

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
9 April 2009 (09.04.2009)

PCT

(10) International Publication Number
WO 2009/043191 A2

(51) International Patent Classification:

A61K 39/00 (2006.01) C08F 2/22 (2006.01)
B01J 20/28 (2006.01)

Miriam [DE/CH]; Schaffhauserstrasse 286, CH-8057
Zürich (CH).

(21) International Application Number:

PCT/CH2008/000390

(74) Agent: BREMI, Tobias; Isler & Pedrazzini AG, Gotthard-
strasse 53, Postfach 1772, CH-8027 Zürich (CH).

(22) International Filing Date:

19 September 2008 (19.09.2008)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

07019511.0 5 October 2007 (05.10.2007) EP

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

(71) Applicant (for all designated States except US): EL-
DGENÖSSISCHE TECHNISCHE HOCHSCHULE
ZÜRICH [CH/CH]; Rämistrasse 101, CH-8092 Zürich
(CH).

(72) Inventors; and

(75) Inventors/Applicants (for US only): MORBIDELLI,
Massimo [IT/CH]; Obsthaldenstrasse 63, CH-8046 Zürich
(CH). BUTTE, Alessandro [IT/CH]; Kelchbachstrasse
5, CH-3904 Naters (CH). MARTI, Nadia [CH/CH];
Winterthurerstrasse 197, CH-8055 Zürich (CH). KÜTHE,

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

Published:

— without international search report and to be republished
upon receipt of that report

(54) Title: METHOD FOR PRODUCING MACRO-POROUS MATERIALS

(57) Abstract: The present document relates to a method for producing macro-porous materials comprising the following steps: a) synthesis of narrowly dispersed cross-linked polymeric particles starting from a monomer and a cross-linker in an emulsion polymerization; b) swelling of the particles with a liquid comprising at least an additional charge of monomer and cross-linker and subsequent destabilisation; c) initiating the reaction of the swelled particles to form a monolithic structure. The corresponding monolithic structures can be very efficiently functionalised if after the synthesis of the polymeric particles in step a) and before the initiation of the reaction of the swelled particles in step c) the polymeric particles are chemically functionalised or prepared for subsequent functionalization of the monolithic structure.



WO 2009/043191 A2

SPECIFICATION

TITLE

Method for producing macro-porous materials

TECHNICAL FIELD

5 The present document relates to a method for producing macro-porous materials, in particular monolithic structures as can be used for chromatographic purposes.

BACKGROUND OF THE INVENTION

Biopharmaceuticals, and particularly monoclonal antibodies, constitute today the basis for the development of the most innovative therapies for a number of diseases, such as
10 cancer. However, the costs of these therapies are exceedingly high, and a significant percentage (50-80%) of the total antibody manufacturing costs is incurred during downstream processing (purification).

Chromatography is the most widely used technology for protein purification. Starting from the '70s, better and better stationary phases for chromatography have been
15 developed, based on properly functionalized porous materials. Emphasis has been placed on improving the surface binding capacity in the frame of various chromatographic modes. In all cases a key role is played by the support, whose porosity has to be properly tuned with respect to two conflicting objectives: a large surface area to maximize binding capacity; and large pores to facilitate mass transport. The
20 resolution of this problem, which is particularly serious in the case of large molecules as proteins, is the key for the development of more efficient chromatographic materials for protein purification.

The problem illustrated above indicates the need for developing techniques to produce porous materials with highly controlled structure which can be used also on a large
25 (industrial) scale. All existing polymeric stationary phases are produced by a one-step-reaction, where a balanced mixture of (co)monomers, cross-linkers and porogens (to create the pores) are used together to form the material. For such reason, the possibility of controlling the material properties is very limited, since the mutual interactions among these components is highly complex.

Recently, a new technique has been introduced, called reactive gelation. According to this technique, the formation of the porous material is divided into a number of separate steps. This allows a much better definition of the material properties and, in particular, of the pore size distribution.

5 A key aspect in the definition of the performance of the supports for chromatography is represented by the surface functionalization. Different chromatography modes exist which are exploiting the differences in charge (ion exchange chromatography), in hydrophobicity (hydrophobic interaction chromatography and reverse phase chromatography), or a mix of the two (e.g., hydrophobic charge induction chromatography). Chromatographic materials are also existing which are performing
10 separation only based on the size of the solutes (size exclusion chromatography), where any interaction with the support must be minimized. Finally, in affinity chromatography specific biologic interactions, such as that between antigen and antibody, enzyme and substrate, or receptor and ligand, are exploited. In all these cases, a precise surface
15 functionalization is of paramount importance for the performance of the material.

As a special functionalization aimed towards separation processes, PNIPAAm chains can be grown from the polymer surface inside the particles or the monolith. Reversible hydrophobicity and hydrophilicity of poly (N-isopropylacrylamide) (PNIPAAm) gels has e.g. been a subject of interest for many years. Owing to its unique properties, these
20 polymeric gels grown from various surfaces have found immense applications especially for biomedical separations. The growth techniques have also undergone tremendous changes in terms of accuracy and control of molecular characteristics. However, the growth of these polymers from spherical substrates is still not as developed as their flat counterparts. Spherical substrates e.g. latex particles offer a
25 unique potential for many applications owing to their high surface area. Recently, a few publications have focused on the use of such spherical particles where aqueous atom transfer radical polymerization (ATRP) was used to grow the PNIPAAm brushes from the emulsifier-free spherical latex particles functionalized with a thin shell of ATRP initiator beforehand.

30 Due to the particular hydrophilic/hydrophobic properties of PNIPAAm, the

chromatographic separations of media like proteins, viruses etc. can be achieved just by changing the network or eluent temperature. In fact, these media are typically adsorbing on hydrophobic surfaces, i.e. for temperatures above, the lower critical solution temperature (LCST) and can desorb when the surface becomes hydrophilic, i.e. below the LCST. This would help in avoiding the use of harsh adsorption and desorption conditions used conventionally which may sometimes affect the quality of the biological media. A qualitative adsorption and desorption behavior of tobacco mosaic virus was successfully shown in a study on the latex particles carrying PNIPAAm brushes around them thus indicating the potential use of monoliths for such purposes.

SUMMARY OF THE INVENTION

In a recent paper the monolith formation from polymer particles by using reactive gelation process is described by the present applicant for the generation of controlled networks based on swelling the latex particles followed by gelation and post gel polymerization (Marti, N.; Quattrini, F.; Butte, A.; Morbidelli, M. *Macromolecular Materials and Engineering* 2005, 290, 221-229). This process has the advantage of providing a large control upon the processes of pore formation and surface functionalization. the same process is used within the scope of this application, and therefore the disclosure of the above publication is explicitly included into this disclosure in as far as the making of that monolithic structure is concerned. We have thus developed a new technique for producing highly controlled porous materials and well defined surface functionalization, referred to as "reactive gelation", which has already been used to produce highly promising materials for chromatographic applications. The key feature of this invention is the separation of the synthesis of the porous support into three well distinguished (and well established) steps (steps a) – c) as given below), each of them having a number of parameters by which it is possible to independently tune the process in combination with a functionalisation or preparation for functionalisation of the final structure. This separation of the process proved to be essential to reach a large degree of control upon the material structure and properties.

This disclosure claims and represents the first example of functionalization of such a monolithic structure e.g. with polymer brushes of a monolith produced by reactive

gelation. The swelling deswelling kinetics and hence the ability to adsorb and desorb the biological entities of the PNIPAAm chains grown from the networked particles are studied quantitatively to show the unexpected properties of the final material.

The objective of the present invention is therefore to provide an improved macro-porous (monolithic) material, for example for use as, a chromatographic separation medium,
5 but also for other uses like insulation materials, filter materials, solid-phase extraction, solid-phase synthesis, gas storage etc..

The method is essentially based on the method as described in the literature given above which is included into the present specification, and comprises at least the following
10 individual steps in given order:

- a) synthesis of dispersed cross-linked polymeric particles, preferably with a narrow particle size distribution, starting from a monomer and a cross-linker in an emulsion polymerization;
- b) swelling of the particles with a liquid comprising at least an additional charge of
15 monomer and cross-linker and subsequent destabilisation;
- c) initiating the reaction of the swollen particles to form a monolithic structure.

In order to functionalise monolithic structures, it has previously either been proposed to functionalise the final monolithic structure or to include the functionalising agent into the starting material of the initial step. The former however leads to an unsatisfactory
20 functionalisation, and the latter leads to a waste of functionalising agent into the mass of the particles forming the monolithic structure.

The present invention thus proposes to eliminate the above problems by, after the synthesis of the polymeric particles in step a) and before the initiation of the reaction of the swelled particles in step c), chemically functionalise or prepare for functionalisation
25 the polymeric particles. It is fully unexpected that this approach actually works as the conditions in steps b) and c) and the surface of the particles are generated in step a) have to match in order to lead to a final monolithic structure with the desired porosity and the desire to mechanical strength. Correspondingly therefore it is unexpected that it is indeed possible and even very successful to introduce the functionalising agent or the

agents to prepare for functionalisation after the making of the particles, correspondingly not penetrating unnecessarily into the body of the particles where these agents will not have any beneficial effect, and before the initiation of the so-called reactive gelation process. Indeed it would be expected that the formation of the monolithic structure, i.e. the attachment of the particles to each other, would be negatively affected by such a functionalisation, or the functionalisation would be lost as the chemical reactivity of the functionalisation would be destroyed during the reactive gelation. Indeed the latter can be made sure in that chemically speaking the functionalisation or preparation for functionalisation is chemically orthogonal to the conditions during the reactive gelation, meaning that it is possible to find a set of functionalisation systems/conditions and reactive gelation system/conditions which allow the proposed process. Some examples of such combinations will be given below.

According to a preferred embodiment of the proposed method, the polymeric particles are functionalised or prepared for subsequent functionalisation between steps a) and b) on the surface only in individual additional step a1). The specific provision of an individual step allows the separate control of the conditions and allows an optimum control of all parameters of the final monolithic structure.

Preferably, in step a1) the particles are functionalised on their surface or prepared for functionalisation on their surface by an additional layer of polymer, preferably cross-linked polymer comprising the functionalisation or the chemical preparation for the functionalisation. In step a1) the surface of the polymeric particles can be for example reacted with another monomer or oligomer comprising the functionalisation or the chemical preparation for the functionalisation in the presence a cross-linker, preferably in the additional presence of the same monomer as of the cross-linked polymeric particles, to form a cross-linked (co)polymeric shell around the particles, preferably with a thickness in the range of 5-40 nm.

According to a further preferred embodiment, the polymeric particles are functionalised or prepared for subsequent functionalisation during the swelling in step b), preferably by adding another monomer or oligomer comprising the functionalisation or the chemical preparation for the functionalisation to the swelling liquid. It should be noted

that functionalisation/preparation for functionalisation can be carried out in the above proposed additional step a1) as well as during the swelling step b).

According to a further preferred embodiment, the other monomer or oligomer is a bifunctional monomer, where one functionality is the vinyl group, and a second
5 functionality is either carrying ion-exchange groups, hydrophobic moieties, reactive groups for covalently binding ligands such as affinity ligands, reactive groups for starting grafting reactions by ATRP, or is used to later introduce the same types of functionalization.

The bifunctional monomers are preferably chosen among styrene, ring substituted
10 styrenes, substituted acrylates and methacrylates, wherein the substitution preferably includes the following groups: chloromethyl, alkyl chains, hydroxyl, t-butyloxycarbonyl, halogen, nitro, amino group, protected hydroxyls or amino groups, glycidyl, pyrrolidone groups, bromopropionyloxy groups.

Preferably, between step a) and before the initiation of the reaction of the swelled
15 particles in step c) the polymeric particles are chemically prepared for subsequent functionalisation of the monolithic structure, and the monolithic structure is subsequent to step c) fully or at least partly functionalised in an additional step, preferably by grafting the monolithic structure with a functionalising unit, like a unit providing reversible hydrophilicity and hydrophobicity like PNIPAAm. Preferably generally a unit
20 providing reversible hydrophilicity hydrophobicity like NIPAAm, or a unit carrying a charge, or a unit carrying an affinity group like Protein A, or a unit modifying the hydrophobicity of the support like hydroxyethyl acrylate.

It is preferred if in step a) cross-linked polymeric latex particles are made based on vinyl monomers, preferably styrene-based and/or acrylic monomers, preferably based
25 on MMA, and wherein even more preferably the particles have a size in the range of 50-200nm, preferably in the range of 100-150nm, with a hard, essentially non-swellable core and a soft, swellable shell with a radial shell thickness in the range of 5-40 nm, wherein even more preferably the particles have a narrow particle size distribution with a value of the FWHH of less than or equal to 10%, preferably in the range of 5-10%.

The monolithic structure can, either directly or after grinding, be used for chromatographic separation purposes e.g. for the separation of biopharmaceuticals, preferably with large molecular weight in the range of 10 000 - 1 000 000 Dalton, particularly monoclonal antibodies. It can equally be used for the separation of ions for analytical purposes and/or as gas storage media.

The present invention furthermore relates to a monolithic structure as obtained in a process as given above .

Furthermore the present invention relates to the use of such a monolithic structure for the separation of biopharmaceuticals, particularly monoclonal antibodies preferably with large molecular weights in the range of 10 000 - 1 000 000 Dalton,.

Further preferred embodiments of the present invention are outlined in the claims and attached.

The porous self-supporting structure thus preferably has the surfaces of the pores modified with functional groups such as ion-exchange groups, hydrophobic or hydrophilic moieties, reactive groups for covalently binding of ligands such as affinity ligands, preferably proteins, enzymes, immunoglobulins, antigens, lectins, sugars, nucleic acids, cell organelles, or dyes, etc.

Possible crosslinkable monomers for the preparation of the functionalization are ethylene glycol dimethacrylate, divinylbenzene, divinylnaphthalene, divinylpyridine, alkylene dimethacrylates, hydroxyalkylene dimethacrylates, hydroxyalkylene diacrylates, oligoethylene glycol diacrylates, vinyl polycarboxylic acids, divinyl ether, pentaerythritol di-, tri-, or tetra methacrylate or acrylate, trimethylopropane trimethacrylate or acrylate, alkylene bis acrylamides or methacrylamides, and combinations of any such suitable polyvinyl monomers.

SHORT DESCRIPTION OF THE FIGURES

In the accompanying drawings preferred embodiments of the invention are shown in which:

Fig. 1 shows SEM micrographs of (a) seed PS particles; (b) seed particles functionalized with a thin layer of surface polymerized BPOEA and DVB

(added as a shot); (c) seed particles functionalized with a thin layer of surface polymerized BPOEA and DVB (added in starved fashion); (d) seed particles functionalized with a thin layer of surface polymerized S, BPOEA and DVB (added as a shot) and (e) seed particles functionalized with a thin layer of surface polymerized S, BPOEA and DVB (added in starved fashion).

Fig. 2 A plot of thickness profile of the PNIPAAm layer around the functionalized PS particles as a function of temperature measured by laser light scattering.

Fig. 3 SEM pictures of polymeric monoliths generated by reactive gelation process: (a) low & (b) high magnification images of the monolith produced from the particles functionalized with a thin layer of polymerized S, BPOEA and DVB (named as monolith 1), (c) low & (d) high magnification images of the monolith synthesized from the original crosslinked PS particles without subsequent functionalization (named as monolith 2).

Fig. 4 (a) Swelling characteristics of monoliths 1 and 2 with respect to temperature (equilibrated for 1 hour at every temperature); swelling = amount of water swelling the PNIPAAm brushes per g of polymer (excluding the water retained in the interstitial spaces); (b) time dependant swelling of the monoliths as a function of time when placed at 10°C instantaneously after equilibrating at 40°C and (c) time dependant deswelling of the monoliths as a function of time when placed at 40°C instantaneously after equilibrating at 10°C. The lines serve only as guide to the eye.

Fig. 5 SEM images of the non-functionalized (a) and of the functionalized material (b).

Fig. 6 Outlet UV signal as a function of the elution time for the LGE experiments by RPC with (a) insulin and (b) calcitonin. In both figures, 30 µl of the two solutions (0.34 g/l each) are injected, followed by 5 min equilibration time with eluent 20% B. Then the eluent composition was linearly changed from 20% B to 100% B using four gradient slopes of 10, 20, 30 and 40 min, respectively. Flow rate: 0.5 ml/min ($u_F \sim 0.66$ cm/min). Temperature: 25°C. Eluent compositions: (A) 181 g/l acetonitrile; (B) 756 g/l acetonitrile. Solid curves: solutes UV

signals; dashed curves: outlet acetonitrile concentration.

Fig. 7 Outlet UV signal as a function of the elution time for the LGE experiments by HIC with (a) IgG and (b) HSA. In the case of IgG, 10 μ l of 8.25 mg/ml solution in 1.0 M NaCl is injected, followed by 5 min equilibration time with 1.5 M Na₂SO₄. The eluent composition was then linearly changed to reach 0.3 M Na₂SO₄. In the case of HSA, 100 μ l of 0.20 mg/ml solution in 2.0 M Na₂SO₄ is injected, followed by 5 min equilibration time with the same eluent. The eluent composition was then linearly changed to reach 0.4 M Na₂SO₄. In both figures, four gradient durations have been used: 10, 20, 30 and 40 min, respectively. Flow rate: 0.5 ml/min ($u_F \sim 0.66$ cm/min). Temperature: 30°C. Solid curves: solutes UV signals; dashed curves: outlet Na₂SO₄ concentration.

Fig. 8 Outlet UV signal as a function of the elution time for the LGE experiments of IgG by wCEXC. On the right axis, the outlet salt concentration is reported. (a) Experiments carried out using increasing gradient lengths (20, 40, 80 and 160 min, respectively). 20 μ l of 1.65 mg/ml solution of IgG in 0.0 M NaCl is injected, followed by 20 min equilibration time. The eluent composition was then linearly changed to reach 1.0 M NaCl. (b) Experiments carried out using increasing injection volumes (10, 20, 40, 80 and 100 μ L). Gradient duration: 40 min. Flow rate: 0.5 ml/min. Temperature: 30°C. Solid curves: solutes UV signals; dashed curves: outlet NaCl concentration.

Fig. 9 SEM micrographs of a monolith comprising of MMA and EGDMA as crosslinker..

Fig. 10 Inverse size exclusion chromatography of the monolith of Fig. 9. Solutions of dextrans with different average molecular weight (0.1 mg/ml) are injected, using water as eluent (50 mM phosphate buffer, pH 8).

Fig. 11 Outlet UV signal as a function of elution time for the LGE experiments by HIC with IgG. Different solutions volumes (49.5 mg/ml solution in 1.0 M (NH₄)₂SO₄) are injected, followed by 5 min equilibration time with 1.0 M (NH₄)₂SO₄. The eluent composition was then linearly changed to reach 0 M

(NH₄)₂SO₄. Flow rate: 0.5 ml/min (uF ~ 0.57 cm/min). Temperature: 30°C. Red curves: outlet (NH₄)₂SO₄ concentration; other curves: IgG UV signal, corresponding to different injection masses of IgG.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 In this application, the production of macro-porous monoliths is described where the surface is functionalized, in one example with a thermo-responsive polymer (NIPAAm) and in another example with acrylic acid (AA). Emulsion polymerization was first carried out to synthesize narrowly dispersed crosslinked polystyrene or PMMA particles. The surface functionalization of these particles was then in case of the first
10 example achieved by copolymerization on the particle surfaces of acrylic end capped ATRP initiator (BPOEA) with divinylbenzene with or without styrene. The monoliths were generated from the functionalized particles as well as the parent particles by swelling the particles with styrene, BPOEA and divinylbenzene followed by gelation with salt and post polymerization. Subsequent grafting of these monoliths with
15 PNIPAAm was successfully achieved by ATRP and their swelling deswelling characteristics quantified. The grafted monoliths represent the special chromatographic separation media where the separation processes can be potentially controlled by the use of temperature solely.

This work thus represents the first example of functionalization with polymer brushes of
20 a monolith produced by reactive gelation. The swelling deswelling kinetics and hence the ability to adsorb and desorb the biological entities of the PNIPAAm chains grown from the networked particles is studied quantitatively.

Experimental, part 1

Materials. Styrene (S, >99.5%), divinylbenzene (DVB, X80%), sodium dodecyl
25 sulphate (SDS, > 98%) and radical initiator (potassium peroxodisulphate, KPS, >99%) were purchased from Fluka (Buchs, Switzerland) and were used as supplied without further purifications. ATRP initiator end capped with an acrylic moiety (2-(2-bromopropionyloxy) ethyl acrylate, BPOEA) was synthesized as reported earlier (Matyjaszewski, K.; Gaynor, S. G.; Kulfan, A.; Podwika, M. Macromolecules 1997,

30, 5192-5194). N-isopropylacrylamide (NIPAAM, 97%) and other reagents to run the ATRP polymerization, namely 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 97%), copper(I) bromide (CuBr, 99.99%), copper(II) bromide (CuBr₂, 99.99%) & powder copper (Cu, 99%, 200 mesh), were procured from Aldrich (Buchs, Switzerland) and used as received. Ultra pure Millipore water was employed in all experiments.

Synthesis of Crosslinked Polystyrene Latex and Surface Functionalization.

The crosslinked polystyrene latex was prepared in a Mettler Toledo reactor (LabMax) as reported earlier using millipore water (240 g), styrene (48 g), divinylbenzene (12 g) and SDS (3, g) followed by addition of 0.3 g of KPS in 10 mL water after a temperature of 70°C was reached (Mittal, V.; Matsko, N. B.; Butte, A.; Morbidelli, M. *submitted for publication in Polymer*). The final solid weight percent of the latex is 20 wt% and the average hydrodynamic diameter of the particles was analyzed by laser light scattering to be 160 nm..

For the surface functionalization, the above synthesized crosslinked polystyrene seed latex (2.6 g) was heated to 70°C at 400 rpm and purged with alternate vacuum/nitrogen cycles. BPOEA (0.21 g) and DVB (0.065g) were either added alone or along with styrene (0.26 g) to the heated latex as a shot followed by KPS solution (0.01 g of KPS in 0.5 mL of water) after 15 min. The reaction was allowed to run for 5 h. In an another trial to investigate the effect of the mode of addition of the monomer feed on the resulting particle morphology, the KPS solution was first added to the heated crosslinked polystyrene latex followed by the addition of monomer feed of BPOEA and DVB with and without styrene in delayed conditions. Finally, the functionalized latexes were washed by repeated ultracentrifugation and resuspension in Millipore water cycles.

Reactive Gelation.

The gelation process consists of latex swelling, gelation and post gel polymerization (Marti, N.; Quattrini, F.; Butte, A.; Morbidelli, M. *Macromolecular Materials and Engineering* 2005, 290, 221-229). For the swelling process, the washed latex was added to a flat bottom glass vial. A monomer mixture consisting of styrene, divinylbenzene

and BPOEA was then added (swelling degree of 10 wt% of the solid fraction, DVB weight fraction 10 wt%, styrene to BPOEA weight ratio of 1). An oil soluble radical initiator, AIBN (1 wt% of the monomer weight), was also added together with monomer feed. The latex was degassed and allowed to swell under stirring for 4 h. After swelling,
5 a solution of NaCl (0.25 mol/L) was added to the latex under vigorous stirring. The dry solid fraction of the final monolith to be achieved was adjusted always to 10 wt% by adjusting the amount of NaCl solution. The stirrer was then removed and the gel was left at room temperature for further 8-10 hours. The polymerization of the swollen gel was initiated subsequently by placing the vial in an oil bath maintained at 70°C. The
10 reaction was allowed to continue for 24 h. The porous monolith structure was then removed from the vial and dried in air at room temperature.

ATRP.

ATRP of N-isopropylacrylamide was first carried out on the latex particles functionalized with BPOEA according to the procedure reported in the literature
15 (Kizhakkedathu, J. N.; Norris-Jones, R.; Brooks, D. E. *Macromolecules* 2004, 37, 734-743; Kizhakkedathu, J. N.; Takacs-Cox, A.; Brooks, D. E. *Macromolecules* 2002, 35, 4247-4257), in order to confirm the generation of brushes from these particles first. The crosslinked polystyrene particles functionalized by forming a shell of styrene, BPOEA and DVB (added in starved conditions) were used for this study. NIPAAM (0.21 g, 1.9
20 mmol), HMTETA (11.3 mg, 49 μ mol), CuBr (2.37 mg, 16 micro-mol), CuBr₂ (0.81 mg, 3.6 μ mol) and Cu powder (1.46 mg, 23 μ mol) were carefully measured and stirred with 0.4 g of the functionalized latex. This was then carefully degassed by applying alternating vacuum and nitrogen cycles. The reaction was carried out at room temperature and kept under stirring overnight. The so-obtained latex particles carrying
25 the PNIPAAM brushes were washed off any free polymer formed in the solution by centrifugation and resuspension in millipore water.

Growth of PNIPAAM chains from the monoliths was achieved by placing the monoliths in an aqueous solution of required amounts (based on the dry weight of monolith as given above) of NIPAAM, HMTETA, CuBr, CuBr₂ and Cu powder followed by
30 degassing and purging with nitrogen. The monoliths were kept immersed in the

monomer solution overnight at room temperature and were subsequently placed in millipore water 5-7 times to wash off any unreacted monomer.

Electron Microscopy, Laser Light Scattering and Swelling Deswelling Studies.

The surface morphology of the particles was observed in Hitachi field emission in-lens
5 S-900 high resolution scanning electron microscope. The SEM of monoliths was
performed by fixing small pieces of dry monoliths on copper supports followed by
sputter coating with 3 nm platinum. Multiangle dynamic laser light scattering (DLS,
Brookhaven) was used to estimate the size of the polystyrene latex particles. Volume
average mean diameter of the particles was taken. Very dilute particle emulsions in
10 distilled water were measured after equilibration for sufficient amount of time. The size
determination of particles with PNIPAAm chains at different temperatures was
conducted after the specimen has been equilibrated to the set temperature for 45
minutes. Swelling deswelling studies of the PNIPAAm chains grown from the
monoliths was performed by placing the monoliths (cut into cubes of 0.5 cm edge)
15 dipped in water in the baths maintained at controlled temperatures. The monoliths were
then quickly taken out of water, wiped with filter paper and carefully weighed.

Results and Discussion, part 1

Chromatographic supports grafted with PNIPAAm chains can be used for adsorption
and desorption processes driven by temperature only. It not only simplifies the whole
20 separation process, but also ensures better handling of the sensitive biological media. In
order to realize this, free latex particles coated with a thin shell of a functional monomer
carrying an ATRP initiator were first tested for the PNIPAAm grafting.

Emulsion polymerization in the automated reactor led to the narrowly sized crosslinked
polystyrene latex particles with an average hydrodynamic diameter of 160 nm. Figure 1
25 a represents the high magnification image of these particles. The particles are not
perfectly spherical as observed in an earlier study for crosslinked particles produced by
surfactant-free polymerization. As styrene and divinylbenzene were both wholly added
at the beginning of the polymerization, a gradient in the crosslinking degree can be
expected owing to the fact that divinylbenzene reacts faster than styrene in

copolymerization conditions. Therefore, under these conditions, the surface of the particles is softer (less crosslinked) than the core.

Functionalization of the surface of the crosslinked polystyrene particles was achieved by co-polymerizing an acrylic end capped ATRP initiator (BPOEA) and divinylbenzene, with or without styrene. These shell forming monomers were fed either as a single shot or under delayed conditions in order to analyze the effect of these process changes on the resulting particle surface and size. Figures 1b-e detail the SEM micrographs of these particles. With the exception of the final particle size, there is no other visible difference in the morphology of the functionalized particles. The presence of emulsifier in the present conditions provides colloidal stability as well as a better compatibility between the copolymer chains and the seed particles. Average particles size of 190 nm was observed when no styrene was used, whereas a size of 210 nm resulted in the presence of styrene in the monomer feed. Thus the particles functionalization technique could be achieved which responds positively to any monomer feed ratios thus allowing us to change the amount of ATRP initiator, and therefore its surface density, according to the requirement of the process.

Grafting of PNIPAAm chains from the free particles was performed first to confirm the growth of brushes. The thoroughly washed latex particles functionalized with a thin shell of styrene, BPOEA and divinylbenzene (added in delayed mode) of Figure 1e were selected as a trial. The latex was washed rigorously after the PNIPAAm grafting and dilute solution of this latex was analyzed with laser light scattering at different temperatures. Figure 2 shows the plot of thickness of grafted layer as a function of temperature. The size of the grafted layer decreases with temperature and almost coils completely as soon as the gel temperature exceeds the lower critical solution temperature of $\approx 32^{\circ}\text{C}$. The result confirms the successful growth of PNIPAAm brushes from the free particles which as the next step was replicated on the particles joined together in the network.

Two different starting latexes were employed for the monolith generation. The first latex was the crosslinked polystyrene particles carrying a thin shell of polymerized styrene, BPOEA and divinylbenzene (particles of Figure 1e; network named as monolith

1). The second latex used for monolith generation was the thoroughly washed parent crosslinked polystyrene seed particles themselves (particles of Figure 1 a; network named as monolith 2). In both cases, the latex particles were swollen with an additional load of styrene, BPOEA and divinylbenzene, which was followed by gelation and post gel polymerization. The use of these two different latexes allowed us to analyze the effect of initially present ATRP initiator on the particle surface apart from the one added during swelling, on the final grafting of PNIPAAm chains and hence their characteristics.

Figure 3 shows the high and low magnification images of monoliths 1 and 2. Both the monoliths have porous structure and the primary particles are still visible, though these seem to be partly fused together in monolith 2. The particles in monolith 1 are well separated from each other. This is due to the fact that these particles have been coated with an additional layer of crosslinker and other monomers during the functionalization step. The presence of a crosslinked surface unfavors the particle interpenetration during gelation. On the other hand, the crosslinked polystyrene particles in monolith 2 have softer shell, as noted above, which swelled more during the swelling step and allowed a partial fusion of the particle surfaces. This is spite of the presence of BPOEA in the swelling feed which is less compatible with polystyrene. Nevertheless, smooth particle surfaces are still observable in the monolith, with a complete absence of secondary nucleation. Moreover, due to the higher degree of interpenetration of the particles in monolith 2, this resulted in a very rigid final structure.

The generated monoliths were porous enough to graft PNIPAAm on the networked particles by ATRP. Figure 4a shows the swelling properties of grafted PNIPAAm brushes on the monoliths. Both the curves show sharp deswelling on exceeding the lower critical solution temperature. Below the LCST, monolith 1 is slightly higher in swelling extent as compared to monolith 2. This effect could be explained considering that in monolith 1 the emulsion particles were already functionalized with the ATRP initiator, and thus, the grafting density in this monolith is higher. Both the curves converge to the same value above the LCST. In Figure 4a, the monoliths were equilibrated for an hour at every temperature. However, the rate of response of the

monoliths to temperature changes is equally necessary to be analyzed in order to quantify the efficiency of these monoliths as supports for chromatographic separations. In fact, it is believed that that transition from the hydrophobic to hydrophilic state should take place in a time which is smaller than or comparable to the characteristic time of the purification process. Figure 4b and c show the kinetics of monolith swelling and deswelling when the monoliths were brought from 10°C to 40°C and vice versa. It is clear from the plots that the swelling and deswelling of the PNIPAAm chains was not instantaneous in these monoliths, whereas this was qualitatively observed to be very fast in the case of free particles. However, the brushes were swollen to a high degree in less than 30 minutes and after 90 minutes there was no further change in the swelling at all. Deswelling behavior was similar but faster, so that deswelling was almost complete in less than 30 minutes, with subsequent minor changes when kept further at 40°C. This indicates that the grafted PNIPAAm chains have the ability to swell and deswell even when grown in constrained environment and confirms the ability of the generated monoliths to be used as a special support for the chromatographic separations totally driven by temperature.

Conclusions

Following a previous work from our group where free latex particles have been used as a support to grow PNIPAAm brushes with a controlled polymerization technique (ATRP), in this work, it has been shown that the same result can be obtained in constrained environments, as typical of macro-porous monoliths. The monolithic structure has been obtained using the "reactive gelation" technique. This technique not only allows a precise control of the macro-porous structure, as also evident from the microscope images contained in this work, but most importantly it allows a precise control of the surface functionalization of the latex and the resulting monoliths. The resulting structures show similar behavior to free particles once they are functionalized with PNIPAAm brushes. The reduced swelling capabilities and the slower kinetics in swelling and deswelling is probably to be ascribed to the constrained environment of the monolith, as opposed to free particles. Therefore, it is believed that this material shows an immense potential in chromatographic separations of bio-molecules driven by

temperature changes only.

Experimental, part 2

In the previous part, the “reactive gelation” technique has been introduced and explained. In particular, it has been discussed in Marti, N.; Quattrini, F.; Butte, A.; Morbidelli, M. *Macromolecular Materials and Engineering* 2005, 290, 221-229, how it is possible to tune the different characteristics of the macro-porous monolith (pore size distribution, pressure drop, specific surface, etc.). As discussed above the most technologically relevant application of these materials is the chromatographic purification of bio-molecules. This is particularly evident when considering the case of the production and purification of monoclonal antibodies (MAb). In this part it is shown how monolithic columns produced by “reactive gelation” are performing and their characteristics once used for the chromatography of bio-molecules. In particular, in the following two types of monolithic columns will be discussed. Both starting latexes have been produced by batch emulsion polymerization. The resulting morphology of the primary particles presents a soft (i.e., low crosslinker) surface, which allows a large rearrangement of the gel structure and the formation of large pores. Moreover, due to the large interpenetration of the particle shells, the resulting monolith is very rigid and stable. The first monolith, is made of styrene-divinylbenzene (S-DVB, 10% DVB in weight) latex particles. No further surface functionalization has been added on the particle surface. Accordingly, the material surface is purely hydrophobic and can be used to perform reversed phase chromatography (RPC), and hydrophobic interaction chromatography (HIC). The second material according to the invention has the same composition of the primary particles, but these were successively functionalized with acrylic acid (AA) during swelling, so to introduce carboxylic groups on the monolith surface. It was used as weak cation exchanger in cation ion exchange chromatography (CIEX).

	S-DVB Monolith	S-DVB-AA Monolith
<i>Primary Particles</i>		
Diameter (nm)	150	100
S:DVB Composition (w%)	90:10	90:10
<i>Latex Swelling</i>		
Swelling Amount ^(a)	15	15
S:DVB:AA Composition (w%)	75:25:0	50:25:25
<i>Final Monolith</i>		
Porosity	0.67	0.37
Diameter (mm)	12.0	12.0
Length (mm)	12.7	6.2
Volume (ml)	1.43	0.71

Table 1: Main characteristics of the non-functionalized (s-DVB) and of the functionalized (S-DVB-AA) monoliths. (a): Ratio with respect to the polymer mass.

Results and Discussion:

- 5 In this section, a discussion on the structural characteristics of the two materials is presented. In Table 1, the main characteristics of the synthesis of the monolith and of the final column are summarized.

In Figures 5(a) and (b), the scanning electron microscopy (SEM) images of the two materials is first presented. In both cases, the monolith presents a relatively
10 homogeneous structure. Most important, it is hardly possible to recognize the primary particles. These have most likely assembled into clusters of particles, as typical for “reactive gelation” monoliths in which primary particles have been formed by batch emulsion polymerization. As a consequence, relatively large pores have been created, whose dimension is in the micron range, which are fundamental to ensure a low
15 pressure drop along the monolithic column. In the case of the S-DVB-AA monolith (material 2), the material reveals a higher heterogeneity in the polymeric matrix. Few

parts of the skeleton are recovered with small particles, whose size is similar to that of the primary particles (about 100 nm). This is probably due to the addition of acrylic acid during post-polymerization.

The pore size distribution of the two monoliths has been determined by inverse size exclusion chromatography (ISEC) experiments and presents no size exclusion in the range of 1 000 - 1 000 000 Dalton.

Performance of the Hydrophobic Monolith (S-DVB)

In the following, the chromatographic performance of the S-DVB material is discussed. Two types of applications have been chosen: (i) reversed phase chromatography (RPC) and (ii) hydrophobic interaction chromatography (HIC). RPC is a highly popular chromatographic technique and operates on the principle of hydrophobic interactions, which result from repulsive forces between a relatively polar solvent, a relatively non-polar solute, and a non-polar stationary phase. The driving force in the binding of the solute to the stationary phase is the decrease in the area of the non-polar segment of the solute molecule exposed to the solvent. The hydrophobic effect is decreased by adding more non-polar solvent into the mobile phase, thus shifting the partition coefficient such that the solute can be desorbed.

Calcitonin will be used as model peptide to test RPC. It has a molecular weight of 3432 g/mol. In addition to this, insulin will be also studied. It has a molecular weight of 5808 g/mol.

The term HIC was introduced to describe the chromatographic separation mode wherein a sample is loaded on a column in a high-salt buffer (usually $(\text{NH}_4)_2\text{SO}_4$) and eluted by a decreasing salt gradient. The HIC of two model proteins will be studied: immunoglobulin G (IgG) and human serum albumin (HSA). IgG has a molecular weight of 150 kg/mol and a hydrodynamic radius of 5.2 nm. HSA has a molecular weight of 67 kg/mol and a hydrodynamic radius of 3.6 nm.

Reverse Phase Chromatography

In order to verify the adsorption and desorption conditions for the column, few injections of calcitonin and insulin were first carried out at isocratic condition. It was

found that both the solutes start to be completely adsorbed at an acetonitrile concentration in the eluent of about 309 g/l. Total elution was instead obtained at about 430 g/l acetonitrile. Therefore, the following two eluent compositions were chosen as mobile phases in the further linear gradient experiments: (A) 89.4 % w/w water, 10.4 % w/w acetonitrile, 0.2 %w/w H₃PO₄, corresponding to 181 g/l acetonitrile; and (B) 47.5 % w/w water, 52.2 % w/w acetonitrile, 0.3 % w/w H₃PO₄, corresponding to 756 g/l acetonitrile. Linear gradient elution (LGE) experiments were done to study the adsorption behavior of both Calcitonin and insulin under linear (diluted) adsorption conditions. These experiments served also as a test to check the reversibility of the adsorption process, the recovery of the injected solutes and the reproducibility of the elution conditions over time. LGE experiments were carried out at a constant temperature of 25°C and a flow rate of 0.5 ml/min. Two solutions of calcitonin and insulin were prepared using 20% eluent B, having a concentration of 0.34 g/l each. 30 µl of the two solutions were injected in the column, followed by 5 min equilibration time with 20% eluent B. Then the eluent composition was linearly changed from 20% eluent B to 100% eluent B using four gradient slopes of 10, 20, 30 and 40 min, respectively. The resulting chromatograms are plotted versus the elution time in Figures 6.

From Figure 6 (a) where the insulin elution is shown, it can be observed that insulin is completely adsorbed after the injection and start eluting only when the gradient starts at a acetonitrile concentration of about 400 g/l. No double peaks or peak shoulders can be observed, although for the least steep slope, the elution peak becomes significantly broader. Similar observation can be made in the case of the Calcitonin LGE experiments (cf Figure 6(b)). In this case, it can be observed that calcitonin elutes slightly earlier than insulin, again showing a regular elution profile.

These impressions are confirmed from the estimation of the Henry coefficients from the Yamamoto equation.

Hydrophobic Induced Chromatography

As already done in the case of RPC, few injections of IgG and HSA were first carried out at isocratic condition to determine the adsorption and desorption conditions of the column. It was found that IgG fully adsorbs when using a Na₂SO₄ concentration of 1.5

M, whereas flow-through conditions are obtained at 0.5 M. HSA requires a larger salt concentration for adsorption (> 2.0 M Na_2SO_4), whereas for salt concentrations smaller than 1.5 M no adsorption can be observed. For this reason, the following two eluent compositions were chosen as mobile phases in the following linear gradient experiments: (A) water, 2.0 M Na_2SO_4 , 50 mM phosphate buffer, pH 8.0; and (B) water, 0.0 M Na_2SO_4 , 50 mM phosphate buffer, pH 8.0. Although larger salt concentrations are sensibly improving the HSA adsorption conditions, it has been decided to limit the salt concentration to 2.0 M, since at this concentration no salt crystallization can be observed at room temperature. Moreover, it should be noted that a pH value equal to 8.0 has been chosen for both HSA and IgG. This pH is slightly larger than the pI values of both proteins. Accordingly, this choice should minimize the cation exchange interactions of the two proteins with the few sulfonic groups left of the material surface after the production of the primary particles by emulsion polymerization (potassium persulfate is used as radical initiator).

Linear gradient elution (LGE) experiments under diluted conditions are shown in Figures 7(a) and (b) for IgG and HSA, respectively. LGE experiments were carried out at a constant temperature of 30°C and a flow rate of 0.5 ml/min. In the case of IgG, 10 μl injections of a 8.25 g/l solution were carried out in 1.0 M NaCl solution (50 mM phosphate buffer). In fact, it was not possible to dissolve IgG in the same eluent used for loading conditions because little precipitation of IgG often occurred. Four different gradient lengths have been chosen: 10, 20, 30 and 40 min, corresponding to about 5, 10, 15 and 20 column volumes, respectively. The gradient started from a concentration equal to 1.5 M of Na_2SO_4 and finished at a 0.3M concentration.

It can be seen IgG completely adsorbed on the monolith during the loading (note that IgG is not retained using 1.0 M NaCl) and that the adsorption is reversible, with a complete recovery of the injected IgG (within the experimental error). As in the case of RPC experiments, some tailing can be seen in the elution peak, especially at long gradient elutions.

Similar results have been obtained when using HSA.

The fact that the column has a very large resolution capacity is confirmed by the

measure of the dynamic binding capacities.

Performance of the Functionalized Monolith (S-DVB-AA)

In the following, the performance of the functionalized material is discussed. These material is carrying carboxylic groups on the surface coming from the presence of acrylic acid. Accordingly, this material resembles a classical weak cation exchange chromatography support. This is a common operation in the separation of proteins and, in particular, of monoclonal antibodies. For this reason, the chromatographic purification of IgG will be discussed in the following.

Weak Cation Exchange Chromatography (wCEXC)

In order to determine adsorption and desorption conditions, few injections at isocratic condition were performed using the following two solutions as mobile phases: (i) eluent A: 50 mM sodium phosphate, pH 7.0, 0.0 M NaCl; (ii) eluent B: 50 mM sodium phosphate, pH 7.0, 1.0 M NaCl. Injections consisted of a 20 μ L antibody solution (1.65 mg/mL) in eluent A. It was found that IgG is completely retained when using eluent A.

When using eluent B (flow-through conditions), IgG is only partly eluting, while most of the sample irreversibly adsorb onto the monolith. Such behavior is typically explained by an incomplete functionalization of the hydrophobic S-DVB backbone of the monolith with AA and, thus, by the presence of a strong hydrophobic interaction with IgG. A first solution to the previous problem was found by adding 0.4 g/l of surfactant (SDS) to both the eluents A and B. The added amount of SDS is under the critical micellar concentration of the surfactant to avoid the formation of micelles and, thus, of inclusion effects. When repeating the injections using the new eluents, complete retention was observed for eluent A, whilst flow-through conditions were obtained for eluent B. This observation seems to support the conclusion that adsorption at 1.0 M NaCl is due to non-specific binding. In fact, SDS can cover all the hydrophobic surfaces on both the monolith and the IgG and prevent any interaction between them. In spite of this, eluents A and B containing SDS can now be used to perform gradient elutions.

LGE experiments were done to check the reversibility of the adsorption process of IgG and the reproducibility of the elution conditions over time. In Figure 8 (a), the recorded

UV signal as a function of time is shown for four different LGE experiments with different duration of the gradient (20, 40, 80 and 160 min, respectively). In the same figure, the outlet salt concentration is shown. In each experiment, 20 μ L of a 1.65 g/L IgG solution in eluent A were injected. Injection was then followed by 20 min
5 equilibration with eluent A, prior starting the gradient. It can be observed that all the IgG is eluted during the gradient, at a salt concentration which ranges from 0.2 M for the longest gradient to about 0.26 M for the shortest ones. Same elution conditions were obtained after carrying out frontal analysis experiments (discussed later) and after column regeneration with 0.1 M NaOH (2 hours contact time at 0.25 ml/min).

10 In Figure 8 (b), the UV signal as a function of time is shown for LGE experiments using increasing injection volumes (10, 20, 40, 80 and 100 μ L of a 1.65 g/L IgG solution in eluent A). Elution conditions are identical to those of Figure 8 (a) with a gradient duration of 40 min. It can be observed that the peak is not moving to smaller elution times, as typical of overloaded conditions. A small peak can be observed at the elution
15 volume corresponding to the bed volume. This peak is to be ascribed to the (small) difference in composition between the injection and the eluent, i.e. to the presence of small impurities in the original IgG solution.

Conclusions

In this part, the application of two monoliths produced by the “reactive gelation”
20 technique to the chromatographic purification of bio-molecules is studied. The first monolith is characterized by a fully hydrophobic (styrene-divinylbenzene) matrix, whereas the second monolith has been later on functionalized with acrylic acid in order to add carboxylic groups on the monolith surface. The two materials are characterized by large flow-through pores ($> 1 \mu$ m) and, therefore, by large permeability values. As a
25 result, the monoliths could be operated at large interstitial velocities (up to 2.65 cm/min) and short residence times (28 s). Two important features of these monoliths are the absence of size exclusion effects and the large resolution. Polymer standards with hydrodynamic radii up to 15 nm have been injected without observing any peak shift. This size is much larger than that of typical bio-molecules, as proteins. Moreover, no
30 peak broadening is observed when increasing the molecular weight of the standards,

which indicate the absence of diffusion limitations and, thus, large resolution. This result is also confirmed by the value of the number of equivalent theoretical plates of the column, which is comparable to similar commercial materials.

Previous materials have been used for the purification of two model (poly-)peptides by reversed phase chromatography, and for the purification of two model proteins by both hydrophobic interaction and cation exchange chromatography. In all these cases, the material capacity is relatively low, due to the small surface available for adsorption. This is particularly true for peptide purification, while in the case of proteins the capacity values are already satisfactory. When the comparison is made on the capacity per surface area, the monoliths appear to have a very good performance. The most important result is represented by the absence of diffusion limitation. In fact, the studied monoliths could be operated at very large flow rates without loss of capacity. It is also worth mentioning that no changes in the monolith behavior was observed after sanitization. Previous characteristics, namely the large permeability, no size exclusion and no diffusion limitation, make the monoliths produced by "reactive gelation" ideal for bio-molecule separation.

Experimental, part 3

A typical recipe for cross-linked PMMA monoliths shall be given below:

Latex Formation by Ab-Initio Emulsion Polymerization

The monomer (methyl methacrylate, MMA, 36.95 g), the cross-linking agent (ethylene glycol dimethacrylate, EGDMA, 18.05 g) were initially mixed together and added to a water solution (335 g of water) containing an anionic surfactant (sodium dodecyl sulfate, SDS, 1.25 g). The mixture was emulsified by vigorous stirring and transferred to the reactor, where it was purged with 5 cycles of vacuum and nitrogen. The mixture was heated to 55°C and a water mixture containing a water soluble initiator (0.25 of potassium persulfate, KPS, in 5 g of water) added to the mixture by a syringe. The mixture was let polymerizing until 80% conversion was reached. At this point, a continuous addition of two separate mixtures to the reactor was started, which lasted for 4 h, while the temperature was kept at 55°C. The first mixture is a water mixture of

surfactant (0.52 g SDS, 35 g water); the second a monomer mixture (49.50 g MMA, 0.50 g EGDMA). After the end of the addition, the polymerization temperature was raised to 75°C for 2 h, after which the polymerization was stopped by introducing air and bringing the temperature down to 25°C. The final dry fraction of the latex was 21.1%. The particles have a final diameter of 69.2 nm and a polydispersity of 0.05, as measured by dynamic light scattering.

Latex Swelling and Gelation

The latex was initially swollen at room temperature for 2h by a monomer mixture (90% MMA, 10% EGDMA). The amount of monomer added is equal to 20% of the latex dry content. Then, a sodium chloride solution (NaCl, 0.075 M) was added to the latex dropwise until the final dry fraction of the reached 10% w/w. The latex was left at room temperature for 12 h until complete gelation occurred.

If appropriate, only NaCl is used to carry out the gelation. In the case too much salt is required, additional divalent salts can be added to the salt mixture in a low fraction, which may include calcium and magnesium chloride.

Latex Post-Polymerization

The glass containing the gel was purged with 5 cycles of vacuum and nitrogen and then immersed into a oil bath kept at 55°C. The polymerization was left running for additional 24 h. After this, the gel was removed from the glass and let drying for 48 h at air and room temperature.

The resulting monolith structure is shown in Fig. 9.

Typical second recipe for cross-linked PMMA monoliths:

Latex Formation

The latex was initially formed using a miniemulsion polymerization. The monomer (MMA, 40.50 g), the cross-linking agent (EGDMA, 9.50 g), a hydrophobic (hexadecane, HD, 2.00 g, to produce the initial emulsification) and an oil-soluble initiator (azobisisobutyronitrile, AIBN, 1.00 g) were initially mixed together and added to a water solution (365 g of water) containing an anionic surfactant (SDS, 1.50 g). The

mixture was emulsified in a ultra-sonic bath for 5 min and then transferred to the reactor, where it was purged with 5 cycles of vacuum and nitrogen. The mixture was heated to 55°C and let polymerizing until 80% conversion was reached. At this point, a continuous addition of two separate mixtures to the reactor was started, which lasted for 5 8 h, while the temperature was kept at 55°C. The first mixture is a water mixture of surfactant (0.52 g SDS, 35 g water); the second a monomer mixture (49.50 g MMA, 0.50 g EGDMA). After the end of the addition, the polymerization temperature was raised to 75°C for 2 h, after which the polymerization was stopped by introducing air and bringing the temperature down to 25°C. The final dry fraction of the latex was of 10 18.6%. The particles have a final diameter of 116 nm and a polydispersity of 0.05, as measured by dynamic light scattering.

The initial cross-linking degree of the core is 19%, while the cross-linking degree of the shell is 1.0%.

Latex Swelling and Gelation

15 The latex was initially swollen at room temperature for 2h by a monomer mixture (90% MMA, 10% EGDMA). The amount of monomer added is equal to 20% of the latex dry content. Then, a sodium chloride solution (NaCl, 0.20 M) was added to the latex drop-wise until the final dry fraction of the reached 10% w/w. The latex was left at room temperature for 12 h until complete gelation occurred.

20 Latex Post-Polymerization

The glass containing the gel was purged with 5 cycles of vacuum and nitrogen and then immersed into a oil bath kept at 55°C. The polymerization was left running for additional 24 h. After this, the gel was removed from the glass and let drying for 48 h at air and room temperature.

25 Monolith Structure and Performance:

The so-obtained monolith was characterized in a chromatographic apparatus (see Figs 10 and 11). The monolith has a length of 6.2 mm and a diameter of 12.0 mm, corresponding to a volume of 0.71 cm³. Inverse size exclusion chromatography was used to determine the pore size distribution. Solution of dextrane polymers (0.1 mg/ml,

20 mM phosphate buffer, pH 8) of different molecular weight were injected through the monolith, at a superficial velocity of 0.57 cm/min (volumetric flow rate = 0.5 cm³/min). The total porosity was measured using the smallest dextrane polymer (5 000 g/mol), which resulted in a value of 68%. From the injection of the other dextrans, it can be
5 noted that size exclusion is present to some extent.

In the same column, different volumes of an immuno-globulin G solution in water (IgG, 49.5 mg/ml, 20 mM phosphate buffer, 1 M (NH₄)₂SO₄, pH 8) were injected into the monolith, which was previously equilibrated with 10 volumes of a salt solution (eluent A, 1 M ammonium sulfate, (NH₄)₂SO₄, 50 mM phosphate buffer, pH 5). After 5 min
10 of equilibration, a salt gradient was carried out using a second water solution (eluent B, 0 M (NH₄)₂SO₄, 50 mM phosphate buffer, pH 8), during which the IgG was eluted. The gradient lasted 20 min and it was followed by 20 min washing with eluent B.

Typical recipe for functionalized cross-linked PMMA monoliths

Different recipes have been tried with success, which include the addition of a
15 functional monomer (hydroxyethyl acrylate, HEA, or glycidyl methacrylate, GMA, or N-vinyl pyrrolidone, N-VP) to the latex formation (only during the shell formation/addition), to the swelling step and to both. In all this cases, the fraction of functional monomer in the monomer mixture never exceeded 10% w/w, while the amount of cross-linker (EGDMA) was always kept constant, as described in the recipe
20 above.

CLAIMS

1. Method for producing macro-porous materials comprising at least the following individual steps in given order:

- 5 a) synthesis of dispersed cross-linked polymeric particles starting from a monomer and a cross-linker in an emulsion polymerization;
- b) swelling of the particles with a liquid comprising at least an additional charge of monomer and cross-linker and subsequent destabilisation;
- 10 c) initiating the reaction of the swelled particles to form a monolithic structure;

wherein

after the synthesis of the polymeric particles in step a) and before the initiation of the reaction of the swelled particles in step c) the polymeric particles are chemically functionalised or prepared for subsequent functionalization of the monolithic structure.

15

2. Method according to claim 1, wherein the polymeric particles are functionalised or prepared for subsequent functionalisation between steps a) and b) on the surface only in an individual additional step a1).
3. Method according to claim 2, wherein in step a1) the particles are functionalised on their surface or prepared for functionalisation on their surface by an additional layer of polymer, preferably cross-linked polymer comprising the functionalisation or the chemical preparation for the functionalisation.
- 20
4. Method according to claim 3, wherein in step a1) the surface of the polymeric particles is reacted with another monomer or oligomer comprising the functionalisation or the chemical preparation for the functionalisation in the presence a cross-linker, preferably in the additional presence of the same monomer as of the cross-linked polymeric particles, to form a cross-linked (co)polymeric shell around the particles, preferably with a thickness in the range
- 25

of 5-40 nm.

5. Method according to claim 4, wherein the other monomer or oligomer is an acrylic end capped initiator, preferably an acrylic end capped ATRP initiator or preferably selected from N-vinyl-2-pyrrolidinone (or 1-vinyl-2-pyrrolidone), 2-Hydroxyethyl acrylate, 4-Chlorostyrene (or vinyl benzene chloride), glycidyl methacrylate (or 2,3-epoxypropyl methacrylate) and the 2-(2-bromopropionyloxy) ethyl acrylate (BPOEA) as well as generally bifunctional monomers wherein one functionality is a vinyl group, and a second functionality is either carrying ion-exchange groups, hydrophobic moieties, reactive groups for covalently binding of ligands, chosen among styrene, ring substituted styrenes, substituted acrylates and methacrylates, wherein the substitution preferably includes the following groups: chloromethyl, alkyl chains, hydroxyl, t-butyloxycarbonyl, halogen, nitro, amino group, protected hydroxyls or amino groups, glycidyl, pyrrolidone groups, bromopropionyloxy groups. .
6. Method according to any of the preceding claims, wherein the polymeric particles are functionalised or prepared for subsequent functionalisation during the swelling in step b), preferably by adding another monomer or oligomer comprising the functionalisation or the chemical preparation for the functionalisation to the swelling liquid, wherein preferably the other monomer is an a bifunctional monomer, wherein one functionality is the vinyl group, and a second functionality is either carrying ion-exchange groups, hydrophobic moieties, reactive groups for covalently binding of ligands such as affinity ligands, reactive groups for starting grafting reactions by ATRP, or is used to later introduce the same types of functionalization, and wherein most preferably the bifunctional monomers are chosen among styrene, ring substituted styrenes, substituted acrylates and methacrylates, wherein the substitution preferably includes the following groups: chloromethyl, alkyl chains, hydroxyl, t-butyloxycarbonyl, halogen, nitro, amino group, protected hydroxyls or amino groups, glycidyl, pyrrolidone groups, bromopropionyloxy groups.
7. Method according to any of the preceding claims, wherein between step a) and

before the initiation of the reaction of the swelled particles in step c) the polymeric particles are chemically prepared for subsequent functionalisation of the monolithic structure, and wherein the monolithic structure is subsequent to step c) functionalised in an additional step, preferably by grafting the monolithic structure with a functionalising unit, preferably a unit providing reversible hydrophilicity hydrophobicity like NIPAAM, or a unit carrying a charge, or a unit carrying an affinity group like Protein A, or a unit modifying the hydrophobicity of the support like hydroxyethyl acrylate.

8. Method according to any of the preceding claims, wherein in step a) cross-linked polymeric latex particles are made based on styrene and/or acrylic monomers, preferably based on PMMA, preferably with a crosslinking degree for the core in the range 5 to 80, preferably 10 to 30, and for the core in the range of 0.01 to 10, preferably 0.1 to 1, and wherein even more preferably the particles have a size in the range of 50-200nm, preferably in the range of 100-150nm, with a hard, essentially non-swellable core and a soft, swellable shell with a thickness in the range of 5-40 nm, wherein even more preferably the particles have a narrow particle size distribution with a value of the FWHH of less than or equal to 10%, preferably in the range of 5-10%, and wherein an additional amount of crosslinker can be added during the swelling, in between 5 to 50, preferably 15 to 35 parts.
9. Method according to any of the preceding claims, wherein the monolithic structure is either directly or after grinding used for chromatographic separation purposes.
10. Method according to any of the preceding claims, wherein two latexes with different functionalization, and if desired with different particle sizes, are mixed in step a1) before swelling to have a mixed chromatography mode like ion exchange and HIC.
11. Monolithic structure as obtained in a process according to any of the preceding claims.

12. Monolithic structure according to claim 11, wherein distribution of the structure is bi- or multi-modal.
- 5 13. Use of a monolithic structure according to claim 11 or 12 for the separation of biopharmaceuticals, preferably with large molecular weight in the range of 10 000 - 1 000 000 Dalton, particularly monoclonal antibodies, wherein preferably the monolithic structure is either directly or after grinding used for said chromatographic separation purposes.
- 10 14. Use of the monolithic structure according to claim 11 or 12, directly or after grinding, for the separation of ions for analytical purposes.
15. Use of the monolithic structure according to claim 11 or 12, directly or after grinding, as gas storage media.

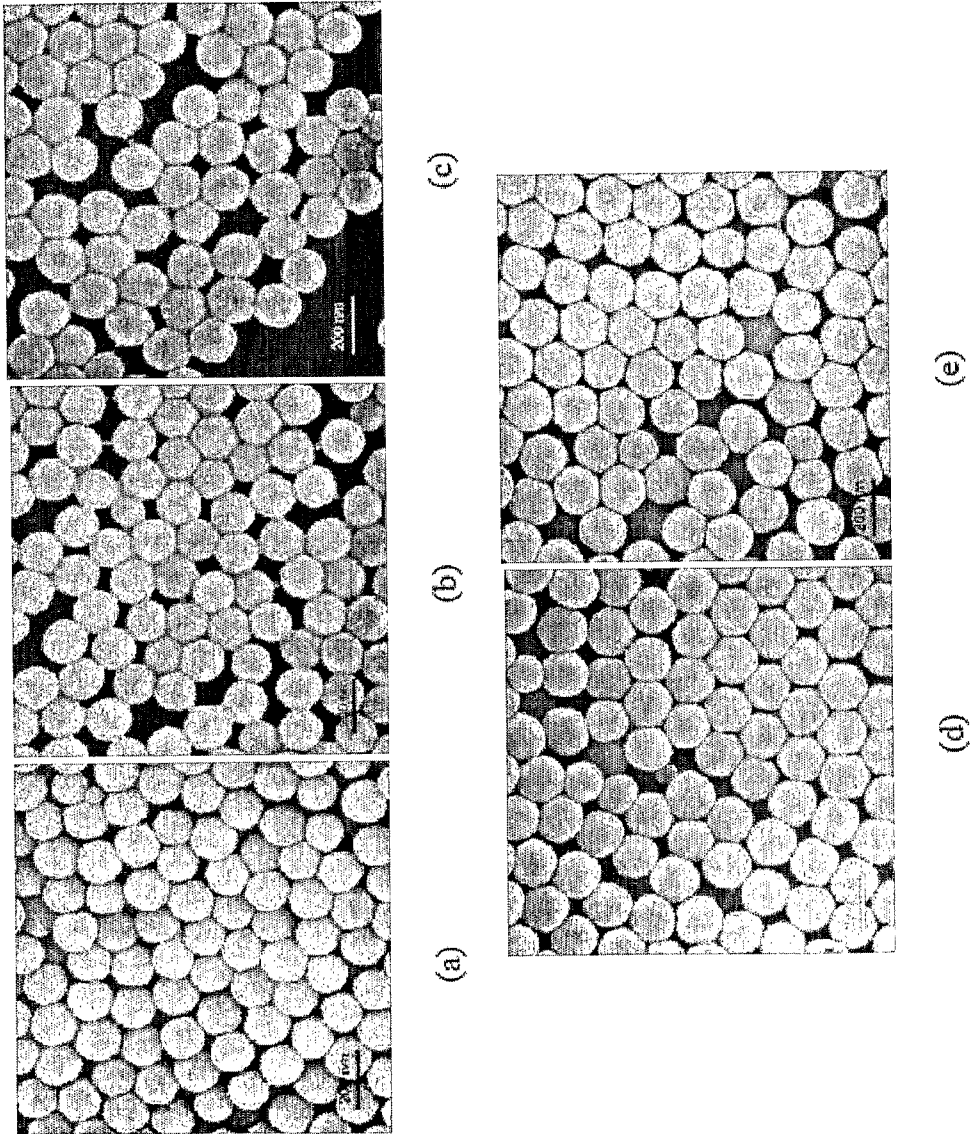


Fig. 1

2/11

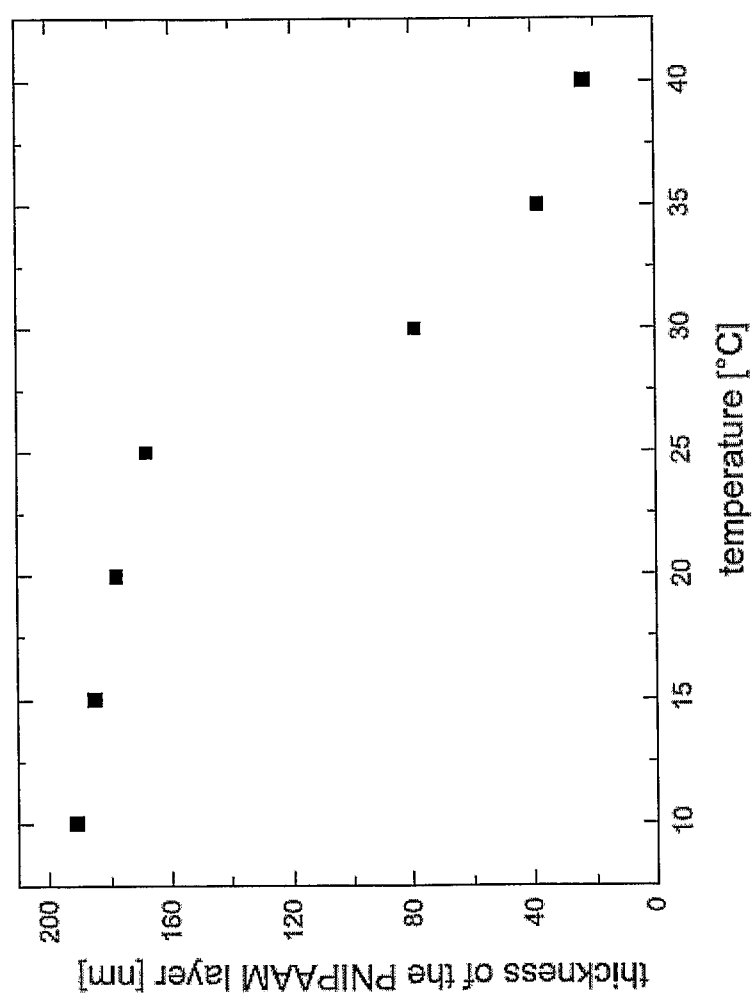


Fig. 2

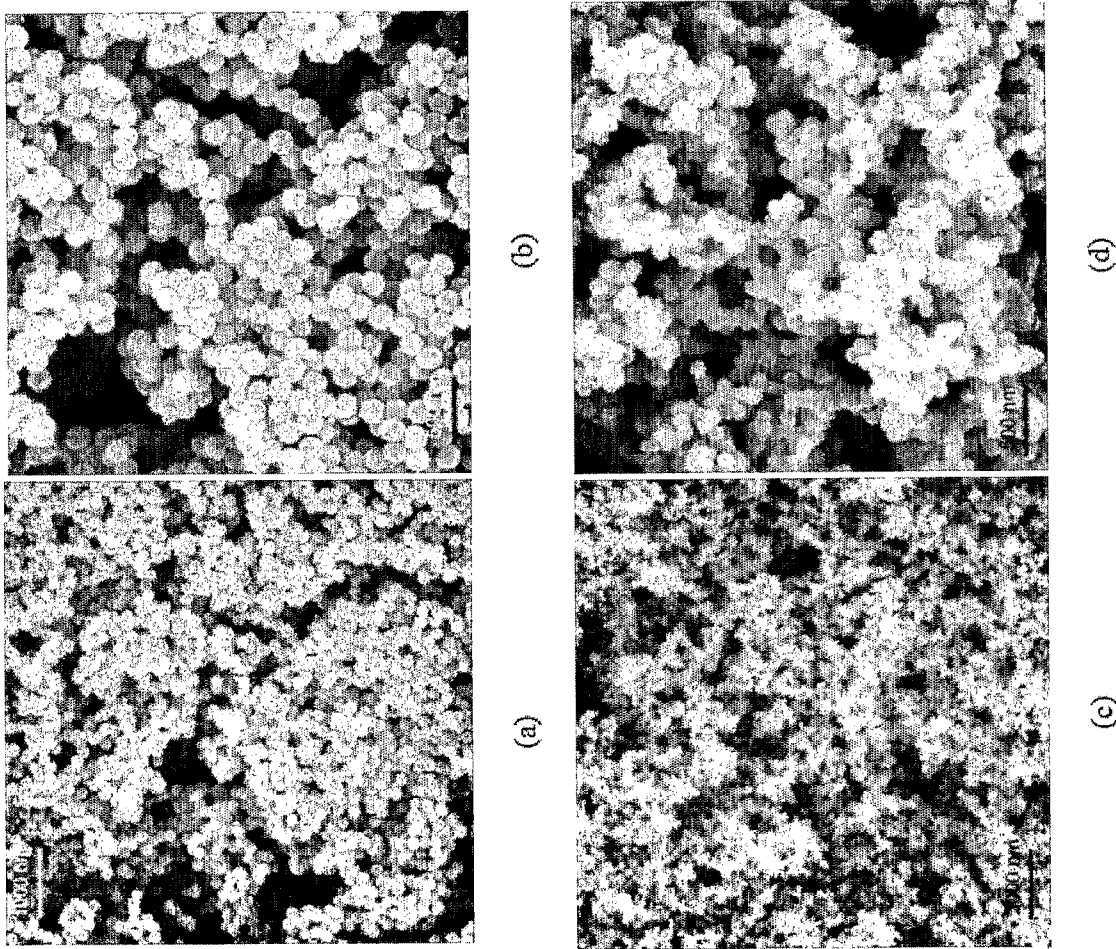


Fig. 3

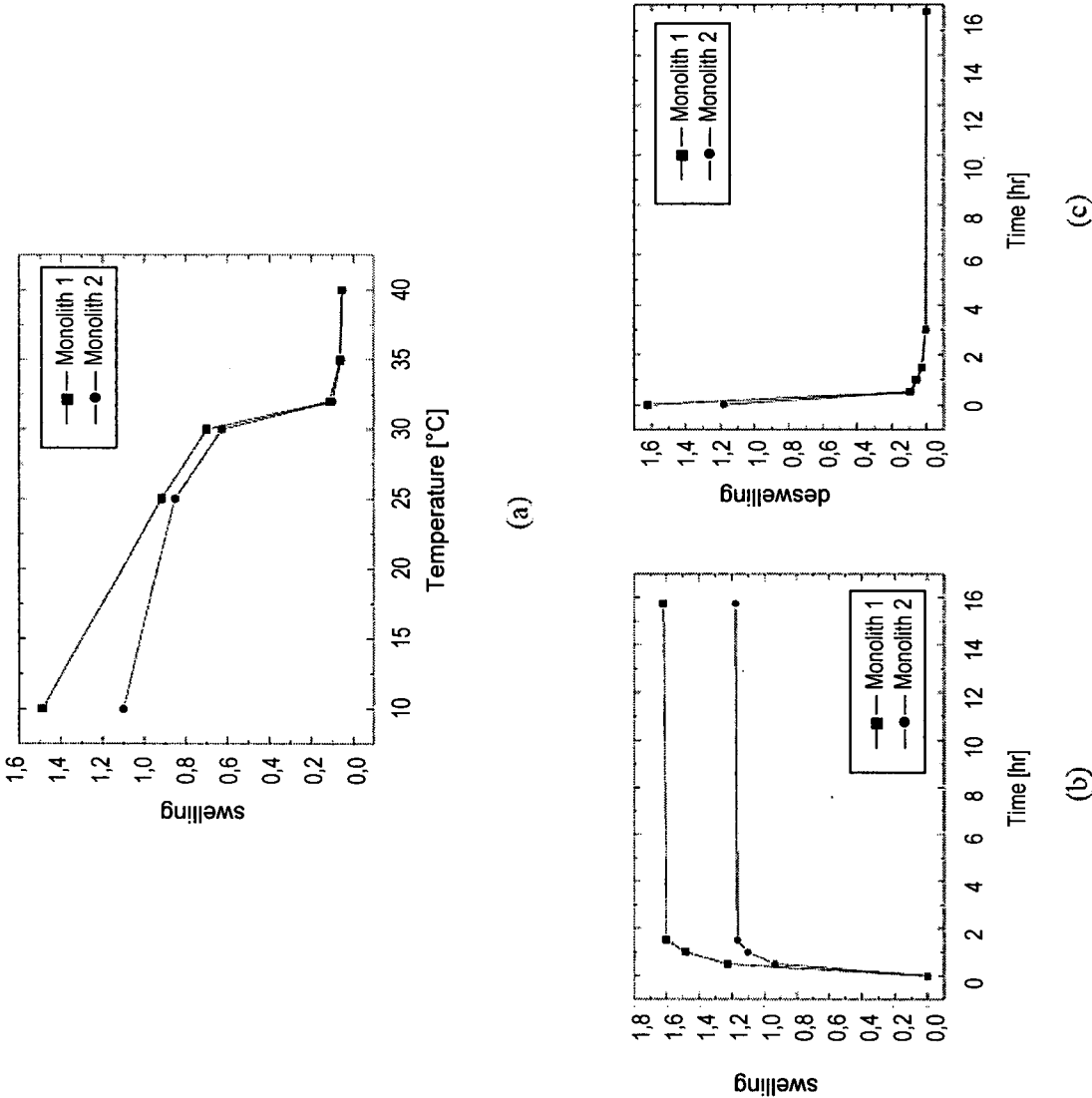


Fig. 4

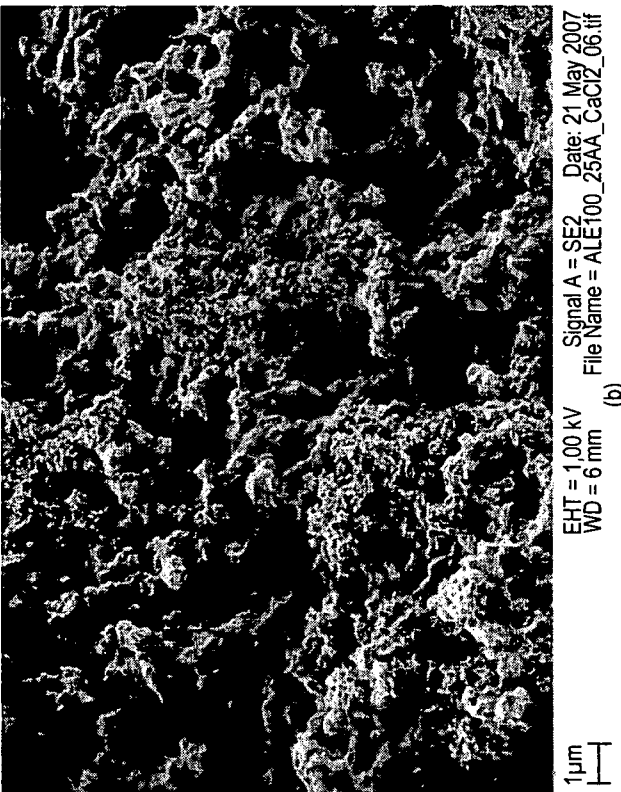
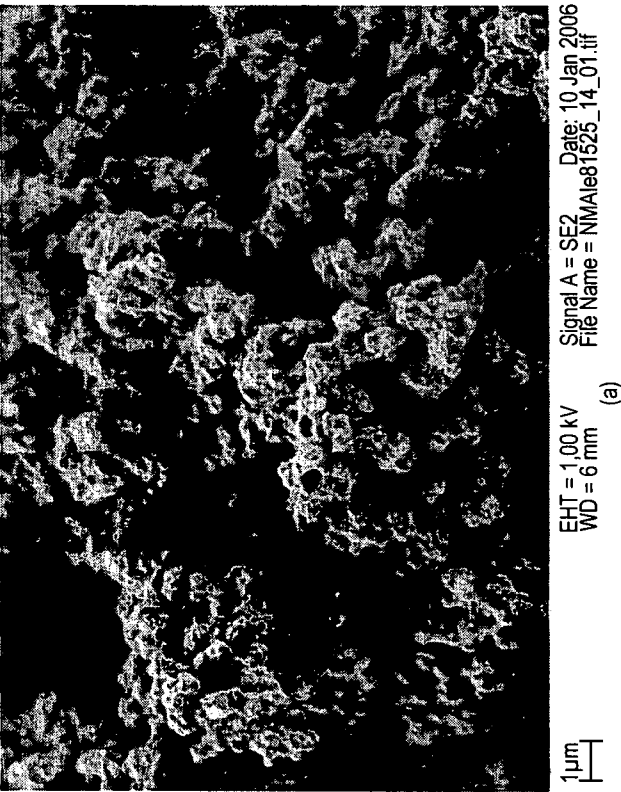


Fig. 5

Fig. 6

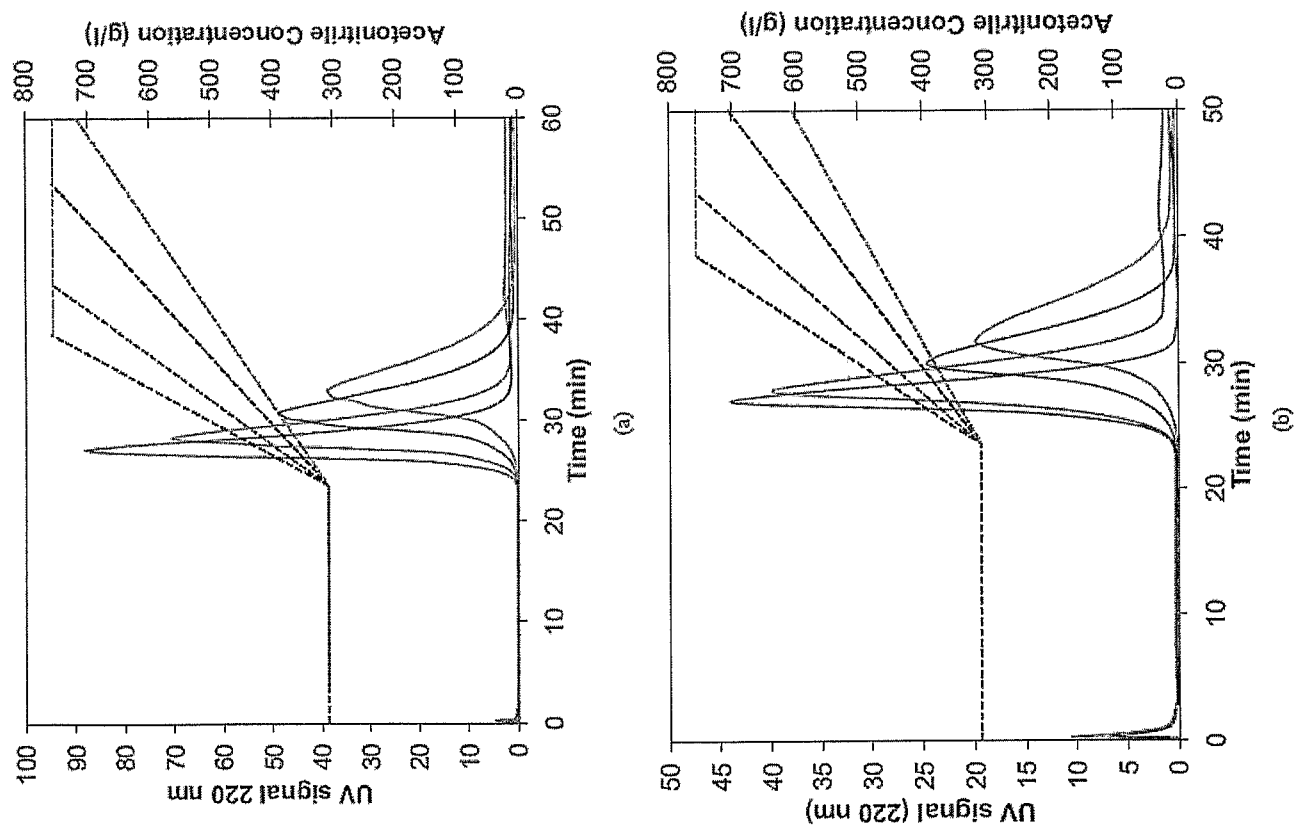


Fig. 7

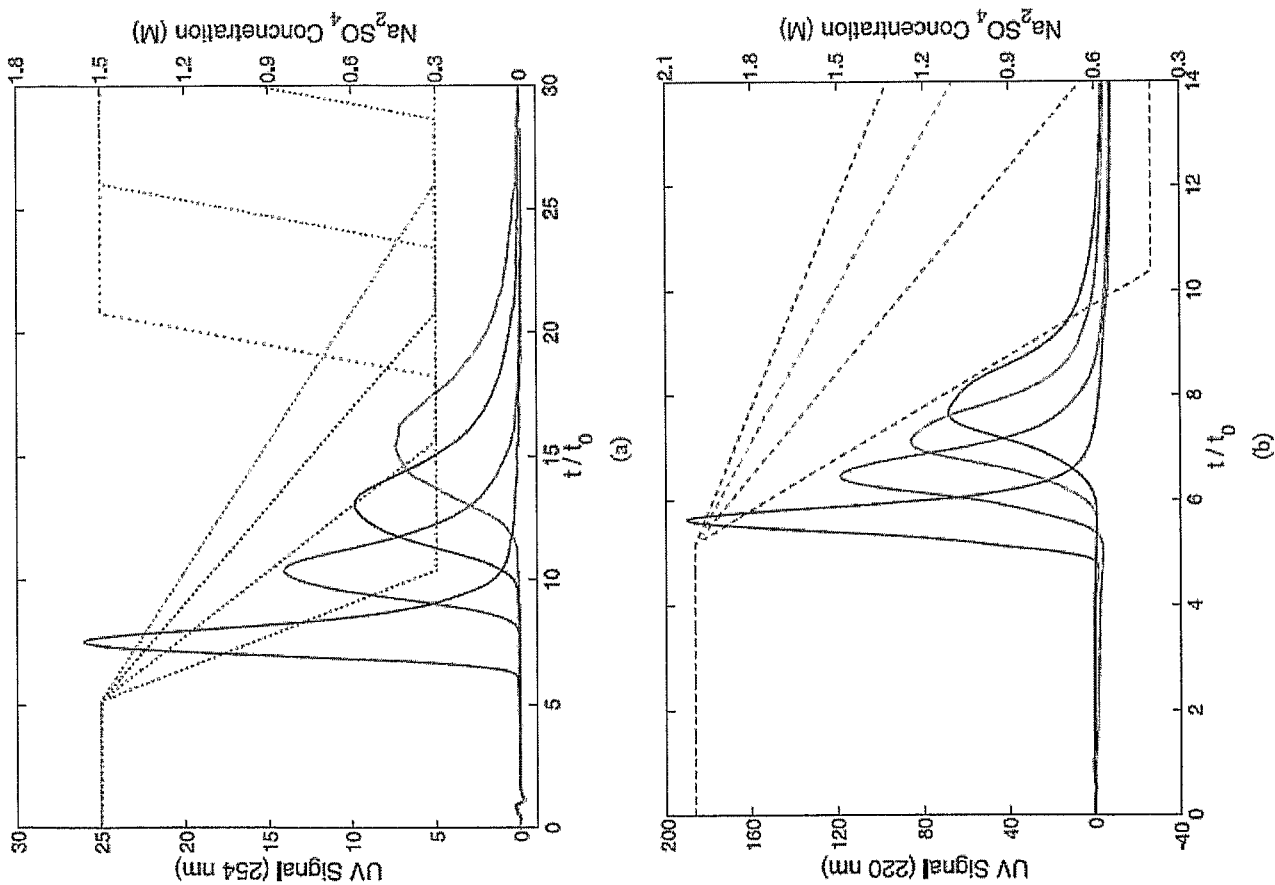
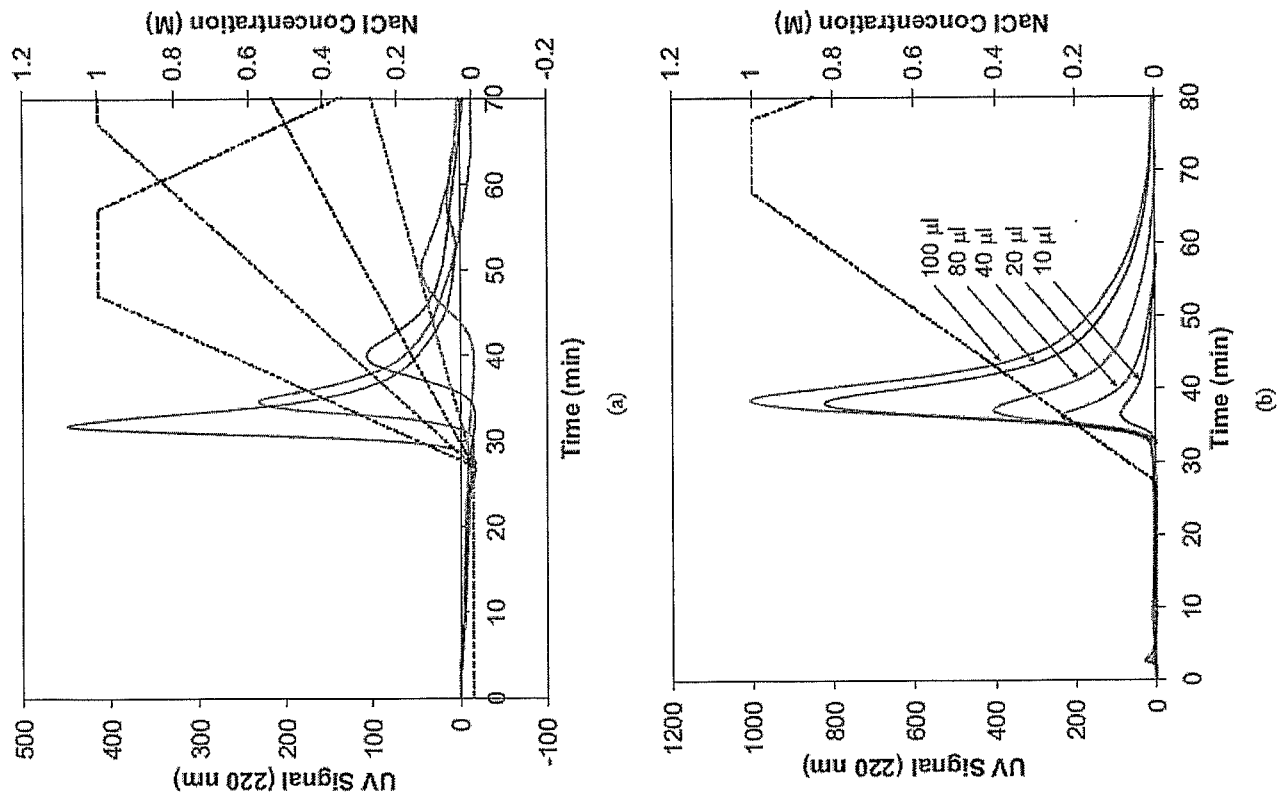


Fig. 8



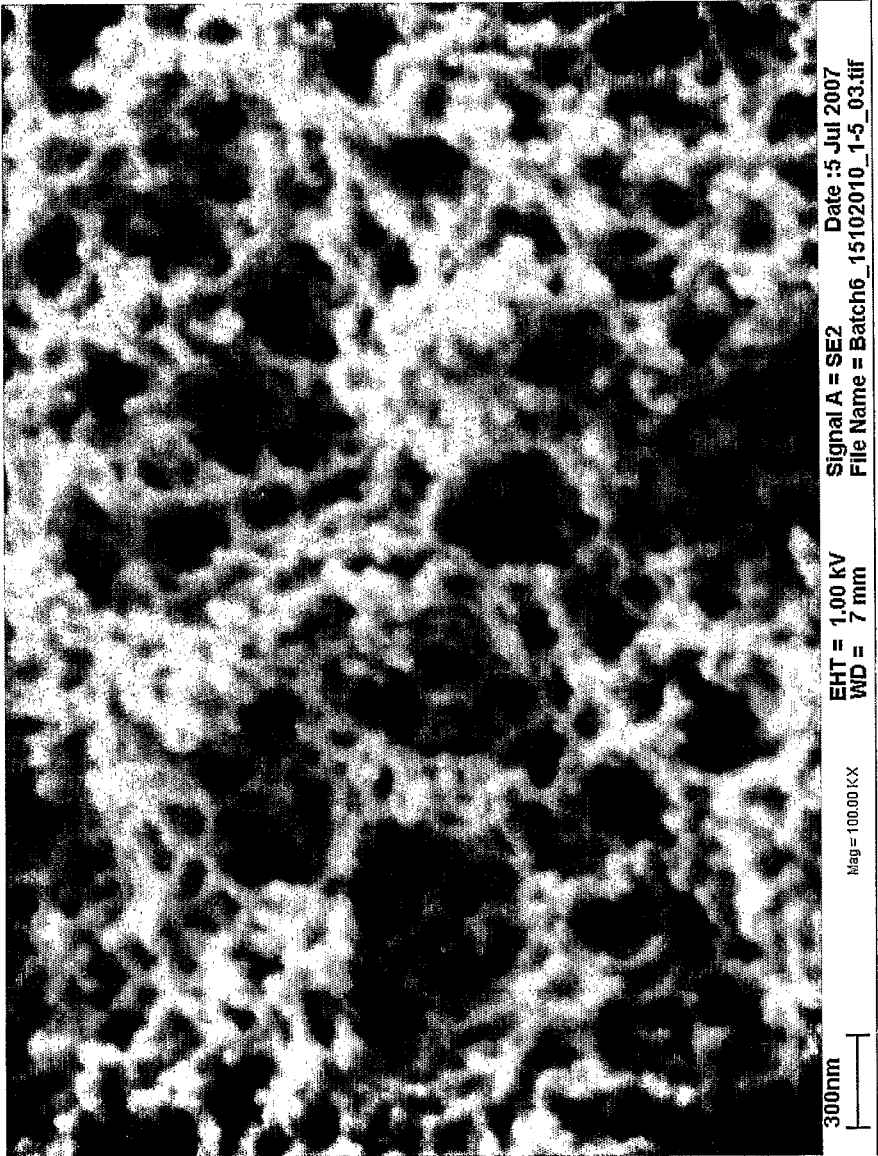


Fig. 9

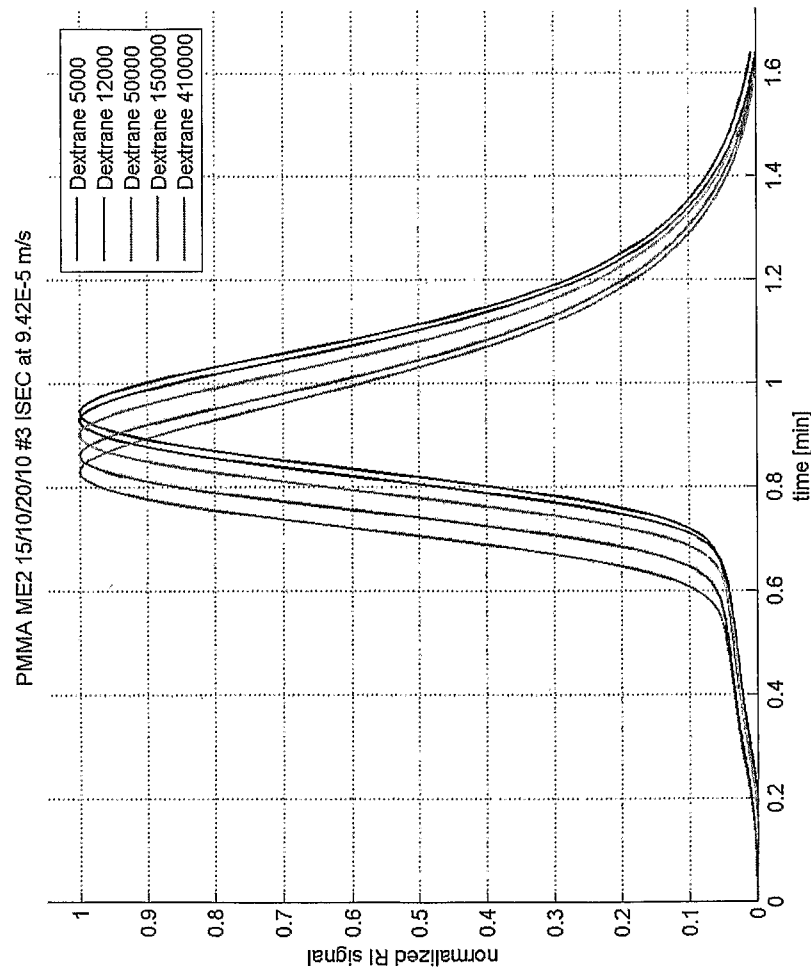


Fig. 10

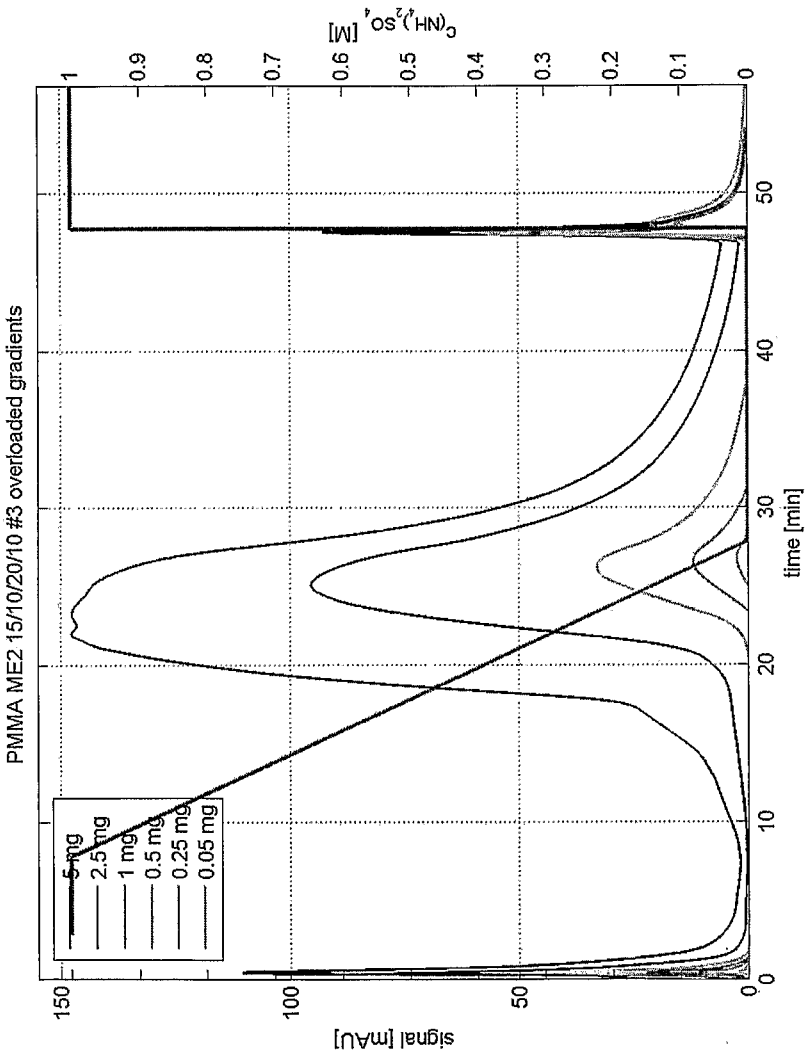


Fig. 11