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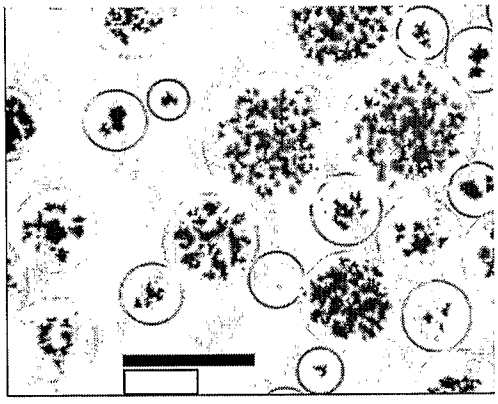
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MAGNETIC BEADS



(57) Abstract: The present invention relates to magnetic beads in the form of composite beads with an inner core of metal particles, which are coated with an inert synthetic polymer and thereafter a hydrophilic porous polymer, preferably dextran. This provides porous biocompatible beads without metal leakage. This construction also allows for simple and convenient handling of cell expansion media by magnetism.

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Title: Magnetic beads

Field of the invention

The present invention relates to magnetic beads suitable for, for example, cell expansion.

Background of the invention

Stem cell therapy is compelling because it promises potential treatments for many of today's most devastating diseases, including diabetes, cardiac disease, cancer, metabolic diseases and diseases related to ageing, such as Alzheimer's disease and osteoarthritis.

A critical enabling technology for the success of cell therapy is ESC (embryonic stem cell) expansion. Commercially available ESC expansion media include Cytodex 1 and Cytodex 3 from Amersham Biosciences AB.

Recently an increased number of products referred to as magnetic beads and a number of products for efficient handling of these products have been presented. Magnetic bead technologies are used for diverse purposes such as isolating nucleic acids and proteins as well as viruses and whole cells. The adaptability and speed of this technique makes it ideal for high-throughput applications e.g. in 96 wells micro titre plates. The technique is also applicable for large scale applications, such as chromatography applications in liquid magnetically stabilised fluidised beds.

The magnetic beads are most commonly used in combination with attached ligands having affinity for different substances. The most commonly encountered examples are metal chelating ligands (of IMAC type) intended for use in combination with His-tags and glutathione intended for use in combination with GST (Gluthathione S transferase). Other examples are a variety of different IgG's with different specificity.

Preparation of beads encapsulating metallic materials and applications of magnetic beads has been described previously. Preparing magnetic beads where the bead is built up of different layers of material has also been presented earlier.

US 5,834,121 describes composite magnetic beads. Polymer coated metal oxide particles that are encapsulated in a rigid and solvent stable polymer of vinyl monomers in order to retain the metal oxide particles during harsh conditions. The primary beads are enclosed in a micro porous polymer bead which is capable of swelling in organic solvents and allowing for further functionalisation in order to be useful for organic synthesis. This procedure is aiming for hydrophobic beads.

US 6,204,033 describes preparation of polyvinyl alcohol-based magnetic beads for binding bio molecules. Preparation of magnetic beads by polyvinyl alcohol in water containing magnetic particles. The final beads contain hydroxyl functionalities that can be further derivatized in order to couple bio molecules. It is claimed that these magnetic beads can be grafted with vinyl monomers carrying various functional groups.

EP 0179039 describes polymer coated metal surfaces. Dextran carrying imino diacetate groups are allowed to attach to a metal surface. Several rounds of activation and coupling of dextran is required to build up a particle. To the dextran various ligands can be attached.

In spite of the relatively large number of magnetic beads described today, there is a need of improved magnetic beads for cell expansion. There is a need of biocompatible magnetic beads intended for cell expansion/cultivation which are free of metal leakage that might have a negative effect on the cells. Especially, it is desired to enable the expansion of ESC`s in the defined differentiation status and to separate the cells from the micro carrier/surface. There is also a need of providing micro carriers/surfaces in a user-friendly and safe format.

Summary of the invention

The present invention provides cross linked dextran beads which may be provided with desired surface properties for, for example, cell expansion and which at the same time possess magnetic properties. These beads are obtained by emulsification using cellulose acetate butyrate, cellulose acetate, cellulose propionate or cellulose acetate phthalate as emulsifier. This emulsifier is after cross linking found in the surface of the bead giving the dextran bead in part the character of a cellulose bead. This bead is further derivatised depending on the intended purpose.

These magnetic beads offer the potential for handling the material by magnets e.g. for pull down during washing procedures or transfer of beads and cells to new vessels.

This invention provides micro carriers/surfaces which enable the expansion of ESC`s in a defined differentiation status combined with the technology to separate the cells from the micro carrier/surface. Furthermore, the micro carriers/ surfaces are user-friendly and in a safe format.

The present invention relates to a novel construction that provides a magnetic beaded material constructed in such a way that low metal leakage is combined with a hydrophilic, biocompatible outer core suitable for, for example, cell expansion.

According to the present invention magnetic metal oxide particles are coated in an inert synthetic polymer and subsequently the particles are coated with a porous outer layer of dextran. This coating procedure provides magnetic beads with low risk of leakage of metal ions even at harsh conditions, in combination with a hydrophilic, bio compatible outer layer.

Thus, in a first aspect the present invention provides magnetic beads, comprising metal particles made of, for example, metals, metal oxides and/or alloys. The magnetic beads comprise a coating of an inert synthetic polymer surrounding the metal particles and an outer coating of a porous layer surrounding the inert coating(s), wherein the outer coating is produced using an emulsifying agent that in part gives the bead the character of a cellulose bead.

Preferably the emulsifying agent is chosen from cellulose acetate butyrate, cellulose acetate, cellulose propionate or cellulose acetate phthalate, most preferably cellulose acetate butyrate.

The magnetic core of the magnetic bead comprises at leas one magnetic particle and may comprise 2-5 coated magnetic particles enclosed in each bead.

Preferably, the inner coating is made of cross linked polystyrene, for example poly(divinyl benzene), but other synthetic polymers such as cross linked poly(methacrylates) or polyacrylates can be used. This coating prevents metal leakage from the magnetic metal particles.

According to the invention the outer coating is made of a natural or synthetic hydrophilic polymer. Hydrophilic properties are very important for obtaining higher absorption capacity, biocompatibility, and prevention of unspecific interactions.

Preferably, the outer coating is made of dextran. Other examples are agarose and carbohydrate polymers, such as cellulose. Further alternatives of hydrophilic coatings are poly(vinyl alcohol) or polyacrylamides.

The particle diameter of the total bead is 5-1000 µm, preferably 20 -400 µm, most preferably 50 -150 µm.

In a preferred embodiment the inner coating is made of poly(divinylbenzene) and the outer coating is made of dextran using and emulsifying agent that in part gives the bead the character of a cellulose bead.

Preferably, the outer coating is provided with ligands having affinity for a desired biomolecule. Alternatively, the outer coating is provided with a surface suitable for cell expansion, such as a suitable coating and/or suitable ligands. Preferred examples are a collagen coating and low charge DEAE ligands.

In a preferred embodiment of the invention, the magnetic metal particles are made of Fe_3O_4, the inner coating is made of poly(divinylbenzene), the outer coating is made of dextran. The pore size of the bead composite is 1 nm-50 µm, preferably 50-500 nm.

In a second aspect, the invention relates to a method of producing magnetic beads, comprising the following steps

a) treating magnetic metal, metal oxide or alloy particles with an amphiphilic agent such as SDS or oleic acid;

b) adding a polymerisable monomer and a radical initiator to the treated magnetic particles;

c) emulsifying the monomer/particle mixture in an aqueous phase and polymerising the monomer by increasing the temperature to obtain polymer-coated magnetic particles;

d) adding a hydrophilic polymer to the polymer-coated magnetic particles;

e) emulsifying the polymer-coated magnetic particles into the hydrophilic polymer; and

f) derivatizing the outer layer of the hydrophilic polymer.

The derivatisation in step f) may be with any desired ligand or with any other suitable surface modification for the desired purpose, such as cell expansion purposes.

In the above method it is preferred that, the magnetic metal oxide particles are Fe_3O_4, the chemically inert polymer is poly(divinylbenzene), and the hydrophilic polymer is dextran, and that the emulsifying agent in part render the bead the character of a cellulose bead.

In a third aspect, the invention relates to use of the magnetic beads described above for separating, concentrating or analysing a biomolecule. The biomolecule may be selected from a peptide, protein, carbohydrate, nucleic acid, plasmid, virus or cell.

In a preferred embodiment, the magnetic beads are used for cultivating cells after suitable derivatisation of the outer layer. The invention is not restricted to cultivation of any special kind of cell and may for example be selected from the group consisting of mammalian cells, stem cells or bacterium. The preferred cells are stem cells, such as embryonic stem cells.

Brief description of the figures

Fig. 1 shows poly(DVB)- particles with encapsulated magnetic beads; and

Fig. 2 shows dextran beads with encapsulated beads according to Fig. 1.

Detailed description of the invention

Two different magnetic materials have been used in the currently prepared magnetic dextran beads. One is magnetite particles without any coating (Figure 1, left) and the other is magnetite particles that have been coated with DVB, using an emulsification procedure (Figure 1, right). The former material offers a straight forward approach and is apparently simple to handle during the dextran emulsification procedure, but the final material will most likely be afflicted with metal leakage. The DVB encapsulated material will offer a final material less prone to metal leakage but introduces one extra step to the preparation procedure.

The present inventors have found that encapsulated magnetic materials can be introduced into hydrophilic, porous materials such as dextran. In a preferred embodiment, the magnetic material is encapsulated in small cross linked polystyrene beads that are used as core particles in the preparation of dextran beads.

This approach results in beads that are chemically stable towards metal leakage and at the same time posses an outer layer that offers a more suitable environment for cell expansion.

EXPERIMENTAL PART

1. Materials and Methods

Synthesis of magnetic poly(divinyl benzene) particles

5 g of iron oxide powder (particle size < 5 µm) is added to 50 mL of oleic acid in an Ehrlenmeyer flask. The flask is left on a shaking table at room temperature for an hour. The iron oxide is allowed to sediment, and as much as possible of the oleic acid is removed by decantation.

0.4 g 2,2'-azobis(2-methylbutyronitrile) (AMBN) is dissolved in 20 g divinyl benzene (DVB), tech. 80%, and after complete dissolution of the initiator, the iron oxide particles are added.

A 4 % Methocel K-100 (w/v) solution is prepared in advance.

85 g of the methocel solution is added to a 250 mL three-necked round-bottom flask, followed by the organic phase prepared as above. The stirring speed is set at 175 rpm. After 30 minutes the reactor is immersed in an oil bath set at 70 degrees, and the polymerisation reaction is left overnight.

The product particles are sedimented a number of times in water, to remove fines. The particles are then washed on a glass filter with water, 5 M HCl and ethanol. No yellow colour (indicating iron leakage) was observed during the acid wash.

According to the invention, the method used for the preparation of magnetic poly(divinyl benzene) beads is suspension polymerisation. An important step in the preparation is that the magnetic entity, such as iron oxide powder, is pre-treated with an amphiphilic agent, such as oleic acid, which will render the material more hydrophobic so as to be dispersable in the divinyl benzene phase during synthesis.

This synthesis method uses emulsification of an oil-in-water suspension. This method results in a highly magnetically active material where the magnetite (Fe_3O_4) particles, are encapsulated within the bead (Figure 1). This means that the risk of leakage at acid pH is minimised, since the poly(divinyl benzene) is chemically inert at all pH commonly used in chromatography (pH 1-14). This material is suited as the basis for further coating with a hydrophilic polymer, e.g. dextran or a hydrophilic synthetic polymer, resulting in a magnetic material encapsulated in the chemically stable poly(DVB)-material and with an external hydrophilic layer (Figure 2).

Hydrophilisation of magnetic poly(divinyl benzene) beads

5 g of 2,2'-azobis(dimethylbutyronitrile) (Fluka 11596) was dissolved in 200 g of diethylene glycol monovinyl ether. Nitrogen was flushed through the solution for 30 minutes. 15 g of magnetic poly(divinylbenzene) beads as prepared in example 1 were added.. The temperature was raised to70 °C and the reaction was left overnight. The magnetic particles were washed with a 50 % (v/v) ethanol in water solution.

Encapsulation of iron oxide powder (particle size <5 µm) in dextran beads

A solution of 209 ml water, 9.4 g sodium hydroxide, 0.46 g sodium borohydride and 94.3 g Dextran TF (molecular weight 150000-250000g/mol) (GEHC) was heated to 50°C and thereafter mixed with 20 g iron (II, III) oxide powder (Aldrich, particle size <5 µm).

A solution of 12.0 g cellulose acetate butyrate (can be obtained from several commercial sources, e.g. Aldrich) and 200 ml dichloroetane, was heated to 50°C in a 1 litre reactor. The

stirring speed was set to 100 rpm. The mixture above was added to this solution and thereafter the speed was increased to 160 rpm. When an acceptable particle size, as judged by ocular analysis using a microscope, was achieved, 12.8 ml epichlorohydrin (can be obtained from several commercial sources, e.g. Aldrich) was added. The reaction was left over night at the same stirring speed and temperature.

The formed beads are then washed repeated times with acetone and thereafter with water.

Encapsulation of magnetic DVB beads in dextran beads

A solution of 105 ml water, 4.7 g sodium hydroxide 0.23 g sodium borohydride and 47.1 g Dextran TF (molecular weight 150000-250000g/mol) was heated to 50°C and thereafter mixed with 6 g dry sucked magnetic DVB particles as prepared in example 2.

A solution of 6.0 g cellulose acetate butyrate and 100 ml dichloroetan, was heated to 50°C in a 1 litre reactor. The stirring speed was set to 100 rpm. The mixture above was added to this solution and thereafter the speed was increased to 125 rpm. When an acceptable particle size, as judged by ocular analysis using a microscopy, was achieved 6.4 ml epichlorohydrin was added. The reaction was left over night at the same speed and temperature.

The formed beads are then washed repeated times with acetone and thereafter with water.

Thereafter the emulsion was cooled and the beads were allowed to gel. The beads were washed with water and ethanol and enriched using a magnet. Approximately half of the dextran beads formed contained magnetic DVB beads. These dextran beads contain at least one inner bead of magnetic DVB, preferably 2-5.

Further derivatisation of the dextran beads

The outer dextran layer is also suited for further derivatisation with any desirable compound that fulfils the needs for the intended application, i.e. cell expansion.

For cell expansion purposes, the derivatisation is preferably with collagen or low density DEAE ligands, according to conventional methods.

Claims

1. Magnetic bead, comprising magnetic particle(s) enclosed by a coating of an inert synthetic polymer and an outer coating of a porous layer, wherein the outer coating is cross-linked and produced using an emulsifying agent that is found in the surface of the bead after cross-linking.

2. Magnetic bead according to claim 1, wherein the magnetic particle(s) are made of magnetic metal, metal oxide and/or alloy particles.

3. Magnetic bead according to claim 2, wherein 2-5 coated magnetic particles are enclosed in each bead.

4. Magnetic bead according to claim 1, 2 or 3, wherein the emulsifying agent is cellulose acetate butyrate, cellulose acetate, cellulose propionate or cellulose acetate phtalate

5. Magnetic bead according to one or more of the above claims, wherein the inner coating is made of cross linked polystyrene, poly(methacrylates) or polyacrylates.

6. Magnetic bead according to one or more of the above claims, wherein the outer coating is made of a natural or synthetic hydrophilic polymer.

7. Magnetic bead according to claim 6, wherein the outer coating is made of, dextran, cellulose, agarose, poly(vinyl alcohol) or polyacrylamides.

8. Magnetic bead according to one or more of the above claims, wherein the particle diameter is 5-1000 µm, preferably 20 - 400 µm, most preferably 50-150 µm.

9. Magnetic bead according to one or more of the above claims, wherein the inner coating is made of crosslinked polystyrene, preferably poly(divinylbenzene), and the outer coating is made of dextran.

10. Magnetic bead according to one or more of the above claims, wherein the outer coating is derivatised.

11. Magnetic bead according to claim 10, wherein the outer coating is derivatised with low density DEAE-ligands or a collagen coating.

12. Magnetic bead according to one or more of the above claims, wherein the

magnetic metal particles are made of Fe_3O_4, the inner coating is made of poly(divinylbenzene), the outer coating is made of dextran.

13. Method of producing magnetic beads, comprising the following steps
a) treating magnetic metal, metal oxide or alloy particles with an amphiphilic agent;
b) adding a polymerisable monomer and a radical initiator to the treated magnetic particles.
c) emulsifying the monomer/particle mixture in an aqueous phase and polymerising the monomer by increasing the temperature to obtain polymer-coated magnetic particles.
d) adding a hydrophilic polymer to the polymer-coated magnetic particles; and
e) emulsifying the polymer-coated magnetic particles into the hydrophilic polymer to form magnetic beads.

14. Method according to claim 13, comprising a further step f) derivatizing the outer layer.

15. Method according to claim 13 or 14, wherein the magnetic metal oxide particles are Fe_3O_4, the chemically inert polymer is divinylbenzene, and the hydrophilic polymer is agarose.

16. Method according to claim 13, 14 or 15, wherein the emulsifying agent is selected from cellulose acetate butyrate, cellulose acetate, cellulose propionate or cellulose acetate phthalate

17. Use of the magnetic particles or beads according to one or more of the claims 1-12 for expanding cells.

18. Use according to claim 17, wherein the cell is a mammalian cell or a bacterium.

19. Use according to claim 18, wherein the cell is a human stem cell.

20. Use of the magnetic particles or beads according to one or more of the claims 11-12 for separating, concentrating or analysing a biomolecule.

21. Use according to claim 20, wherein the biomolecule is selected from a peptide, protein, carbohydrate, nucleic acid, plasmid, virus or cell.

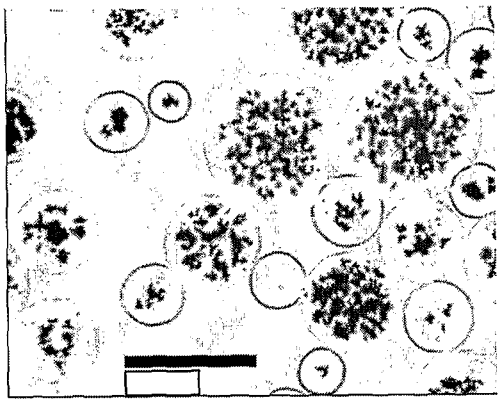

Figure 1

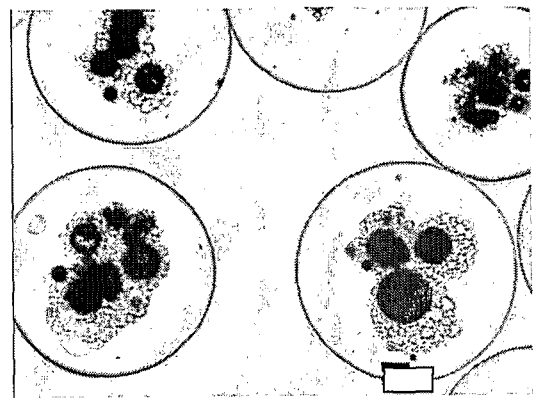

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2007/000273

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: G01N, H01F, C12N

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, WPI DATA, PAJ, TXTE, MEDLINE, BIOSIS, EMBASE, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5834121 A1 (SUCHOLEIKI, IRVING ET AL), 10 November 1998 (10.11.1998), column 4, line 40; column 5, line 6 - line 13; column 5, line 25 - line 40, column 6, line 5	1-3,5-8,10
Y		4,6-7,9, 11-21
	--	
Y	WO 2004056473 A1 (AMERSHAM BIOSCIENCES AB), 8 July 2004 (08.07.2004), the abstract; page 8, paragraps 2 and 5, page 16, line 19 - line 21; page 16, paragraph 3	4,6-7,9, 11-21
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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
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"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
5 July 2007	09 -07- 2007
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz/ELY Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2007/000273

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 8303426 A1 (GAMBRO LUNDIA AB), 13 October 1983 (13.10.1983), page 4, line 26 - line 34, claim 9, abstract --	1-21
A	US 4272510 A1 (SMITH, KENDALL O. ET AL), 9 June 1981 (09.06.1981), column 3, line 50 - line 59; column 3, line 62; column 4, line 17 - line 44, claim 1 --	1-21
A	DATABASE WPI Week 198748 Derwent Publications Ltd., London, GB; Class A97, AN 1987-339074 & JP 62 244438 A (KASHIMA SEKIYU KK), 24 October 1987 (1987-10-24) abstract --	1-21
A	AJAY KUMAR GUPTA ET AL, "Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications", Biomaterials, 2005, vol. 26, page 3995 - page 4021, page 4000, left column, third paragraph - page 4001, left column, first paragraph; figure 5; page 4002 - 4003, "3.2 Surface modification with polymeric stabilizers" --	1-21
A	BASINSKA, TERESA, "Hydrophilic Core-Shell Microspheres: A Suitable Support for Controlled Attachment of Proteins and Biomedical Diagnostics", Macromol. Biosci., 2005, vol. 5, page 1145 - page 1168, page 1151, left column --	13-16
A	EP 0855441 A2 (DIRECTOR OF NATIONAL INSTITUTE OF ANIMAL INDUSTRY), 29 July 1998 (29.07.1998) -- --------	17-21

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C12N 11/10 (2006.01)
B22F 1/00 (2006.01)
C12N 11/08 (2006.01)
C12N 5/00 (2006.01)
G01N 33/543 (2006.01)
H01F 1/06 (2006.01)

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Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2007/000273

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.: 1-5, 8, 10-11, 17-21 (all partially)
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:

Present claims 1-5, 8, 10-11, 17-21 relate, in respect of the wording "porous layer, wherein the outer coating is cross-

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:

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

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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.

Box II.2

linked and produced using an emulsifying agent that is found in the surface of the bead after cross-linking.", to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out mainly for those parts of the claims which appear to be supported and disclosed, namely hydrophilic polymers in general, and especially dextran but also the compounds mentioned in the description page 3, seventh paragraph.

US	5834121	A1	10/11/1998	NONE			
WO	2004056473	A1	08/07/2004	AU	2003287139	A	14/07/2004
				CA	2508271	A	08/07/2004
				EP	1572351	A	14/09/2005
				GB	0229696	D	00/00/0000
				JP	2006510482	T	30/03/2006
				US	20060065583	A	30/03/2006
WO	8303426	A1	13/10/1983	EP	0116550	A	29/08/1984
				JP	59500399	T	15/03/1984
				SE	8201972	A	30/09/1983
				US	4687748	A	18/08/1987
US	4272510	A1	09/06/1981	US	4292920	A	06/10/1981
EP	0855441	A2	29/07/1998	JP	10179165	A	07/07/1998