chromatography medium which is functionalised with one or more moieties which are negatively charged, one or more moieties which are positively charged, one or more proteins, mimetic or synthetic ligands that mimic the action of protein ligands, peptides, antibodies or fragments thereof, dyes, histidine, groups containing a metal cation, or hydrophobic groups. Examples of such groups are defined further below. Suitable reagents for introducing such groups will be evident to the skilled person. 2-chloro-N,N-diethylamine hydrochloride (DEACH) is preferred as the reagent, particularly when the functionalised chromatography medium is for use in an anion exchange chromatography method.

Chromatography media and methods

The products of the process of the present invention are functionalised chromatography media, i.e. chromatography media that have been modified

chemically to render them suitable for use in one or more chromatography methods. Specific chemical modifications are discussed in more detail below. In general terms, such chemical modification changes the chemical and/or physical properties of the chromatography medium. This in turn affects how the chromatography medium behaves when used in a chromatography method. The modifications may, for example, change the polarity, hydrophobicity or biological binding properties of the functionalised chromatography medium compared to its unfunctionalised form.

The chromatography media are typically in the form of membranes. Such membranes are suitable for use in membrane chromatography methods. Membrane chromatography methods are well known to the person skilled in the art and are discussed in "Membrane Processes in Biotechnologies and Pharmaceutics" ed. Catherine Charcosset, Elsevier, 2012, the entirety of which is incorporated herein by reference.

Typically, the functionalised polymer chromatography media are suitable for use in chromatography methods chosen from ion exchange chromatography, affinity capture chromatography and hydrophobic chromatography. In operation, such chromatography methods involve passing a mobile phase containing desired molecule over an adsorbent phase, here the functionalised chromatography media. The adsorbent phase is typically chosen such that the desired molecule is retained on it in preference to other components also present in the mobile phase.

Typically, the polymer chromatography medium is functionalised with DEAE or CM groups. Generally, the polymer is cellulose and the chromatography medium is functionalised with DEAE or CM groups. Thus, the functionalised chromatography medium may be cellulose derivatised with DEAE or CM groups.

Ion exchange chromatography is a technique for separating molecules, typically ions or polar molecules, based on their ionic charge. Functionalised chromatography

media for use in such methods therefore contain one or more moieties which are positively or negatively charged. Positive and/or negative charges in functionalised chromatography media are usually balanced with one or more counter ions. Ion exchange chromatography involves one or more of cation exchange chromatography and anion exchange chromatography.

Functionalised chromatography media for use in cation exchange chromatography contain one or more moieties which are negatively charged. Typical negatively charged moieties include one or more carboxylate, sulphonate or phosphonate groups, or mixtures thereof, i.e. the moieties typically contain one or more $-COO^-$, $-SO_3^-$, or $-P(OH)_2O^-$ groups, or mixtures thereof. Typical functionalised chromatography media for use in cation exchange chromatography contain one or more $-O-CH_2COO^-$, $-CH_2COO^-$, $-SO_3^-$, $-CH_2CH_2CH_2SO_3^-$, $-CH_2CH_2SO_3^-$, or $-P(OH)_2O^-$ moieties.

Functionalised chromatography media for use in anion exchange chromatography contain one or more moieties which are positively charged. Typical positively charged moieties include one or more quaternary amine groups. Typical functionalised chromatography media for use in anion exchange chromatography contain one or more $-N^+(CH_3)_3$, $-N^+(C_2H_5)H$, $-CH_2CH_2N^+(C_2H_5)H$, $-CH_2CH_2N^+(C_2H_5)_2(CH_2CH(OH)CH_3)$, $-O-CH_2CH_2-N^+(CH_3)_3$, $-CH_2CH_2N^+(CH_3)_3$, or $-CH_2CH_2N^+(CH_3)_2H$ moieties.

Affinity capture chromatography is a technique for separating molecules based on their affinity to particular ligands, usually but not always biological ligands. This method may, for example, rely on the attractive forces between antibodies and antigens or enzymes and substrates. Functionalised chromatography media for use in affinity capture chromatography typically contain one or more moieties chosen from one or more proteins, peptides, antibodies or fragments thereof, dyes, histidine, or groups containing a metal cation. Alternatively, functionalised chromatography media for use in affinity capture chromatography may contain mimetic or synthetic

ligands that mimic the action of protein ligands.

Typical proteins for use in affinity capture chromatography are well known to the person skilled in the art and include Protein A, Protein G and Protein L.

Typical antibodies and fragments thereof for use in affinity capture chromatography are well known to the person skilled in the art and include IgG.

Typical dyes for use in affinity capture chromatography are well known to the person skilled in the art and include Yellow HE-4R, Red HE-3B and Cibacron Blue F3G.

Typical groups containing metal cations for use in affinity capture chromatography are well known to the person skilled in the art. Such groups typically contain a chelating agent to immobilize metal cations. The metal cation is typically chosen from copper, nickel, zinc and cobalt cations, preferably Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}.

Hydrophobic interaction chromatography is a technique for separating molecules based on their hydrophobicity. Functionalised chromatography media for use in such methods therefore contain one or more moieties which contain one or more hydrophobic groups. Typical hydrophobic groups include propyl, butyl, phenyl, and octyl groups.

The processes claimed in the present invention for preparing functionalised chromatography media typically involve introducing one or more moieties into a chromatography medium such that the resultant functionalised product comprising the one or more moieties is suitable for use as a chromatography medium in a chromatography method. Typical moieties, media, reagents and methods are as defined above. The one or more moieties are introduced by reacting a reagent with one or more functional groups contained on the one or more polymer nanofibres or one or more non-woven sheets, each comprising one or more polymer nanofibres,

which have typically been pressed, heated, deprotected and/or activated. Typical functional groups include hydroxyl, amino and carboxyl groups.

The one or more functional groups may be activated prior to reaction with a reagent. Conventional activation methods known in the art may be employed. Thus, in the case where the functional group is an hydroxyl group, such a group may be activated by treating with carbonyl diimidazole (CDI), bisoxiranes, cyanuric acid, N-hydroxy succinimide esters (NHS) or 2-fluoro-1-methyl pyridinium toluene-4 sulphonate (FMP). In the case where the functional group is an amino group, such a group may be activated by treating with epichlorohydrine, glutaraldehyde or epoxide. In the case where the functional group is a carboxyl group, such a group may be activated by treating with CDI or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

A skilled person can choose suitable reagents to introduce particular moieties into particular polymers, for example on the basis of the functional groups contained in those polymers. Typical reagents include 2-chloro-N,N-diethylamine hydrochloride (DEACH).

Particular embodiments of the process of the invention

In its broadest sense, the present invention provides processes for preparing functionalised chromatography media, which process involves treating one or more polymer nanofibres with a combination of physical and chemical processing steps to yield a functionalised product that is suitable for use as a chromatography medium in a chromatography method.

Typically, the one or more polymer nanofibres are as defined herein. The one or more polymer nanofibres may be provided as one or more non-woven sheets, each comprising one or more polymer nanofibres.

Typically, the physical processing steps are the steps of pressing and heating as defined herein.

Typically, the chemical processing steps are the steps of contacting with a reagent as defined herein.

Typically, the chromatography method is as defined herein.

In a preferred embodiment, the one or more polymer nanofibres are pressed and heated. This improves the structural properties of the resultant functionalised polymeric chromatography medium. Thus, in this preferred embodiment the present invention provides a process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres as defined herein, (II) pressing the one or more polymer nanofibres as defined herein, (III) heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, and (IV) contacting the pressed and heated product with a reagent as defined herein which functionalises the product of step (III) as a chromatography medium as defined herein.

In this preferred embodiment, the step of providing one or more polymer nanofibres typically comprises providing one or more non-woven sheets, each comprising one or more polymer nanofibres.

In a further preferred embodiment, the step of functionalising with a reagent is carried out in a batchwise fashion. This increases the binding capacity of the resultant functionalised polymeric chromatography medium. Thus, in this further preferred embodiment, the present invention provides a process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres as defined herein, (II) optionally pressing the one or more polymer nanofibres as defined herein, (III) optionally heating the one or more

polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, and (IV) contacting the product of step (I), (II) or (III) in a batchwise fashion at least two times with a reagent as defined herein which functionalises the product of step (I), (II) or (III) as a chromatography medium as defined herein.

In this further preferred embodiment, the step of providing one or more polymer nanofibres typically comprises providing one or more non-woven sheets, each comprising one or more polymer nanofibres.

This further preferred embodiment preferably comprises (I) providing one or more polymer nanofibres as defined herein, (II) pressing the one or more polymer nanofibres as defined herein, (III) heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, and (IV) contacting the product of step (III) in a batchwise fashion at least two times with a reagent as defined herein which functionalises the product of step (III) as a chromatography medium as defined herein.

This further preferred embodiment more preferably comprises (I) providing one or more non-woven sheets, each comprising one or more polymer nanofibres as defined herein, (II) pressing the one or more non-woven sheets as defined herein, (III) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, and (IV) contacting the product of step (III) in a batchwise fashion at least two times with a reagent as defined herein which functionalises the product of step (III) as a chromatography medium as defined herein.

In a yet further preferred embodiment, the step of functionalising with a reagent is carried out by convective flow. Such a process is typically more efficient than a standard diffusive process. Thus, in this further preferred embodiment, the present

invention provides a process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres as defined herein, (II) optionally pressing the one or more polymer nanofibres as defined herein, (III) optionally heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, (IV) placing the product of step (I), (II) or (III) in a holder as defined herein, and (V) causing a reagent as defined herein to flow through the holder so that the reagent flows in contact with the product of step (I), (II) or (III) which functionalises the product of step (I), (II) or (III) as a chromatography medium as defined herein.

This yet further preferred embodiment preferably comprises (I) providing one or more polymer nanofibres as defined herein, (II) pressing the one or more polymer nanofibres as defined herein, (III) heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, (IV) placing the product of step (III) in a holder as defined herein, and (V) causing a reagent as defined herein to flow through the holder so that the reagent flows in contact with the product of step (III) which functionalises the product of step (III) as a chromatography medium as defined herein.

This yet further preferred embodiment more preferably comprises (I) providing one or more non-woven sheets, each comprising one or more polymer nanofibres as defined herein, (II) pressing the one or more non-woven sheets as defined herein, (III) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more polymer nanofibres as defined herein, (IV) placing the product of step (III) in a holder as defined herein, and (V) causing a reagent as defined herein to flow through the holder so that the reagent flows in contact with the product of step (III) which functionalises the product of step (III) as a chromatography medium as defined herein.

It is preferred that the functionalised polymer chromatography medium is a

functionalised cellulose chromatography medium.

In a most preferred embodiment, the present invention provides a process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non-woven sheets as defined herein, each comprising one or more cellulose acetate nanofibres as defined herein, (ii) pressing the one or more non-woven sheets as defined herein, (iii) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres as defined herein, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose as defined herein, and (v) contacting the thus-obtained product in a batchwise fashion between two and four times with a reagent as defined herein which functionalises the product of step (iv) as a chromatography medium as defined herein.

In a further most preferred embodiment, the present invention provides a process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non-woven sheets as defined herein, each comprising one or more cellulose acetate nanofibres as defined herein, (ii) pressing the one or more non-woven sheets as defined herein, (iii) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres as defined herein, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose as defined herein, (v) placing the thus-obtained product in a holder as defined herein, and (vi) causing a reagent as defined herein to flow through the holder so that the reagent flows in contact with the product obtained in step (iv) which functionalises the product of step (iv) as a chromatography medium as defined herein.

Functionalised chromatography medium of the invention

The present invention also provides a functionalised chromatography medium which is obtainable by the process of the present invention.

Also provided is provides a functionalised chromatography medium which is obtained by the process of the present invention.

The functionalised chromatography medium of the present invention typically has a porosity of from 0.1 to 1.0μm, preferably from 0.3 to 0.9 μm, more preferably from 0.4 to 0.8 μm, even more preferably from 0.5 to 0.7 μm, yet more preferably from 0.6 to 0.7 μm, for example from 0.6 to 0.65μm.

The functionalised chromatography medium of the present invention has a density of from 250 to 750 kg/m^3, preferably from 350 to 650 kg/m^3, in some circumstances from 450 to 550 kg/m^3.

Typically, the functionalised chromatography medium of the present invention has a thickness of 0.05 to 10mm, for instance 0.1 to 5mm.

Preferably, the functionalised chromatography medium of the present invention is functionalised so that it is suitable for use in a chromatography method as defined herein, for instance ion exchange chromatography, affinity capture chromatography and hydrophobic chromatography.

The functionalised chromatography medium of the present invention is typically in the form of a membrane.

Chromatography cartridge of the invention

The present invention also provides a chromatography cartridge. The chromatography cartridge of the present invention comprises one or more functionalised chromatography media of the present invention. Alternatively, the chromatography cartridge of the present invention is obtainable by carrying out the process of the present invention and incorporating the thus-obtained product into a cartridge.

Also provided is a process for preparing a chromatography cartridge which comprises carrying out the process of the present invention and incorporating the thus-obtained product into a cartridge.

The chromatography cartridge is typically suitable for use in chromatography, preferably a chromatography method as defined herein.

A chromatography cartridge of the present invention typically comprises one or more functionalised chromatography media of the present invention within a holder, for example a holder as defined above. The holder is typically cylindrical.

Typically, the chromatography cartridge comprises one or more functionalised chromatography media of the present invention stacked inside a cylindrical holder.

Typically, the chromatography cartridge comprises two or more functionalised chromatography media of the present invention. Typically, the chromatography cartridge comprises up to twenty functionalised chromatography media of the present invention.

Typically, the chromatography cartridge also comprises one or more frits within the typically cylindrical holder. Frits are well known to the person skilled in the art and

refer to rigid porous structures, typically rigid metal or ceramic porous structures. Frits are typically included in a chromatography cartridge to improve flow distribution through the cartridge and/or to support the one or more functionalised chromatography media of the present invention. Pores in typical frits have diameters from 1 to 20μm, preferably from 1 to 10μm, more preferably from 3 to 7μm.

Typically, the chromatography cartridge also comprises one or more inlet fluid distribution means and/or outlet fluid collection means. Such means are well known to the person skilled in the art.

Chromatography method of the invention

The present invention also provides use of a functionalised chromatography medium of the invention or a chromatography cartridge of the invention in chromatography, particularly in a chromatography method as defined herein.

The present invention also provides a process for isolating one or more biological molecules from a mobile phase, which process comprises contacting one or more biological molecules in a mobile phase with a functionalised chromatography medium of the invention or a chromatography cartridge of the invention. The chromatography medium or chromatography cartridge binds preferentially to the one or more biological molecules in the mobile phase, typically in preference to other components (for instance other biological molecules) also present in the mobile phase. This can be carried out in accordance with conventional methods known for the bind phase of such chromatographic methods.

Thus, typically, this chromatographic process is an ion (anion or cation) exchange, affinity capture or hydrophobic interaction chromatography process.

Thus, the present invention provides a chromatography process which comprises the above step. Typically, the chromatography process is carried out in accordance with a chromatography method as defined above.

The chromatography process typically comprises a further step of recovering the one or more biological molecules from the functionalised chromatography medium or chromatography cartridge. This step can typically be effected by contacting the functionalised chromatography medium or chromatography cartridge to which is adsorbed the one or more biological molecules with an elution buffer. This can be carried out in accordance with conventional methods known for the elute phase of such chromatographic methods. Thus, the process is typically a bind-elute chromatographic method.

Between the bind and elute steps, the process may further comprise a step of washing the functionalised chromatography medium or chromatography cartridge of the invention to which is adsorbed the one or more biological molecules. This washing step is carried out to remove any components which are not bound to the functionalised chromatography medium or chromatography cartridge. This can be carried out in accordance with conventional methods known for the washing phase of such chromatographic methods.

After the elute step, the process may further comprise a step of regenerating the functionalised chromatography medium or chromatography cartridge of the invention. Typically this is effected by contacting the functionalised chromatography medium or chromatography cartridge from which the one or more biological molecules have been eluted with a buffer. This can be carried out in accordance with conventional methods known for the regeneration phase of such chromatographic methods.

Typically, the one or more biological molecules are chosen from proteins, polypeptides, antibodies, amino acids, viruses and nucleic acids, including, for

example, recombinant proteins, monoclonal antibodies, viral vaccines and plasmid DNA.

Typically, the chromatographic process employs a simulated or actual moving bed system. Thus typically, the process comprises introducing the one or more biological molecules in a mobile phase into one or more simulated or actual moving bed chromatography apparatuses having a plurality of linked chromatography columns, which chromatography columns contain as adsorbent the functionalised chromatography medium of the present invention.

Any known simulated or actual moving bed apparatus may be used to carry out the chromatographic process, provided that it comprises, as adsorbent, the functionalised chromatography medium of the present invention.

Simulated and actual moving bed chromatography are known techniques, familiar to those of skill in the art. The principle of operation involves countercurrent movement of a liquid eluent phase and a solid adsorbent phase. This operation allows minimal usage of solvent making the process economically viable. Such separation technology has found applications in diverse areas including purification of biological molecules using membrane adsorbents.

A simulated moving bed system consists of a number of individual columns containing adsorbent which are connected together in series. Eluent is passed through the columns in a first direction. The injection points of the feedstock and the eluent, and the separated component collection points in the system are periodically shifted by means of a series of valves. The overall effect is to simulate the operation of a single column containing a moving bed of the solid adsorbent. Thus, a simulated moving bed system consists of columns which, as in a conventional stationary bed system, contain stationary beds of solid adsorbent through which eluent is passed, but in a simulated moving bed system the operation is such as to simulate a continuous

countercurrent moving bed.

An actual moving bed system is similar in operation to a simulated moving bed system. However, rather than shifting the injection points of the feed mixture and the eluent, and the separated component collection points by means of a system of valves, instead a series of adsorption units (i.e. columns) are physically moved relative to the feed and drawoff points. Again, operation is such as to simulate a continuous countercurrent moving bed.

The following Examples illustrate the invention.

Materials and equipment

The following materials, equipment and techniques were employed unless stated otherwise

BSA protein Bovine Albumin Serum Fraction V, >96% with molecular weight of ~66 kDa and all other chemicals were purchased from Sigma–Aldrich Co. (Sigma–Aldrich Company Ltd. Dorset, UK) of the highest purity available and used without further purification, unless stated otherwise.

Cytochrome c, from Equine Heart, ≥90% with molecular weight of ~12 kDA was purchased from Merck Chemical (Merck Serono Ltd. Middlesex, UK).

Preparative Example 1

Nanofibre membrane preparation

A 0.20g/mL solution of cellulose acetate, with a relative molecular mass of 29,000g/mol, was dissolved in acetone/dimethylformamide/ethanol (2:2:1). Electrospinning was carried out in a Climate Zone climate control cabinet (a1-safetech Luton, UK) to allow temperature and humidity control of the ambient conditions. Optimized conditions from **O. Hardick, et al, J.Mater. Sci. 46 (2011) 3890** were used to produce non-woven sheets of electrospun cellulose acetate nanofibres with low distribution of fibre diameters, average thicknesses of 20 microns and average area densities of $10g/m^2$.

Once electrospun, fifteen non-woven sheets of nanofibres with a face surface area of $100cm^2$ were layered and pressed in a manual hydraulic press at a pressure of 1MPa for two minutes. After pressing, the sheet of material was immediately placed in a pre-heated oven at 213°C for 5 minutes. The pressed and heated material was then

cut into multiple 25mm diameter discs.

Example 1

Chemical modification by convection

A cellulose acetate nanofibre disc was prepared as above and modified chemically to yield anion exchange surface functionality. A disc having a thickness of 0.188mm and total volume 0.1mL was modified as set out below.

The nanofibre disc was packed into a filter holder prior to derivatisation. Deacetylation was carried out using 30mL of0.1M NaOH in DI water:ethanol (2:1), which was pumped through the disc in a cyclical manner using a Dionex, P680 HPLC pump at a rate of 25mL/min for 24 hours. The disc was then rinsed with 300mL DI H2O at a rate of 25mL/min. Anion-exchange surface functionality was then imparted by cycling 20mL warm (40°C) 15% 2-chloro-N,N-diethylethylamine hydrochloride 99% (DEACH) aqueous solution through the disc at 40mL/min for 10 min. The disc was then removed from the filter holder housing and left for 30 seconds to drip dry before placing into 20mL hot (80°C) 0.5M NaOH in a 50mL sample tube on a shaker table with gentle agitation for 10 min. Finally the disc was rinsed in multiple volumes of DI H2O and left to dry before use.

Example 2

An experiment was carried out as set out in Example 1 above, except that the cellulose acetate nanofibre disc used had a thickness of 0.376mm and total volume 0.2mL.

Comparative Example 1

Chemical modification by diffusion

A cellulose acetate nanofibre disc was prepared as above. A disc having a thickness of 0.188mm and total volume 0.1mL was modified as set out below.

The disc was placed in a 50mL sample tube containing 30mL 0.1M NaOH in DI water:ethanol (2:1) for 24 hours on a laboratory shaker table to deacetylate the cellulose acetate to form regenerated cellulose. The disc was then rinsed thoroughly in 10 x 30mL volumes of DI water for 5 minutes each on the shaker table. Anion-exchange surface functionality was then introduced by placing the rinsed disc into a sample tube with 20mL warm (40°C) 15% 2-chloro-N,N diethylethylamine hydrochloride 99% (DEACH) aqueous solution for 10 min. The adsorbent was removed and allowed to drip dry for 30 seconds before being placed in 20mL hot (80°C) 0.5M NaOH in a new sample tube on the shaker table for 10 min. Finally the disc was rinsed in multiple volumes of DI H2O and left to dry before use.

Comparative Example 2

An experiment was carried out as set out in Comparative Example 1 above, except that the cellulose acetate nanofibre disc used had a thickness of 0.376mm and total volume 0.2mL.

Example 3

Analysis of bioseparation performance

Nanofibre discs prepared and modified in accordance with Examples 1 and 2 and Comparative Examples 1 and 2 were analysed to compare the protein binding

performance of equivalent mass and volume nanofibre membranes derivatised by the two different methods. This was to determine the extent of the modification of the surface area presented by these nanofibre systems.

Experiments were conducted using an AKTA Basic (GE Healthcare Life Sciences, Buckinghamshire, UK) with online measurement of UV absorbance (280nm), pH, and conductivity.

Nanofibre discs prepared and modified in accordance with Examples 1 and 2 and Comparative Examples 1 and 2 were equilibrated with 5mL 20mM Bis-Tris, pH 5.3 wash buffer at a rate of 480 cm/h and then loaded with 1mL of a two component protein solution containing 1mg/mL BSA and 0.25mg/mL Cytochrome C. 5mL wash buffer was then passed through the adsorbent before 5mL 0.4M NaCl 20mM Bis-Tris, pH 5.3 elution buffer was introduced. The eluted capacity was then analysed using Unicorn 5.0 software as measured by the integration of the peak area.

A mixture of BSA and Cytochrome C was used to take advantage of their different isoelectric points and therefore suitability for separation by ion-exchange chromatography. Cytochrome C has a pI of 10.0 while BSA has a pI of 4.7 in water at 25°C. This means that in a Bis-Tris buffer solution at pH 5.3 the Cytochrome C will have a net positive charge and will not bind to the weak anion exchange surface of the DEAE adsorbent. In contrast, at this pH above the pI of BSA, BSA will have a net negative surface charge and therefore will bind to the DEAE adsorbent. As the salt concentration is increased during elution the interaction between the negative surface charge of the BSA and the anion exchanger is out-competed by the salt ions and so the BSA is removed from the adsorbent and collected.

The performance of the adsorbent was analysed over a number of operational cycles to determine reproducibility with regard to the lifetime of the adsorbents. The Table below sets out the average binding capacities for the discs tested.

Sample	Thickness of adsorbent (mm)	Volume of adsorbent (mL)	Binding capacity (mg/mL)
Example 1	0.188	0.1	5.64±0.10
Comp Ex 1	0.188	0.1	5.56±0.40
Example 2	0.376	0.2	6.46±0.44
Comp Ex 2	0.376	0.2	5.88±0.56

For both thicknesses of nanofibre discs, the convective modification process gave a higher binding capacity than the diffusive modification process. This effect was more pronounced for the thicker disc.

The ability of chemical reagents to reach functional surfaces of the polymer system was also observed to depend on the thickness of the nanofibre membrane. Thus, for the thicker nanofibre membranes tested there was a significantly improved binding capacity observed for the DEAE nanofibre adsorbents that were derivatised through convective flow. This suggests that for protocols investigated, diffusion is insufficient for the chemical reagents to reach all binding surface areas.

Example 4

Repeated diffusive chemical modification

Anion-exchange surface derivatisation of cellulose nanofibre discs was carried out as described above in Comparative Example 2, i.e. using a cellulose acetate nanofibre disc having a thickness of 0.376mm and total volume 0.2mL. The chemical modification was then repeated from the point of DEACH introduction to the end of the protocol.

Thus, the disc obtained in Comparative Example 2 was placed into a sample tube with

20mL warm (40°C) 15% 2-chloro-N,N-diethylethylamine hydrochloride 99% (DEACH) aqueous solution for 10 min. The adsorbent was removed and allowed to drip dry for 30 seconds before being placed in 20mL hot (80°C) 0.5M NaOH in a new sample tube on a shaker table for 10 min, then rinsed in multiple volumes of DI H2O.

Example 5

An experiment was carried out as in Example 4, except that the chemical modification was repeated a further time.

Thus, the disc obtained in Example 4 was placed into a sample tube with 20mL warm (40°C) 15% 2-chloro-N,N-diethylethylamine hydrochloride 99% (DEACH) aqueous solution for 10 min. The adsorbent was removed and allowed to drip dry for 30 seconds before being placed in 20mL hot (80°C) 0.5M NaOH in a new sample tube on a shaker table for 10 min, then rinsed in multiple volumes of DI H2O.

Example 6

An experiment was carried out as in Example 5, except that the chemical modification was repeated a further time.

Thus, the disc obtained in Example 5 was placed into a sample tube with 20mL warm (40°C) 15% 2-chloro-N,N-diethylethylamine hydrochloride 99% (DEACH) aqueous solution for 10 min. The adsorbent was removed and allowed to drip dry for 30 seconds before being placed in 20mL hot (80°C) 0.5M NaOH in a new sample tube on a shaker table for 10 min, then rinsed in multiple volumes of DI H2O.

Example 7

Discs produced in Examples 4 to 6 and Comparative Example 2 were analysed for binding capacity using the protocol set out above in Example 3. The pressure drop for each disc was also determined.

The Table below sets out the average binding capacities and pressure drops for the discs tested.

Sample	Chemical modifications	Pressure drop (MPa)	Binding capacity (mg/mL)
Comp Ex 2	x1	0.210	5.88±0.56
Example 4	x2	0.205	13.31±3.11
Example 5	x3	0.245	14.8±0.38
Example 6	x4	0.205	15.88±0.73

These results show a clear improvement in functional group substitution with repeated derivatisation protocol steps. Overall a 270% improvement in binding capacity was observed for the adsorbents tested using repeated protocol steps during derivatisation. Pressure drop was not affected by the repeated modification.

Structural stability of the repeat derivatisation DEAE nanofibre adsorbents did not appear to be affected and this was confirmed by reproducibility studies which showed constant performance over 50 cycles of typical operation.

Figure 1 shows the flowthrough of cytochrome C + unbound BSA as the first peak during loading of a 2-component mixture and the elution of BSA as the second peak. The average result for 50 equivalent binding runs is plotted ±1 standard deviation of the sample population (shown by dashed curves around the main curve of the same colour). These results show the reproducibility over the 50 equivalent binding runs

and show that for the conditions chosen the nanofibre adsorbents performed reproducibly, capturing and eluting 99% of the BSA loaded. The standard deviation was calculated to show that the nanofibre adsorbent operates consistently.

CLAIMS

1. A process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non-woven sheets, each comprising one or more cellulose acetate nanofibres, (ii) pressing the one or more non-woven sheets, (iii) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose, and (v) contacting the thus-obtained product in a batchwise fashion between two and four times with a reagent which functionalises the product of step (iv) as a chromatography medium.

2. The process according to claim 1, each step of contacting with a reagent comprising (a) contacting with the reagent, (b) isolating the product of step (a) from the reagent, (c) optionally treating the product of step (b) with aqueous alkali, and (d) optionally washing the product of step (b)/(c) with water.

3. The process according to claim 1 or 2, step (v) comprising

- (1) (a1) contacting the product of step (iv) with the reagent, (b1) isolating the product of step (a1) from the reagent, (c1) optionally treating the product of step (b1) with aqueous alkali, and (d1) optionally washing the product of step (b1)/(c1) with water, and
  (2) (a2) contacting the product of step (b1)/(c1)/(d1) with the reagent, (b2) isolating the product of step (a2) from the reagent, (c2) optionally treating the product of step (b2) with aqueous alkali, and (d2) optionally washing the product of step (b2)/(c2) with water; or
- (1) (a1) contacting the product of step (iv) with the reagent, (b1) isolating the product of step (a1) from the reagent, (c1) optionally treating the product of step (b1) with aqueous alkali, and (d1) optionally washing the product of step (b1)/(c1) with water,

(2) (a2) contacting the product of step (b1)/(c1)/(d1) with the reagent, (b2) isolating the product of step (a2) from the reagent, (c2) optionally treating the product of step (b2) with aqueous alkali, and (d2) optionally washing the product of step (b2)/(c2) with water, and

(3) (a3) contacting the product of step (b2)/(c2)/(d2) with the reagent, (b3) isolating the product of step (a3) from the reagent, (c3) optionally treating the product of step (b3) with aqueous alkali, and (d3) optionally washing the product of step (b3)/(c3) with water; or

- (1) (a1) contacting the product of step (iv) with the reagent, (b1) isolating the product of step (a1) from the reagent, (c1) optionally treating the product of step (b1) with aqueous alkali, and (d1) optionally washing the product of step (b1)/(c1) with water,

(2) (a2) contacting the product of step (b1)/(c1)/(d1) with the reagent, (b2) isolating the product of step (a2) from the reagent, (c2) optionally treating the product of step (b2) with aqueous alkali, and (d2) optionally washing the product of step (b2)/(c2) with water,

(3) (a3) contacting the product of step (b2)/(c2)/(d2) with the reagent, (b3) isolating the product of step (a3) from the reagent, (c3) optionally treating the product of step (b3) with aqueous alkali, and (d3) optionally washing the product of step (b3)/(c3) with water, and

(4) (a4) contacting the product of step (b3)/(c3)/(d3) with the reagent, (b4) isolating the product of step (a4) from the reagent, (c4) optionally treating the product of step (b4) with aqueous alkali, and (d4) optionally washing the product of step (b4)/(c4) with water.

4. The process according to any one of the preceding claims, each step of contacting with a reagent being for a period of time from 1 to 20 minutes.

5. A process for preparing a functionalised cellulose chromatography medium, which process comprises (i) providing one or more non-woven sheets, each

comprising one or more cellulose acetate nanofibres, (ii) pressing the one or more non-woven sheets, (iii) heating the one or more non-woven sheets to fuse points of contact between sections of the one or more cellulose acetate nanofibres, (iv) treating the pressed and heated product to convert the cellulose acetate to cellulose, (v) placing the thus-obtained product in a holder, and (vi) causing a reagent to flow through the holder so that the reagent flows in contact with the product obtained in step (iv) which functionalises the product of step (iv) as a chromatography medium.

6. The process according to claim 5, step (vi) comprising
- causing a reagent to flow through the holder under pressure; and/or
- causing a reagent to flow through the holder using a pump; and/or
- causing a reagent to flow through the holder in a cyclical manner; and/or
- causing a reagent to flow through the holder for a period of time from 1 to 20 minutes.

7. The process according to claim 6 or 7 additionally comprising the step of treating the product of step (vi) with aqueous alkali, and optionally washing the thus-obtained product with water.

8. The process according to any one of the preceding claims, the functionalised cellulose chromatography medium being suitable for use in a chromatography method chosen from ion exchange, affinity capture or hydrophobic interaction methods.

9. The process according to claim 8,
- the chromatography method being a cationic exchange method, and the chromatography medium being functionalised with one or more carboxylate, sulphonate or phosphonate groups;
- the chromatography method being an anionic exchange method, and the chromatography medium being functionalised with one or more quaternary amino or diethylamine groups, preferably one or more DEAE groups;

- the chromatography method being an affinity capture chromatography method, and the chromatography medium being functionalised with one or more proteins, peptides, antibodies or fragments thereof, dyes, histidine, or groups containing a metal cation; or
- the chromatography method being a hydrophobic interaction chromatography method, and the chromatography medium being functionalised with one or more propyl, butyl, phenyl, or octyl groups.

10. The process according to any one of the preceding claims, wherein one or more hydroxyl groups on the cellulose chromatography medium are functionalised.

11. The process according to any one of the preceding claims, the one or more cellulose nanofibres having a mean diameter of 10nm to 1000nm.

12. The process according to any one of the preceding claims, the step of pressing the one or more non-woven sheets employing a pressure of 0.01 to 5 MPa.

13. The process according to any one of the preceding claims, the step of heating the one or more non-woven sheets employing a temperature of from 200 to 220°C.

14. A process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres, (II) pressing the one or more polymer nanofibres, (III) heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres, and (IV) contacting the pressed and heated product with a reagent which functionalises the product of step (III) as a chromatography medium.

15. A process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres, (II) optionally pressing the one or more polymer nanofibres, (III) optionally heating the

one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres, and (IV) contacting the product of step (I), (II) or (III) in a batchwise fashion at least two times with a reagent which functionalises the product of step (I), (II) or (III) as a chromatography medium.

16. A process for preparing a functionalised polymeric chromatography medium, which process comprises (I) providing one or more polymer nanofibres, (II) optionally pressing the one or more polymer nanofibres, (III) optionally heating the one or more polymer nanofibres to fuse points of contact between sections of the one or more polymer nanofibres, (IV) placing the product of step (I), (II) or (III) in a holder, and (V) causing a reagent to flow through the holder so that the reagent flows in contact with the product of step (I), (II) or (III) which functionalises the product of step (I), (II) or (III) as a chromatography medium.

17. The process according to any one of claims 14 to 16, the functionalised chromatography medium being suitable for use in a chromatography method defined in claim 8 or 9.

18. The process according to any one of the claims 14 to 16, wherein one or more hydroxyl, amino or carboxylic acid groups on the chromatography medium are functionalised.

19. The process according to any one of claims 14 to 16, the polymer being chosen from cellulose, cellulose acetate, polysulfones, polyamides, polyacrylic acid, polymethacrylic acid, polyacrylonitrile, polystyrene, polyethylene oxide, and mixtures thereof.

20. A functionalised chromatography medium obtainable by the process according to any one of the preceding claims.

21. A process for preparing a chromatography cartridge, which process comprises carrying out the process of any one of claims 1 to 19 and incorporating the thus-obtained product into a cartridge.

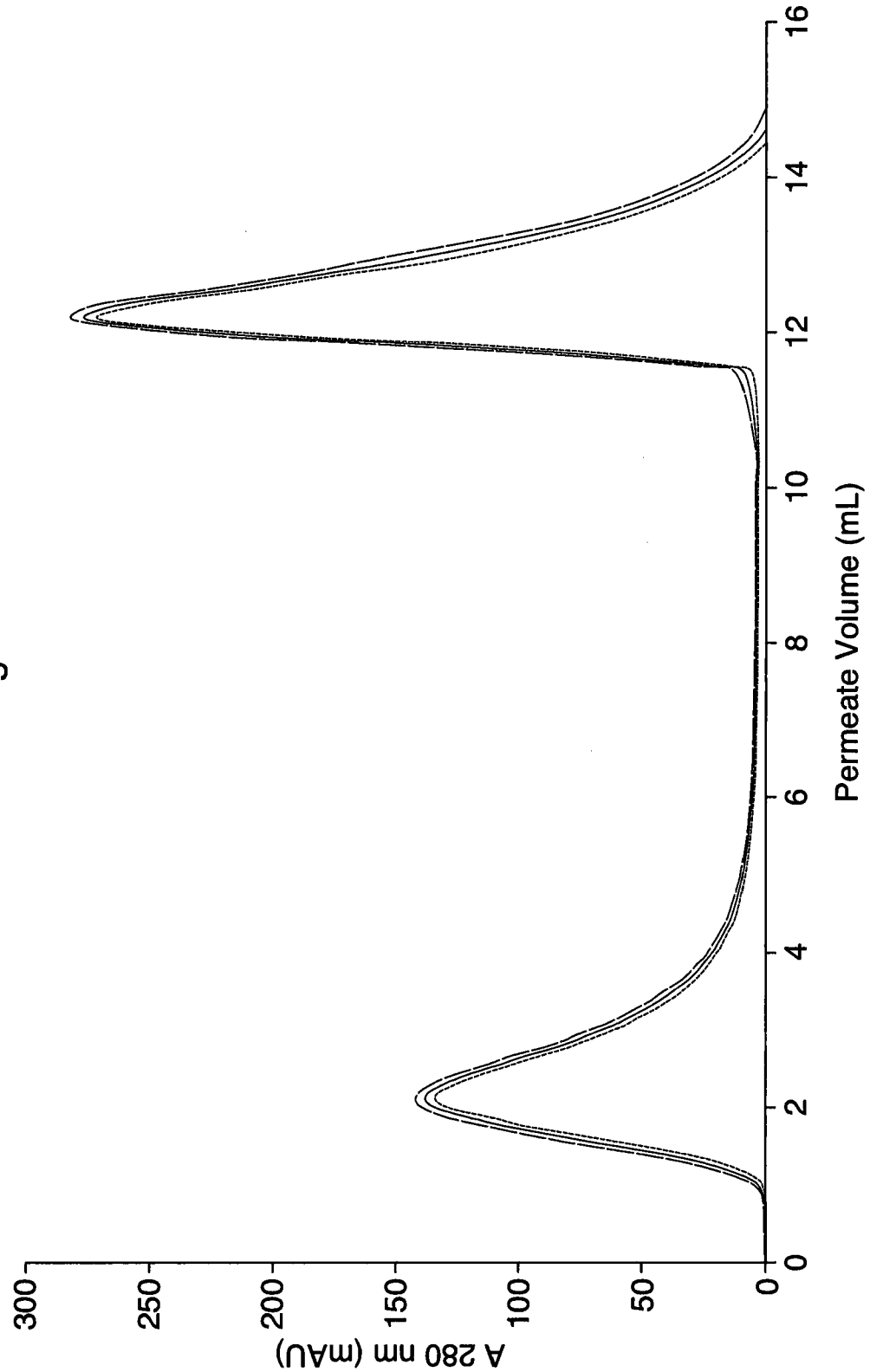
22. A chromatography cartridge which (a) is obtainable by the process of claim 21, or (b) which comprises one or more functionalised chromatography media according to claim 20.

23. Use of a functionalised chromatography medium according to claim 20 or a chromatography cartridge according to claim 22 in chromatography.

24. A process for isolating one or more biological molecules from a mobile phase, which process comprises contacting one or more biological molecules in a mobile phase with a functionalised chromatography medium according to claim 20 or a chromatography cartridge according to claim 22.

25. The process according to claim 24, which is an ion exchange, affinity capture or hydrophobic interaction chromatography process.

Figure 1


A 280 nm (mAU)
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
Permeate Volume (mL)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/052626

A. CLASSIFICATION OF SUBJECT MATTER
INV. B01J20/24 B01D15/36 B01D15/38 B01J20/28 B01J20/285
B01J20/30 C07K1/18 C07K1/22
ADD.
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)
B01J B01D C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MA Z ET AL: "Electrospun cellulose nanofiber as affinity membrane", JOURNAL OF MEMBRANE SCIENCE, ELSEVIER SCIENTIFIC PUBL.COMPANY. AMSTERDAM, NL, vol. 265, no. 1-2, 15 November 2005 (2005-11-15), pages 115-123, XP027869363, ISSN: 0376-7388 [retrieved on 2005-11-15]	20,22-25
Y	abstract Section "2. Experiments"; page 116 - page 118	1-4, 8-13,21
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[X] Further documents are listed in the continuation of Box C.

[X] 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

"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
30 April 2014	21/08/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Klemps, Christian

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZHANG L ET AL: "Fabrication and bioseparation studies of adsorptive membranes/felts made from electrospun cellulose acetate nanofibers", JOURNAL OF MEMBRANE SCIENCE, ELSEVIER SCIENTIFIC PUBL.COMPANY. AMSTERDAM, NL, vol. 319, no. 1-2, 1 July 2008 (2008-07-01), pages 176-184, XP022696405, ISSN: 0376-7388, DOI: 10.1016/J.MEMSCI.2008.03.030 [retrieved on 2008-03-27] Section: "2.2.2. Hydrolysis/deacetylation and surface functionalization"; page 178, right-hand column -----	1-4, 8-13,21
A	WO 2013/068741 A1 (UCL BUSINESS PLC [GB]) 16 May 2013 (2013-05-16) the whole document -----	1-4, 8-13, 20-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2013/052626

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:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional 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.:

1-4(completely); 8-13, 20-25(partially)

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

Information on patent family members

International application No
PCT/GB2013/052626

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013068741 A1	16-05-2013	NONE	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4(completely); 8-13, 20-25(partially)

   directed towards a process for preparing a functionalised cellulose chromatography medium

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2. claims: 5-7(completely); 8-13, 20-25(partially)

   directed towards a different process for preparing a functionalised cellulose chromatography medium

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3. claims: 14(completely); 17-25(partially)

   directed towards a process for preparing a functionalised polymeric chromatography medium

   ---

4. claims: 15(completely); 17-25(partially)

   directed towards a different process for preparing a functionalised polymeric chromatography medium

   ---

5. claims: 16(completely); 17-25(partially)

   directed towards a different process for preparing a functionalised polymeric chromatography medium

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