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Composite Cryogels: Stationary Phase for Separation of Complex Media

Solmaz Hajizadeh

LUND
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Department of Biotechnology
Doctoral thesis
December 2012

Academic thesis, which by due permission of Faculty of Engineering at Lund University, will be publicly defended on Thursday the 6th of December, 2012 at 10.30 a.m. in lecture hall B, at the Center of Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Dr. Aleš Podgornik, Center of Excellence for Biosensors, Instrumentation and Process Control (COBIK) and BIA Separations d.o.o., Slovenia.

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Composite Cryogels: Stationary Phase for Separation of Complex Media

Abstract

Demands for efficient, economical and fast separation and purification methods for various target molecules have led to the development of new techniques and materials for bioseparation processes. Separation can be carried out in both batch and chromatographic modes. Running the separation step in a conventional chromatographic column is more efficient than batch mode, but there are some limitations with this technique for direct capture of targets from complex media or applying it for purification of products larger than 10 nanometres. Therefore there is a need for new materials that can be used for purification of a target directly from particulate-containing fluids in a short time. On the other hand, packing different adsorbent particles in a column or handling them free in suspension might be difficult and challenging due to the high backpressure and risk of leakage, while immobilization of the particle adsorbents on a matrix can reduce these problems.

Cryogels, supermacroporous hydrogels, as a class of monolithic stationary phase are a product of cryogelation technology and have interconnected channels in the micrometre range. Due to the porous structure, free passage of particles is possible and efficient mass transfer and good flow through properties are expected. These polymeric networks can be used as a robust matrix for embedding different types of particles and adsorbents in order to form composite cryogels. Immobilization of particles inside cryogels leads to the creation of greater surface area inside the matrix; thus, greater binding capacity of the gels is achieved. The combination of the porous structure of the matrix and selective adsorption by the adsorbents embedded in the gel form a unique composition that is suitable for separating a wide range of small/large target molecules. Composite cryogels can be prepared by one-step polymerization either from monomer/polymer solutions or particle suspensions under cryotropic conditions. Applying a particle suspension has advantages over a monomer solution when embedding porous adsorbents inside the network, since a polymerization solution can penetrate into the pores and affect the capacity of the adsorbent during the process, while particles are larger than the size of the pores and cannot block them.

Here in this work, composite cryogels were evaluated for water/wastewater treatment and also as an affinity matrix for glycoprotein purification. Activated carbon, molecularly imprinted polymer (MIP) and ion-exchange adsorbents were synthesized to form composite cryogels for environmental applications in this study. Capturing phenol, as a small organic compound, was evaluated with a carbon-composite cryogel formed from a particle suspension due to the highly porous structure of the carbon. A molecularly imprinted polymer technique was also used for fabrication of adsorbents based on inorganic and organic templates for selective removal of bromate and propranolol from aqueous/complex media, respectively. The selectivity of these adsorbents towards the target molecules was high even in the presence of analogue molecules. The selectivity of MIP adsorbents was evaluated with ion-exchange adsorbent for studying the removal of bromate.

The possibility of forming different types of chromatography columns, such as affinity columns, by introducing proper (bio)ligands or functional groups on the cryogels is another feature which makes this material adaptable for different applications. By synthesizing composite cryogels as a form of affinity matrix, the binding capacity of the network will increase due to the higher surface area. This was studied in this work by preparing composite polyvinylalcohol cryogels and immobilizing concanavalin A via epoxy groups. Horseradish peroxidase was selected as a target and its binding/elution was studied in both batch and chromatographic systems. The adsorption isotherm and kinetics of the developed materials were also evaluated in this work. Improving composite cryogel materials with porous/non-porous adsorbents for direct capture of small/large targets from complex media can be valuable for synthesizing more efficient chromatographic supports with high capacity both for academic and industrial applications.

Key words

Composite cryogel; Complex media; Purification; Chromatography; Environmental application; Molecularly imprinted polymer

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کم ماند ز اسرار که معلوم نشد
هفتاد و دو سال فکر کردم شب و روز
معلوم شد که هیچ معلوم نشد

حکیم عمر خیام

Persian Poem of Omar Khayyám
(1048-1311)

Abstract

Demands for efficient, economical and fast separation and purification methods for various target molecules have led to the development of new techniques and materials for bioseparation processes. Separation can be carried out in both batch and chromatographic modes. Running the separation step in a conventional chromatographic column is more efficient than batch mode, but there are some limitations with this technique for direct capture of targets from complex media or applying it for purification of products larger than 10 nanometres. Therefore there is a need for new materials that can be used for purification of a target directly from particulate-containing fluids in a short time. On the other hand, packing different adsorbent particles in a column or handling them free in suspension might be difficult and challenging due to the high back pressure and risk of leakage, while immobilization of the particle adsorbents on a matrix can reduce these problems.

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List of Papers

I. **Hajizadeh S.**, Kirsebom H., Mattiasson B. (2010) *Characterization of macroporous carbon- cryostructured particle gel, an adsorbent for small organic molecules*, Soft Matter, 6, 5562-5569.

II. **Hajizadeh S.**, Kirsebom H., Leistner A., Mattiasson B.(2012) *Composite Cryogel with Immobilized Concanavalin A for Affinity Chromatography of Glycoproteins*, Journal of Separation science, 35, 2978–2985.

III. **Hajizadeh S.**, Xu C., Kirsebom H., Mattiasson B., Ye L. *Solid Phase Extraction of β-Blockers Using Composite Macroporous Hydrogels with Molecular Imprinted Polymers*. (Accepted in Journal of Chromatography A)

IV. **Hajizadeh S.**, Kirsebom H., Galaev I.Yu., Mattiasson B. (2010) *Evaluation of selective composite cryogel for bromate removal from drinking water*, Journal of Separation Science, 33, 1752-1759.

V. Syverud K., Kirsebom H., **Hajizadeh S.**, Chinga-Carrasco G. (2011) *Cross-linking cellulose nanofibrils for potential elastic cryo-structured gels*, Nanoscale Research Letters, 6, 626.

My Contribution to the Papers

I. I performed all the experimental works, contributed in the planning and wrote the first draft of the paper.

II. I performed all the experimental work, participated in the experimental planning and wrote the first draft of the paper.

III. I performed part of the experimental works regarding the material preparation, participated in writing and planning the experiments.

IV. I performed all the experiments, contributed in the planning of the work and wrote the first draft of the paper.

V. I participated by performing some of the experimental works and help with co-writing the paper.

Contents

List of Abbreviations

AAm	Acrylamide
ATP	Adenosine triphosphate
ATPE	Aqueous two-phase extractions
BSA	Bovine serum albumin
CIM	Convective interaction media
CNTs	Carbon nanotubes
Con A	Concanavalin A
E. coli	*Escherichia Coli*
EDCs	Endocrine disrupting contaminants
HEMA	2-hydroxyethyl methacrylate
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HSA	Human serum albumin
IgA, IgG, IgM and IgD	Immunoglobulins A, G, M and D
IMAC	Immobilized metal ion affinity chromatography
ISISA	Ice segregation induced self assembly
L-GLU	L-glutamic acid
MBAAm	*N,N´*-methylenbisacrylamide
MIP	Molecularly imprinted polymers
NIP	Non-imprinted polymers
NIPA	*N*-isopropylacrylamide
PVA	Polyvinyl alcohol
SEM	Scanning electron microscopy
SPE	Solid phase extraction
UV	Ultraviolet

1.Introduction

Rapid improvements in biotechnology during the last 50 years, both in developing new products and new techniques, has had a great impact on the environment and human life. In order to meet and satisfy the requirements of these new products, optimization and modification need to be carried out at all different stages of the production line. Therefore, downstream processing, as one of the major steps in the production line, needs to be improved as well. Downstream processing can be defined as a series of unit operations in sequence resulting in a purified product. Depending on the purpose of the downstream processing, two main factors normally have been considered for optimization: the level of purity and the total cost [1].

Based on the types of microorganism used, the desired target after production will either remain inside the cells or be expressed in the surrounding environment; these are known as intracellular and extracellular products, respectively. Isolation of the desired products is not always the goal in biotechnology. Selective removal of contaminants and by-products from a mixture e.g., for environmental purposes is another example of a separation process. Separation techniques for removing contaminants can be completely different from the methods applied for purification of bioproducts from a mixture [2]. This wide variety of applications requires different separation techniques in the form of batch or continuous modes. Normally the concentration of the target in the mixture is low and in some cases very high purity is demanded, which makes the separation process even more expensive and complex. The different steps in the separation procedure should be optimized and modified in order to be more efficient and economical. In some cases, such as production of antibodies, enzymes or other sensitive bioproducts, short separation times are also crucial in order to maintain a high product quality.

One of the common unit operations in downstream processing for purification of (bio)products widely used in academia and industrial applications is packed-bed chromatography columns. This is known as the

most efficient system for achieving high resolution [3]. Particulate-containing fluids such as fermentation broth and animal/plant cell cultures are typical samples of upstream (fermentation) processes which need to be purified in a downstream process [4]. Therefore, for the purification step, removing undesired solid structures is essential before reaching any type of packed-bed chromatography systems. The inability of packed-bed chromatography columns to directly purify targets from particulate-containing fluids can be considered one of their disadvantages. On the other hand, many of the new biotechnological products are in the nanometre range (20–300 nm) such as vectors for gene therapy, protein assemblies and virus-like particles [5]. Conventional packed-bed chromatography was designed for recovery of proteins or other products less than 10 nm; working with larger particles is not a good match with their diffusion transport mechanism [5]. Alternatives to chromatography, e.g., aqueous two phase extractions (ATPE), crystallization and precipitation have been studied, but the efficiency of these methods in comparison with chromatography systems is normally low.

Integrated, fast, and economical isolation with the desired purity level are the ultimate goals for any separation process. Therefore, great effort has been made to develop new materials and techniques that can meet these criteria at the same time. Expanded bed chromatography and use of monolithic materials as the stationary phase in chromatographic systems are some of the well-known examples of these developments. Due to the presence of large flow-through pores, convective flow is achieved which can significantly increase the mass transfer in monoliths and makes it possible to process particulate-containing fluids at higher flow rates of the mobile phase, in comparison with packed-bed columns, without losing resolution [6]. In this work, the potential of a class of polymeric monoliths known as cryogels has been studied for separation applications in purification of products as well as removal of impurities from complex media.

1.1 Aim and Objectives

Cryogels are polymeric networks with macroporous structures that have found use in different biotechnological and biomedical applications. Cryogels are products of cryogelation technology and have interconnected channels with pore diameters in the range of micrometres. Therefore, free passage of particles is possible and efficient mass transfer and good flow-through properties are expected. The main aim of this research is to investigate the

potential of this polymeric network as the stationary phase for capturing different target compounds from simple and complex media. Complex media (the feed) includes any solutions or suspensions consisting of numbers of different high/low molecular mass compounds or solid particulate materials. Some well-known biological examples of complex media are crude homogenates, blood, urine and plasma. For simplicity in this thesis, complex media refers to any particulate-containing fluid unless otherwise mentioned.

The highly porous structure of cryogels allows higher flow rate and as a consequence of that the mass transfer will be increased which makes them suitable materials for purification of complex media without increasing back pressure. At the same time these pores decrease the volumetric capacity of the materials towards small molecules which can be considered as a drawback for chromatographic systems. In order to overcome this matter, composite cryogels have been developed to increase the surface area by including different particles or adsorbent beads. In this thesis, composite cryogels are defined as any type of cryogels containing particles or built from at least two different types of particles. This work highlights the potential of cryogels in general and composite cryogels in particular and evaluates their applications for purification of simple/complex media. The binding capacity of the gels can be improved by embedding different types of adsorbents or using particle suspension to form cryogels; the results of this work were reported in **papers I, II, III and IV**.

In **paper I,** adsorption of a small organic compound, phenol, from water and milk was studied using carbon-composite cryogels formed from a suspension of *N*-isopropylacrylamide-*co*-allylamine particles. In this paper, higher binding capacities in carbon composite cryogels were achieved by using a particle suspension to produce the gels instead of a simple monomer solution. In this method, pores of activated carbon inside composite cryogels remained unblocked and accessible. The aim in **paper II** was to generate composite cryogels as an affinity matrix with a high binding capacity for capturing glycoproteins. Composite cryogels were synthesized by using polyvinyl alcohol (PVA) particles and porous beads. Concanavalin A (Con A) was selected as a bioaffinity ligand and attached to PVA composite cryogels via epoxy groups. Utilizing synthetic ligands instead of bioligands in order to form an affinity matrix was evaluated in **Paper III.** As described in this paper, molecularly imprinted polymers (MIP) adsorbents were embedded in a PVA composite cryogel to form an affinity matrix for removing propranolol from

water and plasma. Molecularly imprinted nanoparticles with a propranolol-template core and a shell containing amino groups were synthesized by one-pot precipitation polymerization. Solid phase extraction (SPE) of propranolol and other similar β-blockers were evaluated on both MIP composite cryogels and non-imprinted polymer (NIP) composite cryogels. The focus of **paper IV** was on the adsorption of bromate from water using two different types of composite cryogels for selective and non-selective adsorption. Both adsorbents were added to a monomeric acrylamide solution in order to form composite cryogels. A comparison study was done on the capacity and selectivity of MIP adsorbents and an ion-exchange adsorbent synthesized through a sol-gel method. Adsorption isotherms and kinetic modelling were evaluated for both adsorbents in this study. **Paper V** was mainly focused on material development and forming a composite cryogel from modified cellulose nanofibres containing aldehyde groups. This material also has potential in bioseparation.

1.2 Downstream Process for Complex Media

A bioprocess can be divided into two major phases: upstream and downstream processes. From a process point of view, these processes are dependent and have a strong influence on each other; for example, the choice of fermentation substrate can affect the path of separation and vice versa[7]. The downstream process, also known as bioseparation, has undergone development during the last few decades and become more effective due to new technologies. This process is generally divided into different stages: solid-liquid separation, concentration, purification and formulation [8]. Depending on the required purity and type of product, downstream processing may represent a major production cost in biotechnological industries. In some cases the purity of the product is required to be at a very high level, such as in production of pharmaceuticals and vaccines and this definitely has impact on the total cost. Therefore one major challenge is finding a proper route for bioseparation, one that has high capacity and selectivity and meets safety requirements as well as being economical. Bioseparation normally requires multiple operational units in series for proper isolation of the target, which causes loss of product and adds cost to the final processing. A conventional sequence of discrete operational units involved in a standard downstream process is illustrated in Figure 1.1.

Suspensions of prokaryotes or eukaryotes are normally the end product of the upstream processes. The variety of different compounds and the presence of cells, cell debris, substrate media and other available particles makes the separation process complicated due to the complexity of the mixture. Purification and separation of the desired product from these different suspensions/solutions is influenced by many different factors such as the scale of production, process throughput, particle size, impurities and the desired product concentration, which varies from one product to another [9]. Intracellular products need to be extracted from the cells and to achieve this cell disruption is required. Breaking the cells can be done chemically, mechanically or by a combination of both [10]. This step in the downstream process is not needed for extracellular targets (Figure 1.1). If the cells are not the desired product they need to be discarded from the suspension. Removing the cells and other solid compounds can normally be accomplished by filtration or centrifugation based on the type of target molecules and the processing requirements. For example, at a large scale, centrifugation is usually preferred over filtration for removing cells [2]. Concentration is the next step after removing the solid components. The concentration of the target is normally low in the solution; therefore, to minimize and concentrate the solution, different techniques such as precipitation and ultrafiltration can be applied [1] to facilitate the purification stage. Purification can be the most challenging and expensive unit operation in the downstream process. Chromatography has been used as an operational unit in purification for many years. In order to increase the purity and quality of the end product, different types of chromatographic adsorbents may be applied based on the type of the target and downstream setup design. Normally, before the formulation of the product, a polishing step is carried out as a final touch and the solvent is changed if required. The polishing step is not always considered as a separate operational unit in the downstream process and in some cases it can be merged with the purification step. Membrane filtration, diafiltration and size-exclusion chromatography are just some of the methods which are commonly used as a polishing step [1].

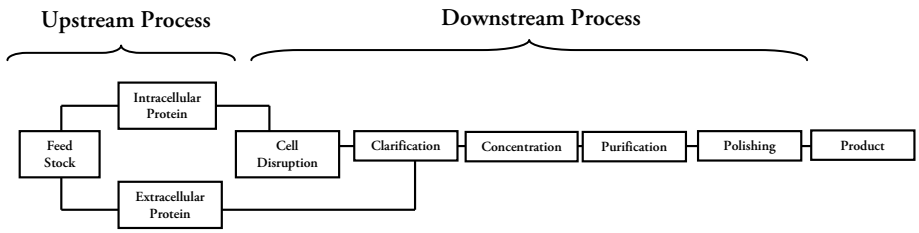

Figure 1.1. Full scheme of conventional biotechnological production procedure.

1.3 Conventional Separation Method for Complex Media and Its Challenges

Packed-bed chromatography is a direct capture technique widely used for recovery of valuable products and is one of the most common techniques applied for purification. In general, chromatography involves four steps: capture (adsorption), washing, elution and regeneration. Adsorption is carried out as a purification method and is usually performed in columns packed with adsorbent beads [11]. Today a wide range of adsorbents is available on the market for specific and non-specific interactions. Some types of adsorbents for specific and non-specific interactions will be discussed briefly in section 1.4. Each adsorbent has been designed for a special purpose such as ion-exchange interaction, hydrophobic interaction, affinity adsorption, size exclusion, etc. The classification of chromatography media (stationary phase) can also be based on their structures (Figure 1.2) [12].

The first adsorbent for protein chromatography was reported in the 1950s, which was a derivatized form of cellulose in bead shape for ion-exchange chromatography [13]. The breakthrough for chromatography was achieved when Sephadex and Sepharose were introduced to the market. This was a revolution in chromatographic techniques for protein separation [14]. They have better flow properties than cellulose beads and are easier to handle and pack in a column. These beads are also soft, therefore the chromatography has be operated at low flow rate[14]. Beads can also be prepared from synthetic polymers and inorganic compounds such as silica [15,16]. The performance of a packed-bed column is dependent on particle size, void fraction between the spherical particles, viscosity and velocity of the solution [17]. The key factors for running a packed-bed column under optimal conditions is to maximize solute capacity and mass transfer, minimize pressure drop, reduce axial dispersion and maintain the flow in a uniform condition [2].

The geometry of the particles and height of the column affect the drop in cross pressure and flow rate [5]. Smaller and more porous adsorbents may have higher capacities in comparison with larger and nonporous particles due to their greater surface area, but by reducing the adsorbent diameter the back pressure in the column will be increased and thus the flow decreased. The limitations of chromatography in packed-bed columns are the batch-mode of operation, complex scale-up and high back pressure [3]. Flow rate, height and diameter of the column and adsorbent properties all need to be optimized to minimize the back pressure of running packed-bed chromatography. Another limitation for packed-bed systems is that particulate-containing fluids such as crude homogenates from fermentation processes cannot be introduced directly to the column. Clarification of feedstock by filtration or centrifugation is necessary (Figure 1.1). This step is crucial in order to apply any complex suspension media, since the particles may become trapped in the system and eventually stop the separation process.

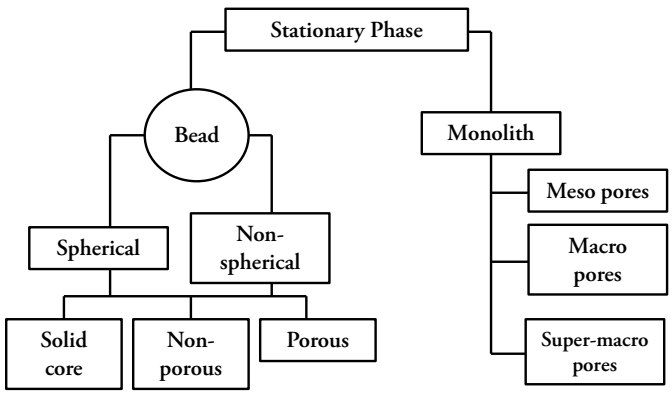

Figure 1.2. Different types of stationary phases for chromatographic columns based on material morphology.

As mentioned in the previous section, purification by itself can be divided into different phases such as primary, intermediate purification and final polishing. Each of these stages requires different treatment and they have their own challenges, especially when it comes to scaling up the system. As an example, the primary stage mostly involves isolation of the target from a clarified solution, therefore a high capacity medium with high operating flow is required [18]. The binding capacity of the medium is more important than the resolution at this stage, while in the intermediate purification steps resolution of the target product becomes a priority. The purposes of polishing

chromatography are achieving high resolution and removal of any trace contaminants. Higher resolution normally can be achieved by using smaller beads in chromatographic systems, therefore they are used in a polishing column [1]. Due to the high back pressure of small beads, a lower flow rate should be applied and that will influence the total processing time.

1.4 Adsorbents for Non-specific and Specific Interactions

Electrostatic-, hydrophobic-, charge transfer-interactions hydrogen bonding or combinations of them cause different types of adsorption, specific or non-specific. The chemical structure of the molecule or the surface chemistry normally determines these types of interactions. Specific interactions indicate adsorption of a target to an immobilized ligand or specially prepared surface due exclusively to (bio)specific interactions, while non-specific adsorption may be regarded as an undesirable reaction in some cases.

Adsorbents can be categorized based on their physical properties such as shape and porosity (Figure 1.2) or based on their surface chemistry. Based on the chemical nature of the adsorbent surface, Kiselev classified adsorbents into three different classes based on their interactions as specific or non-specific adsorbents with different molecules. Adsorbents with no ions or active functional groups on their surface for electrostatic interactions are classified in one group, which mainly relies upon hydrophobic interactions for non-specific adsorption [19]. The two other groups of adsorbents are specific adsorbents having either partial positive or negative charges localized on the surface. A very well known example of group one is activated carbon. Its very high surface area with micro/mesopores inside is the main reason for its usefulness. The surface area of activated carbon is greater than that of silica gel, zeolite or activated alumina [20]. The non-polar surface of activated carbon confers its unique character, which differentiates it from other adsorbents. This property has several advantages such as: a) prior stringent moisture removal is not required for sample treatment. For this reason, it is also widely used as an adsorbent for treating aqueous solution processes; and b) the capacity of activated carbon toward non-polar and weakly polar organic molecules is higher than other adsorbents due to its large and accessible surface area [21]. Activated carbon is widely used in water treatments for capturing organic materials such as phenolic, aromatic and chlorinated compounds, organic pesticides etc. (**Paper I**) [22]. Phenol is known as a good

model compound for organic pollutants in water and wastewater and its adsorption from aqueous solutions on carbon has been extensively studied [21]. Various factors were studied for their impact on phenol adsorption and it was shown that the pH of the solution, type of carbon, carbon surface functionalities etc. all influence the separation process [23]. In **paper I**, activated carbon was chosen for phenol adsorption from water and also from a colloidal solution, milk.

Specific interactions can be formed in addition to non-specific ones, normally by introducing active functional groups (negative or positive charges) on their surface. Here in this thesis, bioligands (Con A) and MIP adsorbent will be discussed as as examples of specific interactions.

1.4.1 Ligands

A substance with unique binding sites which is specially prepared for exclusively (bio)specific adsorption is known as a bioligand. Ligands, with a latin root *ligandum*, for binding, are substances which form interactions with the target molecules. The binding is based on intermolecular forces that normally are reversible. The best example of ligand application in downstream processing is in affinity chromatography, which relies on specific recognition between a ligand (immobilized on the surface of the stationary phase) and a target molecule in a solution. These interactions may have a very high selectivity, making it possible to operate under conditions that reduce non-specific interactions. Both low affinity and high affinity between the ligand and the target can lead to low yield by reducing the binding efficiency and inefficient elution, respectively [24].

Ligands can be divided into biological, synthetic and metal ion groups (Table 1.1). The bioligands most adapted for selective purification of immunoglobulins are proteins A and G. Protein A can bind to immunoglobulins A, G, M and D (IgA, IgG, IgM and IgD) while protein G can interact with different subclasses of IgG antibodies and albumin. Lectins are another group of bioligands that can bind to carbohydrates and glycoproteins. Concanavalin A is one example of a lectin that can be used in affinity chromatography to capture polysaccharides and glycoproteins. It is a homotetramer and each of its subunits is around 25 KDa. At physiological pH, all four identical sites are active and can interact separately with sugar or glycoconjugates [25]. Due to this characteristic, Con A has been used as a suitable ligand for capturing many proteins and substances [26]. This ligand was

used for capturing a glycoprotein, horseradish peroxidase (HRP), in **paper (II)**. Despite the highly selective interaction of bioligands, their high cost, sensitivity to harsh conditions, risk of denaturation, demands of correct orientations and risk of leakage are some of the drawbacks for using them. As an alternative, synthetic or inorganic ligands have attracted much interest since they are potentially inexpensive, chemically well-defined and stable [27]. In comparison, synthetic ones may not have as high selectivity as bioligands towards the target molecules, which is their main disadvantage. Chelating metal ions, hydroxyapatite, dyes and MIP particles are some examples of this group, which have a wide range of applications in affinity chromatography systems [28] (Table 1.1).

Table 1.1. Commonly used ligands and their specificity [24,29].

Type	Ligand	Specificity
Biological	Biotin	Streptavidin, avidin
	Gelatin	Fibronectin
	Heparin	DNA binding proteins, serine protease inhibitors, growth factors, lipoproteins, hormone receptors, DNA, RNA
	Lysine	Plasminogen, rRNA, dsDNA
	Arginine	Serine proteases with affinity for arg, fibronectin, prothrombin
	Lectins	Glycoproteins, polysaccharides, glycolipids
	Calmodulin	Calmodulin binding proteins, ATPase, adenylate cyclase, kinases, phosphodiesterase,
	Protein A	Many IgG subtypes, species dependent, weak interactions with IgA, IgM, IgD
	Protein G	Many IgG subtypes, albumin, species dependent
Synthetic	Cibacron Blue F3G-A	Albumin, kinases, dehydrogenases, enzymes requiring adenylyl-containing cofactors, nicotinamide adenine dinucleotide (NAD^+)
	MIP (adsorbent)	Organic and inorganic compounds
	Polymixin	Endotoxins
	Benzamidine	Serine proteases (thrombin, trypsin, kallikrein)
Inorganic	Metal ions	Metal binding amino acids, proteins, peptides and nucleotides

Although reactive dyes have the ability to react with natural nucleotide-binding sites and that is the main rationale for using them as ligands, their non-specific interactions with other proteins is their weakness. Metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Fe^{3+}) have been used for immobilized metal ion affinity chromatography (IMAC) [30] usually for purification of proteins containing histidine residues, either inherently or via affinity tags.

1.4.2 Molecularly Imprinted Polymer Adsorbent

Molecularly imprinted polymers are another class of materials which has specific adsorption sites for both organic and inorganic target compounds [31]. The technique involves polymerization of functional monomers around the target compound by either non-covalent [32], covalent [33] or metal ion-meditated interactions [34]. The interactions are preserved during the polymerization by co-polymerization with crosslinkers into a highly cross-linked network. After polymerization, the target is extracted and leaves behind artificial receptor structures called "memory sites" with specific shapes and functional groups [35]. The main advantage of this technique, which has been utilized repeatedly in recent years, is the possibility to synthesize adsorbents with selectivity towards a specific target compound. Their stability and the simplicity of the production process are other reasons which make them attractive for many applications such as SPE, sensors, catalysis, capillary electrochromatography, immunoassays and bioanalysis [34,35]. In the early years of this technique, covalent interactions were used excessively between functional monomers and templates. The covalent conjugate was polymerized and the imprinted molecule was removed by chemical cleavage [36]. In the non-covalent approach, electrostatic interactions, hydrogen bonding, π–π bonding and hydrophobic interactions between the template and the functional monomers are used to form molecular assemblies. The latter approach is more flexible in terms of preparation due to the absence of complicated synthetic chemistry as well as the broad selection of functional monomers and possible target molecules available. In addition, imprinted polymers prepared by the non-covalent imprinting approach show much faster rebinding kinetics than those prepared by the covalent approach [37]. Designing MIP adsorbents is influenced by several factors e.g., template properties, selection of functional monomer and cross linker, solvent and polymerization

method [38]. In both approaches (covalent and non-covalent) organic solvents such as chloroform, toluene, or acetonitrile are widely used. These solvents are not environmentally friendly and limit the production of MIP to only small organic molecules [39]. In addition, some of these adsorbents need to be used in aqueous solution after production and the difference in polarity between the preparation solvent (organic) and the adsorption media (water) may affect their structure [40]. Therefore possible reductions in selectivity and molecular recognition can be expected, which is not suitable from an application point of view. Synthesizing MIP in aqueous solution has been considered to overcome these problems. A surface molecular imprinting technique in aqueous solution has been reported for preparation of MIP particles that remove 2,4 dinitrophenol. The particles had high selectivity and their capacity was comparable to chromatography over a C_{18} column via high performance liquid chromatography (HPLC) [40]. The efficiency of a MIP adsorbent is normally evaluated by chromatographic or equilibrium binding assays and comparing its selectivity with non-imprinted polymer (**Papers III and IV**).

Bromate ions are known as a carcinogenic compound and their presence in drinking water needs to be maintained below an accepted standard level (< 10 μg/L). The kidney is a target for both BrO_3^--induced toxicity and cancer and for this reason removal of bromate from drinking water is crucial. Different techniques have been suggested for bromate removal such as nanofiltration, activated carbon, ultraviolet (UV) irradiation, and chemical reduction, but their high cost and non-selective adsorption make them unsuitable for large scale application. As an alternative to these methods, MIP technology has been used for bromate removal. Classical preparation of MIPs was not suitable with bromate as the template due to its insolubility in any organic solvents. Therefore MIP particles were synthesized in aqueous solution using a two-step polymerization based on chitosan as functional polymer and epichlorohydrin as crosslinking agent. Chitosan was selected, due to its nontoxicity and low price, to interact with the template via electrostatic interactions during the preparation (**Paper IV**). Their high selectivity for capturing bromate is the reason for using MIP particles in comparison with other adsorption techniques.

Preparation of MIP adsorbents for removal of pharmaceutical compounds such as propranolol (**Paper III**) and 17 β-estradiol, have also been reported [38,41]. Propranolol-imprinted core-shell nanoparticles were synthesized by a one-pot precipitation polymerization method [42]. In order to introduce

amino functional groups on the hydrophobic particles, a layer of allylamine and NIPA monomers was copolymerized with the residual C=C double bonds of the core particles. The selective adsorption and capacity of these particles were investigated in the presence of other β-blockers (**Paper III**). Removal of propranolol from aqueous solution by MIP particles was greater than 80% in comparison with analogues such as pindolol, atenolol, metoprolol and timolol. The highest level of recovery after exposure to propranolol was reported for metoprolol, which was less than 50%.

1.5 Developed Methods for Separation of Complex Media

Process design has to be optimized to decrease costs and increase the production yield [5]. Increasing the number of sequential unit operations to achieve the desired product translates into a significantly higher overall cost including capital investment, consumables and man-hours needed for each unit. This can be minimized by designing and scheduling an optimal order of unit separations e.g., using a single appropriate type of chromatography column instead of a series of different types of columns. A second approach is indirect purification, by collecting impurities in operational units instead of the target, if it was possible in order to shorten the whole separation line. The most interesting method is integration of two or more processes to create a new unit operation e.g., using an expanded bed [5]. The necessity of using different units for complex media in conventional downstream processes was mentioned earlier (Figure 1.1). Rapid methods to capture targets from media are preferred since short processing times will reduce the risk of denaturation of bioproducts. There are also other techniques which can be applied to separate products from complex media as an alternative to packed-bed chromatography such as ATPE, crystallization, precipitation and three-phase partitioning [3].

As an alternative to packed-bed chromatography, a batch adsorption procedure can be used instead. In this method, adsorbents are added directly to the feedstock. This allows capture of the product in the presence of contaminant particles e.g., cell debris. Batch adsorption can also be carried out with cheaper adsorbents which do not have suitable geometry for use in packed-bed columns [5]. Although this method has advantages over a conventional packed-bed chromatography system, since it can capture the desired product directly from a complex medium, the long processing time is a major drawback for its application. Adding adsorbents to the stirred tank

will act as one theoretical plate, therefore poor efficiency and long processing time are expected [43]. The limitations of packed-bed chromatography and batch adsorption have led to the development of new methods and alternatives for bioseparation. In this thesis, expanded bed and monolithic chromatography will be discussed.

1.5.1 Expanded bed

The drive to integrate downstream processes to reduce the number of unit operations, shorten the processing period, optimize contact time under harsh conditions for sensitive target molecules and decrease the cost led to the development of new techniques such as expanded bed chromatography to capture products. Expanded bed chromatography allows integrating solid–liquid separation and concentration of product by adsorption and partial purification in a single unit operation with quite high efficiency. The process is based on the use of particulate adsorbents that can be expanded in the column. In the expanded bed, the complex media is pumped upward through the column of adsorbents; due to the flexibility of the upper flow adaptor the adsorbents can be expanded in the column, in contrast to a packed-bed column where the upper flow adapter is fixed on top of the adsorbent (Figure 1.3).

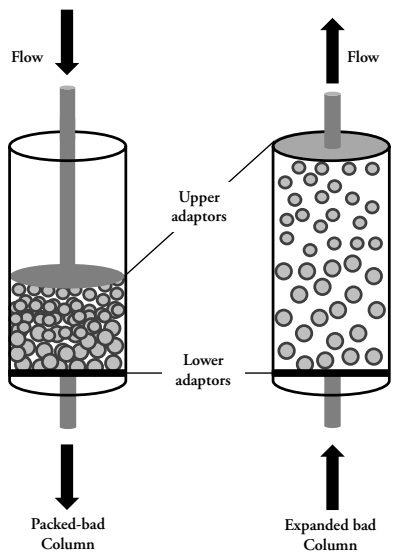

Figure 1.3. Schematic structure of (left column) packed-bed and (right column) expanded bed. Arrows show the direction of the flow in each column.

As a result of the upward flow, the bed expands and the voids between the adsorbent particles increase. This increase in void helps the particulate-containing fluid to pass freely through the column without any entrapment. The principles of expanded bed and fluidized bed are very much alike and their difference lies in the minimal and turbulent mixing, respectively. By applying an expanded bed in downstream process design, clarification, concentration and primary purification can be integrated in a single operational unit and thus it has great potential to save time and reduce overall cost. The operation of an expanded bed includes several phases, as with packed-bed columns (equilibrium, adsorption, washing, elution and regeneration) [44]: 1) adsorbents are expanded and equilibrated by the upward flow of liquid; a stable bed is formed when the level of expansion reaches equilibrium. Particles (usually in the range of 50–400 μm) will be sorted based on their size and density in the column. At equilibrium, smaller/lighter particles can be found in the top fraction and larger/denser ones at the bottom of the column. In term of distribution of the particles, an expanded bed is more stable than a fluidized bed [45]. 2) A complex medium is pumped upward through the column. If the expanded bed was well equilibrated in the first step, the expansion ratio will not change due to the stability of the bed. In this phase, target molecules will be captured by the adsorbents and the rest of the particles and contaminants in the suspension/solution will pass through the bed and the upper adaptor. 3) In the washing stage, the remaining feedstock and particulates that weakly bound to the adsorbents are washed with an upward flow. The washing step in an expanded bed is another important factor that requires extra attention. Due to the presence of small particles such as cell debris in loaded samples, it is crucial to remove all the unwanted compounds during the washing steps in order not to contaminate the target molecule during the elution phase. 4) The elution phase is run in either expanded or packed-bed mode. In the packed-bed mode, adsorbents are allowed to settle and the upper adaptor is fixed on top of them and the flow will go downward, while in the expanded form the elution buffer or solvent is passed through the column in an upward flow. The advantage with using an expanded mode during elution is prevention of particle aggregation, leading to a smooth cleaning process [46]. 5) The regeneration step normally is carried out in packed-bed form. During regeneration, there is a need for verification that all the contaminant particles have been removed from the column during the cleaning phase, and that the characteristics of the adsorbent were not affected.

In summary, an expanded bed can be achieved by stable and controlled fluidization. The stability of the bed is based on flow rate, adsorbent density and particle size distribution. The performance of stable fluidization with no back mixing is comparable to a traditional plug flow column [44]. The distributor directs the flow in the vertical position and prevents any radial flow, while the density and size distribution of the adsorbents ensure minimal local mixing. To increase the density and reduce the particle diameter (in order to increase the surface area and reduce the diffusion length) different methods for fabrication of adsorbents have been suggested: designing a layer of polymer over a small and dense core such as magnetic particles, or forming a porous structure from rigid materials such as zirconia and filling the pores with a biocompatible matrix (agarose) for adsorption. Other ways have also been suggested to minimize the mixing in expanded bed columns, such as using baffles to divide the column into different sections [47], localizing the particles with external forces such as magnetic fields for magnetic particles [48] and using a wide range of particle sizes with different densities [49].

Using an expanded bed column in industrial applications is quite new in comparison with packed-bed chromatography. Both methods have high efficiency and resolution but the packed-bed column is not appropriate for particulate-containing fluids due to clogging problems. On the other hand, designing proper adsorbents for an expanded bed in order to run it under equilibrium conditions is another issue that needs to be considered when using this type of chromatography. Other advantages and disadvantages of the expanded bed method in comparison with packed bed chromatography are summarized in Table 1.2.

Table 1.2. Comparison of packed-bed vs. expanded bed chromatography.

System	Advantages	Disadvantages
Packed-bed	High adsorption and elution efficiency; well studied and widely used at an industrial scale	Cannot operate with particulate-containing fluids
Expanded bed	Operated with particulate-containing fluids; high adsorption and elution efficiency; integration of several steps in the downstream process	Demands use of specific equipment; operates with special adsorbents; lack of data on industrial application; consumes more buffer

Based on the type of adsorbent, an expanded bed can be used for ion-exchange, hydrophobic, metal chelating and affinity chromatography [5]. Streamline is one of the well-known matrices available on the market for ion-exchange and affinity expanded bed adsorption. The adsorption capacities of Streamline based adsorbents for ion-exchange expanded bed chromatography for bovine serum albumin (BSA) and lysozyme were reported to be greater than 110 and 140 (mg/mL), respectively [5]. Heparin, iminodiacetic and protein A were immobilized on the Streamline matrix in order to form affinity adsorbents. Application of expanded beds for recovery of both extracellular and intracellular proteins were also reported for recombinant human serum albumin (HSA) [50] and recombinant anti-HIV Fab-Fragment from *Escherichia coli* (*E. coli*) [51], respectively. There are also reports available on industrial-scale recovery of the antibiotics streptomycin and novobiocin by expanded bed chromatography [52].

1.5.2 Monoliths

Continuous polymer beds, continuous stationary phases, continuous column supports etc. are all other names which have been used in the literature for monoliths [53]. The first time "monolith" terminology was used for protein separation was the early 1990s for a cellulose based material [54]. Monoliths have a Greek root meaning single stone and refers to a support which contains single, continuous and porous materials [53]. The term monoliths will be used in this thesis in order to cover and unify all the materials belonging to this class. The first experiments based on monoliths as size-exclusion columns were reported at the end of the 1960s, but did not show impressive results [6]. Monoliths have been used in different forms and structures since their development e.g., as a catalyst support they can have channels a few millimetres in diameter without interconnections, or in bioseparation applications where they may have interconnected pores on the order of the nano- to micrometre scale [55]. Short processing times, high flow rates and low back pressure have brought them attention for purification in comparison with traditional packed-bed column. An early stationary phase was hydrogels formed by using free-radical polymerization, but they did not allow high flow rates or efficient diffusion [53]. Polyurethane foam was another attempt at monolith preparation that was used in some types of gas chromatography (GC) separation due to its polar surface. Their complicated processing, low permeability, softness, low thermal stabilities compared to

inorganic packing and excessive swelling were their weak points for becoming commercialized [56]. The next generations of monoliths were created using compressed beads (polyacrylamide gel) [57] or macroporous disks for high performance membrane chromatography [58]. Monoliths in rod shape were cast directly in the column to prevent any channelling at the column wall/monolith interface [59]. In the most recent approach, polymerization takes place in the presence of solvents which help form pores inside the end product [60]. These solvents, known as porogens, do not participate in the polymerization process and can be removed from the materials using low pressure due to the presence of interconnected macropores inside the material. Monoliths, their characterization and applications have attracted much interest in recent years, as illustrated by the number of publications about them, which has been increasing dramatically during the last decades [61]. There are different reasons for this great attention: a) they can be prepared in different shapes and forms and b) they render the separation process short because of their low back pressure and high flow rate [62].

Monoliths can be prepared from both organic and inorganic compounds [6]. Preparation of organic based monoliths is easy and straightforward. They are prepared by polymerization of suitable monomers, cross-linkers, and porogens. Polymerization normally takes place in the column where the separation process will be carried out later. The monomer solution is poured into the column, sealed at both ends and triggered by heat or UV light. After polymerization, adaptors are attached to the column and the porogen and non-reacted monomers are removed from the column using solvent [63]. Inorganic monoliths are prepared by a sol-gel method and offer greater mechanical stability than organic monoliths. Organic and inorganic monoliths can be used in different ways such as ion-exchange [64], affinity [62] and reverse phase chromatography [65]. Monoliths typically have lower capacity in comparison with packed-bed columns. Therefore, when used properly they can contribute to simplifying downstream processing (Table 1.3).

The flow transport into the monolith columns are based on convection, while transport into the adsorbent beads in the packed-bed columns is by diffusion (Figure 1. 4) [66]. Due to a latticelike structure of monoliths with a high connectivity the pressure drop is expected to be low by increasing the flow rate, while increasing the flow in the packed-bed column will lead to the high back pressure. It was shown that the mass transfer in monolith columns is independent of the flow velocity. Therefore breakthrough behavior of the monolithic column will not change within certain velocities [66]. Increasing in

the convective transport of the flow in the monoliths as a consequence of their structure enhances the mass transfer and therefore better performance of monoliths is expected since the solute reaches the pores by convection and a faster separation process can be achieved. Gritti *et al.* [67] studied the adsorption/desorption of different small compounds on silica based monolith and packed-bed columns under non-linear operating conditions. The results showed that the apparent surface area of the monolith is higher than that of packed-bed and the adsorption capacity of the monolithic column was approximately 1.4 greater than the packed-bed column. The monolith can easily be operated at a 40% higher flow velocity with having the same efficiency as the conventional column [67].

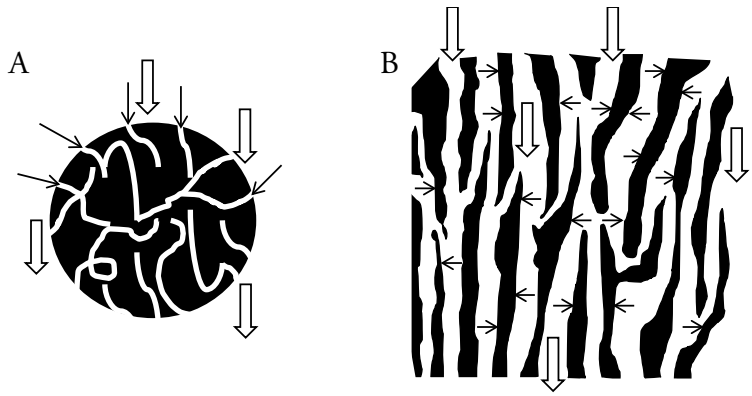

Figure 1.4. Scheme of flow transport inside A) a porous adsorbent in a packed-bed column and B) a selected area of a monolith column. The white arrows show the bulk convective flow and black arrows show the diffusion path.

Passing particulate-containing fluids through some types of monoliths is another feature that makes them more interesting for separation processes. In order to increase the capacity of the monolith support, a particle-fixed monolith can be produced. In this method, conventional packing materials are entrapped and glued together inside a column by a sol-gel solution. There are several reports describing in-situ polymerization of organic monomers in a packed column of silica gel particles for protein separation [68]. Such a column has high stability and its hydrodynamic flow properties are increased due to the presence of organic polymer.

The potential of monoliths in chemical engineering and biotechnology is no longer limited to separation of biomolecules. They have also been used in bioengineering and enzyme reactors, solid phase organic synthesis, flow

injection analysis and bioanalysis [6]. The industrial application of monoliths in the field of separation is not as wide as that of packed-bed columns, but commercial columns have been introduced to the market (Table 1.4) [64].

Table 1.3. Comparison of advantages and disadvantages of conventional packed-bed with monolithic columns.

System	Advantages	Disadvantages
Packed-bed	High adsorption and elution efficiency; well studied and widely used on an industrial scale	Cannot operate with particulate-containing fluids; diffusion is a driving force; slow process
Monolith	May operate with particulate-containing fluids; low back pressure; faster separation; uses convection as driving force; better contact with sample due to geometry of the pores	May have low adsorption capacity and low efficiency

Convective interaction media-disks (CIM-disk) based on polymethacrylate have been used for purification of large plasmid DNAs, with sizes up to 93 kb, for potential use as vectors in multigene delivery applications [69]. The potential of these monoliths for purification of other biomolecules such as antibodies has also been investigated [70]. The Bio-Rad columns have been applied to downstream processing for purification of proteins as well as analytical applications for HPLC [71]. For separation of highly polar compounds such as amino acids, peptides etc. hydrophilic interaction chromatography (HILIC) can be a good candidate. Therefore an inorganic stationary phase such as Chromolith [72] or Onyx [73] can be applied instead of an organic monolith. All these different types of polymeric monoliths show potential for capturing large molecules. Their efficiencies towards separation of small targets are low compared to those of silica-based monoliths due to the smaller surface areas of the former [61]. Using polymeric materials as monoliths has had a great impact on bioseparation and downstream processing. Cryogels, as polymeric networks with supermacroporous structures, belong to this group. A commercial type of cryogel is produced by Protista Biotechnology AB under the name monolithic macroporous polymer structure (MMPS ™).

Cryogels are a supermacroporous polymeric material with controlled porosities synthesized in partially frozen aqueous media and containing

continuous interconnected pores after preparation. Low surface area has been shown to be an issue for cryogels for capturing small molecules and needs to be modified for optimization of separation processes. Cryogels are the main materials studied in this thesis. Forming a composite cryogel as a means to improve the binding capacity of cryogels is suggested and discussed in the next chapter.

Table 1.4. Some of the commercially available monolithic columns on the market with their applications.

Company	Material	Trade name	Shape	Separation method
BIA separation	Polymethacrylate	CIM®	Disks, Rods	Ion-exchange, Reversed phase, Immunoaffinity
Bio-Rad	Polyacrylamide	UNO®	Columns	Ion-exchange
Conchrom	Silica	CB Silica Plate	Disks	Reversed phase, Normal phase
Isco	Polyacrylamide	SWIFT	Rods	Reversed phase, Ion-exchange
LC-Packing/ Dionex	Polystyrene-divenylbenzene	Ultimate	Columns	Reversed phase
Merck	Silica	Chromolith®	Rods	Reversed phase, Normal phase
Phenomenex	Silica	Onyx	Columns, Rods	Reversed phase, Normal phase
Protista Biotechnology AB	Polyacrylamide	MPPS™	Disks	Ion-exchange
Sepragen Corp.	Modified cellulose	Seprasorb	Disks	Ion-exchange

2. Cryogels

Polymeric gel matrices have been used in biotechnology for chromatography, electrophoresis and as carriers for cell immobilization. These gels are three-dimensional networks consisting of polymer and solvent where the macromolecules are connected via chemical or physical bonds and the solvent prevents the collapse of the system [74]. The polymeric network can be formed in macroporous, microporous or nanoporous structures [75]. Hydrogels are a class of gels with a hydrophilic polymeric network and therefore have a highly hydrated structure. Macroporous hydrogels refer to porous materials having pore sizes of greater than 50 nm (based on IUPAC definition) and remain porous even in the dry state [76]. Due to their porous structure, efficient mass transfer, and the possibility to form them from biodegradable and biocompatible materials, macroporous hydrogels have been used widely in biomedical and biotechnological applications. Different techniques have been reported for preparation of macroporous hydrogels e.g., freeze-drying, gas blowing, particle leaching, precipitation polymerization, phase separation and porogenation [77]. In the latest method, the formation of the polymer network occurs in the presence of the porogen and the final pores in the structure appear after extracting the porogen from the material. Another method for synthesizing macroporous hydrogels is the cryogelation technique, which is based on thermally induced solid-liquid phase separation at sub-zero temperature.

Cryogels (from the Greek *κριοσ* (kryos) meaning frost or ice) are an end product of the cryotropic gelation (cryogelation) technique that permits formation of macroporous polymeric materials with controlled porosities [78]. Cryogels can be classified as supermacroporous hydrogels having pore sizes in the range of a few micrometres up to 100 μm. The polymer/monomer solution is frozen at low temperature (sufficient to freeze the sample but not lower than the eutectic point) where crystals form and two phases are segregated; one with a high concentration of monomer/polymer solution (unfrozen liquid microphase) and the ice phase (frozen part). Water has been used as a solvent for production of cryogel in this thesis due to its

environmentally friendly properties as well as being cheaper than other solvents such as dimethyl sulfoxide. Therefore in this system the ice crystals act as porogens and create the porous structure. After allowing sufficient time for cryogelation, cryogels are thawed at room temperature. The ice crystals melt and the result of polymerization will be permanent supermacroprous structures even in the dry state; the geometry of the ice crystals determines the final pore size.

Cryogels normally have a spongy texture with interconnected channels; they will be referred to as macroporous structures in this thesis (Figure 2.1). Unlike foam polymers, the macropores of cryogels are connected due to the mechanism of ice crystal growth. During the freezing procedure, water crystals (= ice) grow until they meet each other. Therefore the pores of the cryogel are continuous and connected. This porous structure allows the passage of complex media such as crude extracts, cells, blood etc. and enables efficient mass transfer and good flow-through properties. It is assumed that macro pores facilitate the transport of compounds subject to reaction/separation while sorption and chemical reactions take place on the surfaces of the walls [5]. It is possible to control the size distribution of the ice crystals by changing the freezing temperature. At lower temperature the number of ice nucleation sites and the amount of water expelled from the unfrozen phase increases, therefore the frozen phase become larger while the unfrozen microphases become smaller with thinner and denser walls [78]. The consequence of smaller pores will be greater hydrodynamic resistance and back pressure towards passing fluids. Therefore selecting the right freezing temperature is one of the key factors in cryogel preparation.

There is no direct method for analysing the porosity of the cryogel in the wet state. Mercury porosimetry is normally applied to determine the porosity, pore size distribution and surface area of rigid materials in the dry state for pores larger than 10 nm. Due to applying high pressure the samples should be rigid, otherwise deformation and damage to the pores cannot be ignored. Cryogels have a soft structure and for this reason this technique is not suitable for measuring their structure. The pore volume of the samples was measured by calculating water and cyclohexane uptake [77]. In this method, the dried gels are placed in cyclohexane solution and weighted after one hour. The increase in the weight of the cryogel is equal to the amount of cyclohexane uptake. Since hydrophilic polymers of cryogel will not swell in cyclohexane, the amount of solvent taken up reflects the pore volume inside the cryogel.

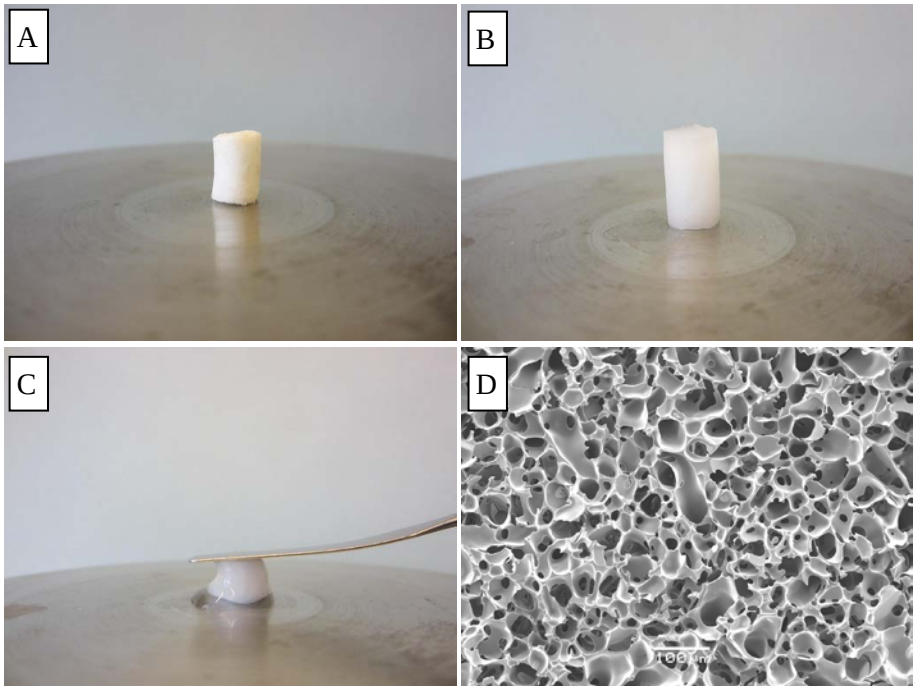

Figure 2.1. Digital photos of polyacrylamide cryogels, A) dried state; B) wet state; C) under mechanical compression in the wet state; D) scanning electron microscopy image of polyacrylamide cryogel showing the macroporous structure of the cryogel.

Scanning electron microscopy (SEM) has also been used to study the pores and wall surface structures, but due to the pretreatment procedure does not provide any data regarding the structure of the cryogel in wet form. In order to prepare the sample for SEM in a completely dry form, freeze-drying or dehydration with serial ethanol solutions (25, 50, 75, 98 and 100%) followed by critical point drying are often used. Both drying techniques can influence the structure of the cryogel. Environmental scanning electron microscopy (ESEM) makes it possible to study the cryogel and its dehydration procedure in the wet state but still not sufficiently well to study the whole structure in three dimensional forms. Confocal laser scanning microscopy (CLSM) is another technique based on two-dimensional images that allows the structure of a cryogel in wet form to be studied. In this method the thin disc of the sample needs to be stained by fluorescent dye before analysis. This technique has been used more in tissue engineering and

cell scaffolds to detect live/dead cells or for studying the secondary structure formed inside the cryogel matrix [77].

2.1 Different Polymerization Techniques for Cryogel Preparation

One of the most common reactions when preparing cryogels is free radical polymerization, which forms a polymer by the successive addition of building blocks (radical monomers). The free radical polymerization reaction needs an initiator molecule that starts the reaction. Free radicals from initiator molecules, also called initiator fragments, activate the monomers by attacking the vinyl groups. Initiation and propagation are considered as the first and second steps in radical polymerization, respectively. The activated monomer reacts with the monomers and chain propagation continues under reaction conditions until two growing chain ends interact with each other and couple together, known as termination phase [79]. Common monomers used for preparation of cryogels by radical polymerization are acrylamide (AAm) [80,81], *N,N*-dimethylacrylamide (DMAAm) [82], *N*-isopropylacrylamide (NIPA) [83,84] and 2-hydroxyethyl methacrylate (HEMA) [85]. These monomers are normally polymerized with *N,N´*-methylenbisacrylamide (MBAAm) as a crosslinker. The presence of crosslinker will prevent formation of a linear polymer in the unfrozen phase and leads to formation of a network. In this work, the free radical polymerization technique was used for the preparation of the AAm cryogel in **paper I** as a backbone matrix. In **paper II,** formation of a composite cryogel from NIPA solution was also based on radical polymerization. Ammonium persulphate (APS) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were used as initiator and reaction catalyst, respectively. Oxygen is a polymerization inhibitor agent and must be removed before the freezing stage in order to guarantee formation of a polymeric network during the cryogelation process. Removing oxygen from the polymerization mixture is normally done by purging nitrogen gas into the mixture or degassing the solution under vacuum. It is essential for cryogel preparation that the polymerization of the monomer solution occurs in the unfrozen phase under partially frozen conditions. Therefore, pre-cooling of the solution on the ice bath before adding initiator is necessary, since low temperature delays the polymerization process and gives sufficient time for the solution mixture to become frozen before the polymerization is propagated.

Free radical polymerization is straight-forward and can be used with various types of soluble monomers. The critical problem in this process is formation of polymer chains, even in low quantity, immediately after adding the redox initiator and before complete freezing of the solvent. This can affect the properties of the final product [86]. To make sure that polymer chains will not be formed before all the solution is frozen, a UV polymerization technique for cryogel preparation was suggested by Petrov *et al.* [87]. In this method, monomer/polymer solution is mixed with a UV initiator and frozen at the desired temperature. Polymerization begins when the frozen solution is exposed to UV light and therefore any formation of polymer chains in the solution will be hindered before freezing. Another advantage of UV polymerization is the extremely short processing time, due to the high polymerization yield of gel formation, which decreases the conventional procedure from 12 hours [88] to 5 minutes [89].

Another technique for preparing supermacroporous hydrogels is chemical crosslinking polymerization. This method has been applied with a number of synthetic and natural polymers such as PVA [88], chitosan and proteins [90,91], respectively. In this method, crosslinking agents such as glutaraldehyde react with the available functional amino groups on the polymers (chitosan, gelatin, fibrinogen and collagen) and lead to the formation of imine bonds. This bond, known as a Shiff base, is not stable and needs to be reduced in order to form a stable amine bond. Sodium borohydride is normally used to reduce Shiff bases and convert free aldehyde groups to alcohol in order to prevent any possible covalent interactions or non-specific adsorption during the separation. The mechanism of chemical crosslinking of PVA is different and the interaction of hydroxyl and aldehyde groups occurs at low pH, where more protons are available and facilitate the crosslinking reaction.

Poly(vinyl alcohol) is one of the best studied hydrophilic polymers in biotechnology and bioseparation due to its characteristics of nontoxicity and biocompatibility. Glutaraldehyde is used extensively for PVA cryogel formation as the reaction is simple to perform and the bonds in the end product are stable [92]. A higher concentration of PVA and crosslinking agent results in greater mechanical stability [88]. There is a significant shrinkage of the sample when using chemical crosslinking polymerization, which can be the result of inter-polymerization inside the gel [55]. Crosslinking polymerization does not require any radical fragments similar to radical polymerization due to the strong bonds formed between the crosslinking agent and the polymer chains. The other dissimilarity between radical polymerization and chemical

crosslinking is the presence of oxygen, which does not affect or hinder crosslinking polymerization. Combinations of gelatin or chitosan with other natural polymers such as agarose and cellulose are other examples of cryogels formed by chemical crosslinking polymerization [93]. These cryogels are mainly used in biomedical applications due to their biocompatibility and nontoxicity.

Chemical crosslinking polymerization of PVA was used in **papers II and III** in order to study adsorption of glycoprotein and propranolol, respectively. In **paper I**, NIPA-*co*-allylamine particles were crosslinked with glutaraldehyde via amine functional groups on the particles. Adding allylamine to the polymerization mixture of NIPA was done in order to introduce functional amino groups on the particles' surfaces. Unlike the PVA system, NIPA-*co*-allylamine is polymerized under neutral pH and does not require any pH adjustment. In **paper V**, crosslinking of oxidized cellulose nanofibrils with NIPA-*co*-allylamine particles and polyethylenimine was studied. Aldehyde groups available on the surfaces of oxidized nanofibers were reacted with amino groups to form stable three-dimensional networks.

Not all formation of cryogels needs to be based on chemical reactions, as discussed earlier. Freeze-thawing of PVA resulted in physical cryogelation [94]. This technique has been well studied and has great potential in biomedical and drug delivery applications due to its formation of a matrix in the absence of any chemical crosslinking agents that might be harmful to cells [95]. The structure and the properties of PVA cryogels formed by freeze-thawing method depends on so many factors such as PVA molecular mass, the degree of deacetylation, initial polymer concentration, presence of additives, nature of solvent, temperature and duration of freezing and thawing [96]. One of the most important parameters in principle is the presence of residual *O*-acetyl (OAc) groups in the polymer after hydrolyzing polyvinyl acetate during PVA preparation. It was reported that a fairly strong PVA cryogel can be prepared from highly deacylated form (deacetylation degree above 97%) and no gel will be formed from the polymer with deacetylation degree less than 90%. Since the OAc residues can interfere with formation of hydrogen bonds in the microcrystalline domains and cause poor gelation [97].

The end product of the cryotropic gelation process through freeze-thawing cycles of PVA solution is a porous structure with a highly interconnected network formed between hydroxyl groups of PVA chains through hydrogen bond interactions. The mechanical properties of the cryogels produced by this method are dependent on the number of freeze-

thaw cycles, the molecular weight of the PVA and degree of its saponification, the freezing temperature and the initial concentration of PVA solution [88,98]. Formation of cryogels via physical crosslinking is strongly dependent on the temperature used. The most efficient temperature for forming PVA cryogel was reported to be below 0 °C (-5 to -1 °C), just low enough to freeze the sample. The temperature of the thawing step should be as low as possible in order to increase intermolecular contacts in the highly viscous liquid microphase [97]. Fabrications of physically crosslinked cryogel from chitosan, agarose, gelatin, potato starch and combinations of these with PVA through freeze-thawing cycles have also been evaluated [99,100]. The morphology of these gels can be different from each other; gelatin cryogel has a spongy structure while PVA is more firm [78]. A physical crosslinking technique was used to fabricate the PVA particles that were used in **papers II** and **III**. PVA solution was added to paraffin oil while it was being stirred in order to form an oil/water emulsion. The mixture was frozen at -20 °C for 24 hours and thawed at room temperature for 4 hours. This cycle was repeated four times until the PVA particles became stable.

2.2 Cryogels from Particle Suspensions

Hierarchical porous structured materials with a bimodal pore size distribution can be produced from particle suspension by ice-templating process (so-called ice-segregation-induced self-assembly, ISISA) approach, using liquid nitrogen followed by freeze-drying in order to remove the solvent and stabilize the final structure [101]. It was shown that the end product of this system can be a hierarchical biohybrid structure with very sophisticated morphology. These materials have porous structures and have shown potential in electronics, chips and sensors, catalysts, chromatography and biomedical applications due to their macroporous structures and large internal surface areas [102]. The potential of this technique in drug delivery, sensors and cell scaffolds have been studied by del Monte and co-workers [103,104]. Post-treatment of these materials with either chemical or thermal modification is required in many cases in order to increase the mechanical stability of the materials in solutions [105]. One of the advantages of these materials is their aligned porous structure, which forms in the direction of freezing with liquid nitrogen [106].

This is a well known technique for synthesis of macroporous structures, but the weak point in its synthesis is the complicated processing, since special

equipment or harsh conditions may be required for cryogel production. Another method for production of cryogels from particle suspensions (synthetic or natural) was introduced by our group via one-step polymerization by crosslinking a particle suspension instead of a monomer/polymer solution under cryotropic conditions to form a macroporous structure consisting of small particles. Chemical crosslinking polymerization with crosslinking agent was applied for this approach. Suspensions of synthetic nano/macro particles having functional amino groups were evaluated for cryogel formation [84]. Fabrication of cryogels from nanoparticles of NIPA-*co*-allylamine by a one-step crygelation was described elsewhere [84] and in **paper I** (Figure 2.2). The size distribution of the particles has an influence on cryogel formation [84]. It has been shown that NIPA-*co*-allylamine cryogel can be prepared from particles with wide size distributions as long as they are mixed together, since smaller particles will act as bridges and help the larger ones to connect (**Paper I**).

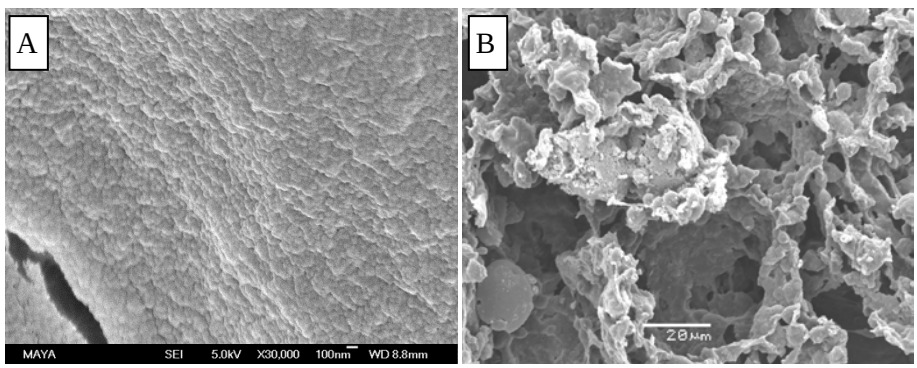

Figure 2.2. Scanning electron microscopy images of NIPA-co-allylamine cryogel: A) forming from nanoparticles with a narrow size distribution of about 125 nm; B) applying microparticles with a wide size distribution of less than 25 µm (**Paper I**).

In **papers II and III**, PVA particles were prepared through freeze-thawing cycles and then crosslinked by glutaraldehyde at low pH. Chemical crosslinking polymerization was chosen since PVA particle suspensions cannot form a continuous secondary network through physical crosslinking by freeze-thawing. The formation of cryogels from PVA solutions is a well studied technique [75] but the same mechanism will not work on a PVA particle suspension. This can be explained by the formation of a stable gel from highly connected crystalline domains of PVA chains in PVA solution

during freeze-thawing techniques. The mechanical and physical characteristics of these gels are well established and are not affected by dispersing them in aqueous solution at room temperature [98]. Therefore, free PVA chains or crystalline domains are not available in a PVA particle suspension to carry out and continue the gel formation around the particles and form a secondary network. Thus chemical crosslinking polymerization was applied in order to produce PVA cryogels from particle suspensions. The morphology and structure of the pores as well as the wall surface of the gels were studied by SEM. The porous structure of the PVA particles was influenced by dehydration (removing water and drying the gel completely) for pre-treatment of the sample for SEM; therefore there was a dramatic size reduction between the PVA particles in wet and dried states, making it difficult to detect the particles in SEM images (Figure 2.3).

To the best of the author's knowledge, there is no report on forming cryogels from only a microorganism mixture by available techniques such as and ISISA approach. The ISISA method has been reported for cell immobilization elsewhere [107]. The harsh conditions and the freeze-drying step in the ISISA technique may render it unsuitable procedure for preparing cryogels based on biological compounds. There is a report on forming porous structures through the ISISA method from carbon nanotube (CNT)/chitosan suspensions. In order to add *E. coli* to the suspension before freezing, the microorganisms were trapped inside glucose-alginate beads as a protective shell and added to the suspension. After polymerization, the beads were dissolved in sodium citrate and the growth of *E. coli* was evaluated on the formed matrix [108]. Synthesizing cryogels from different types of microorganisms was reported by Kirsebom *et al.* [84]. Covalent bonds were formed between aldehyde groups on glutaraldehyde and the functional groups on microorganisms and the end result was stable structures after thawing at room temperature. The mild conditions of the process in comparison with the ISISA method makes it possible to form spongy macroporous structures which do not require any post-treatment [84]. Taking advantage of the viability of the cells after polymerization by this method and combining it with the properties of cryogels makes the gels suitable candidates for bioreactor applications.

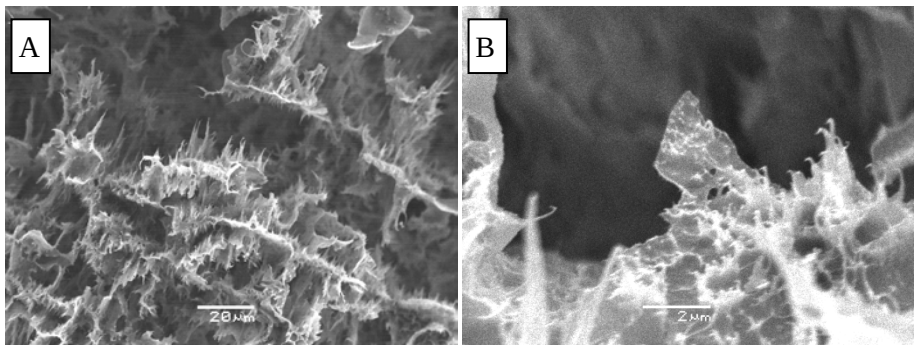

Figure 2.3. Scanning electron microscopy images of PVA cryogel: A) overall view; B) at higher magnification.

2.3 Composite Cryogels

Allowing efficient mass transfer and flow through even in the presence of particulate-containing fluids is one of the attractive features of cryogels due to their macroporous structure. Cryogels have a highly porous structure and their network is limited to the thin walls which are mainly formed around the pores (a few micrometres up to 100 µm); therefore the surface area of the whole structure is low, leading to low capacity, especially towards low molecular weight compounds and proteins [6]. The reactive surface area provided by the matrix for product adsorption governs its capacity of chromatographic separation. Therefore large pores clearly decrease volumetric capacity and may affect the mechanical strength as well [5]. By using different techniques it should be possible to increase the capacity of the cryogel. One way to functionalize the cryogel with desirable segments and increase its binding capacity is by grafting polymer chains on the main backbone of the cryogel. Providing multi-binding positions from a single site, reducing non-specific interaction by decreasing the contact of biomolecules with the support surface, improving the strength of ionic interaction with biocompounds and multilayer adsorption are some of the advantages of the grafting polymer method [109]. Cation- and anion- exchange cryogels for chromatographic purposes can be prepared by grafting functional monomers such as tertiary amines, quaternary amines, carboxylic and sulphonic acid groups [110]. A sequential freeze–thawing approach is another technique for introducing a secondary network with desired functional groups within a preformed cryogel. Different gel precursors, changes in the properties of the cryogels prepared through sequential freeze-thawing and the effects of

crystallization on preformed media were investigated through this new approach [111]. Greater mechanical stability, the required gel surface chemistry and the tissue-like elasticity of double continuous macroporous cryogels allows for the broad design of cryogel scaffolds [112]. Adding an extra network with desired functional groups into the cryogel will also provide more surface area and a potential matrix for bioseparation applications [111].

The other solution to this challenge is preparation of composite cryogels by adding filler particles to develop the cryogel structure with required functionality. These particles (micro/nano particles) normally are introduced into the polymerization mixture before the cryogelation process and lead to the fabrication of macroporous structures with greater surface area by embedding particles or adsorbents inside the gel [113]. Magnetic nanoparticles, MIP particles (**Papers III, IV**), polymer beads (**Paper II**), activated carbon (**Paper I**), cellulose nanofibres (**Paper V**) and CNTs have all been used to form composite cryogels (Figure 2.4) [114].

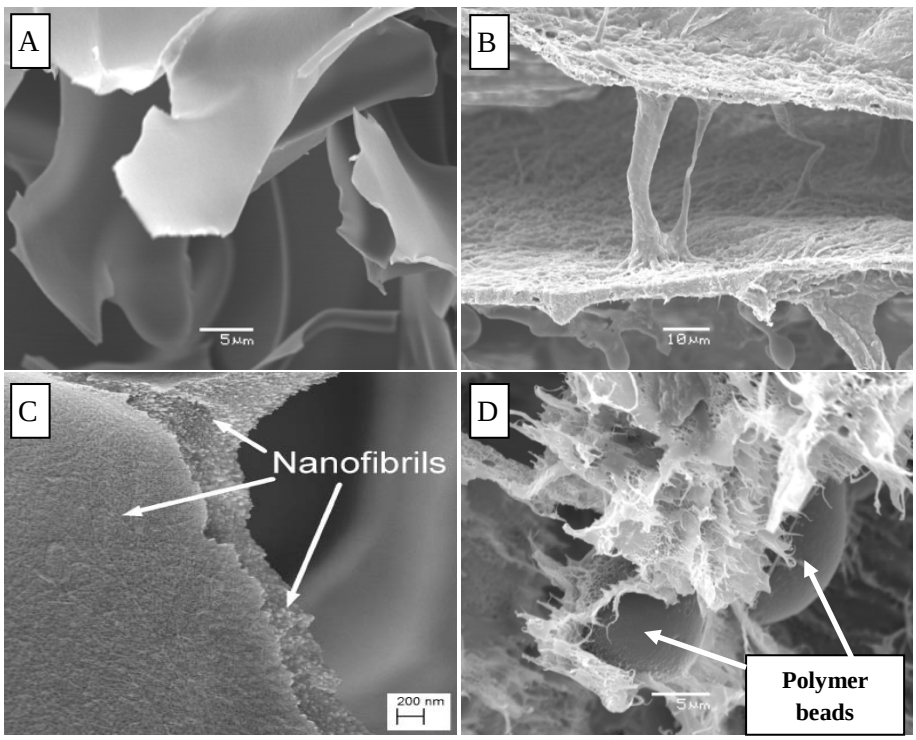

Figure 2.4. Scanning electron microscopy images of A) plain polyacrylamide cryogel; B) polyacrylamide-MIP composite cryogel (**Paper IV**); C) cellulose-NIPA particles composite cryogel (**Paper V**); D) PVA-polymer beads composite cryogel (**Paper II**).

Adsorption of proteins, anions and heavy metal ions has been reported using different types of adsorbents in composite cryogels [115-117]. From a practical point of view, using adsorbent particles in free solution is problematic in terms of their handling. Therefore, immobilizing them in a matrix makes it possible to manage the particles in a better and easier way than in continuous chromatographic systems, especially of the hydrophobic type. Particles and fibres can be added either to the monomer/polymer solution or particle suspension before freezing and embed inside the walls of the cryogel, or they can be immobilized on a premade cryogel via covalent interactions. The latest attempt, to add CNTs into a premade cryogel form, was reported very recently. It was shown that this material has high electrical conductivity at much lower CNTs concentration in comparison with similar reported materials formed with other techniques [118]. Adding particles to a premade cryogel may give higher binding capacity over embedding particles inside the cryogel since more surface area of the adsorbent is exposed to the adsorbate, while particles added to the polymerization mixture before freezing are partially covered by layers of polymer. The disadvantage of immobilization of particles after polymerization can be to increase the risk of their leakage during the process.

2.5 Composite Cryogels Formed with Porous Adsorbent

Chromatography media can be categorized based on their structure into three categories: porous, non-porous and with a solid core (Figure 1.2). All three types have been used either in packed-bed or monolithic forms for adsorption/desorption processes [6,119]. It has been shown that by controlling the surface area and chemical composition down to the nanoscale, it is possible to increase the selectivity and surface area of the adsorbent, [120] which then makes it more suitable and interesting from an application point of view. As long as these types of porous particles are free, then most of their surface will be available to the adsorbate and the maximum level of their capacity can be utilized. Handling these particles either in the form of a free suspension or packed in a column is a challenge. Different issues such as loss of particles and high back pressure, especially with complex media, need to be considered.

Fabrication of composite cryogels by one-step polymerization might attract greater attention in industry and academia. Surface area and the total adsorbent capacity of embedded porous adsorbents such as activated carbon,

functionalized agarose and silica particles inside composite cryogels can be affected by the monomer/polymer solution used during cryogelation. This phenomenon was studied by Lozinskii and his group on PVA composite cryogel preparation with porous dextran particles [121]. The main study was focused on the composite cryogel's properties; it was shown that by increasing the concentration of dextran beads, the strength of the composite cryogel was increased but the porosity of the adsorbent inside the composite cryogel was not studied. The same phenomenon regarding the mechanical properties of cryogels was also observed when adding activated carbon to a NIPA-*co*-allylamine particle suspension (**Paper I**). The mechanical stability of the cryogels was enhanced by increasing the concentration of activated carbon and its structure became more rigid. The porosity of the activated carbon was also investigated in the same paper. The monomer/polymer solution can penetrate inside the pores of the adsorbent due to a capillary effect. During the cryogelation process, these pores act as unfrozen phase and the monomer/polymer solution inside them will be polymerized and therefore block the pores partially or completely and decrease the active surface area (**Paper I**). In order to overcome this problem, two solutions can be suggested for using the cryogelation method via one step polymerization to form composite cryogels with a porous adsorbent. The pores of the adsorbents can be filled by an inert solvent that will not participate in the polymerization process and can be removed after the cryogelation step. By using this strategy, the pores will be protected during the polymerization reaction and will be accessible after that. This approach has not been demonstrated yet and needs to be examined. The second approach is adding the porous adsorbent to a particle suspension instead of a monomer/polymer solution. By using particles larger than the size of the pores in the adsorbent, the risk of penetration of the particles inside the adsorbent channels will be very low and the pores will be accessible after polymerization. The effect of blocking the pores in activated carbon on a separation process was studied in **paper I**. The results of mercury porosimetery showed the difference in carbon porosity between carbon-composite cryogels formed from a monomer solution and from a particle suspension (Figure 2.5 A). By using NIPA and MBAAm solutions to prepare composite cryogels, the porosity of activated carbon could not be detected, while a porosity of the same type and concentration as activated carbon was observed when a NIPA-*co*-allylamine particle suspension was used for composite cryogel synthesis. The porosity of the embedded carbon inside the particle cryogel was approximately in the range of the free

carbon. The second peak detected by mercury porosimetery shows the macroporosity of carbon-composite cryogels (Figure 2.5 B). These data cannot be used as quantitative data for measuring and analysing cryogel pore volumes in the dried state. The softness of the cryogel cannot withstand the high pressure of mercury during analysis and the gel will be deformed. This pressure is not a problem for analysing the carbon particles' porosity, due to their solid nature.

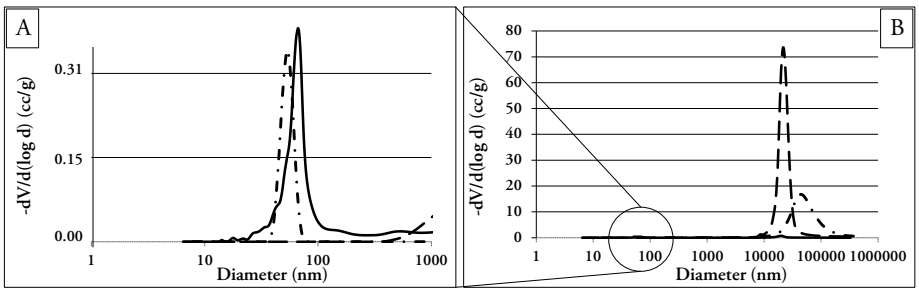

Figure 2.5. Mercury pore size distribution in (A) microporosity of activated carbon particles and carbon composite cryogels; (B) macroporosity of the composite cryogels. Free activated carbon (—); Composite cryogel from monomer solution (— —); Composite cryogel from particle suspension (- . -) (**Paper I**).

Another advantage of using a particle suspension with a (non)porous adsorbent is even distribution of the adsorbent in the end product after polymerization due to the high viscosity of the suspension (**Paper I**). Normally, adsorbent beads have a greater density than water and, due to the viscosity of the monomer/polymer solution being close to that of water, these particles can sediment before freezing is completed. Therefore the concentration of adsorbent gradually decreases from the bottom of a composite cryogel to the top (Figure 2.6), which can increase the risk of leakage of the adsorbent from the gel.

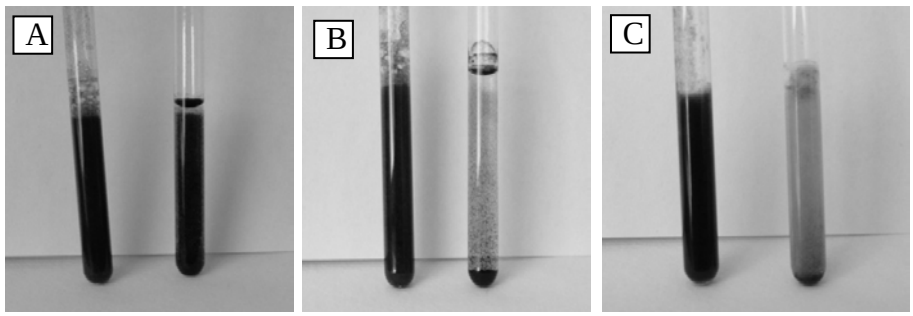

Figure 2.6. Digital photos of distribution of activated carbon particles in polymer mixture and gels: A) immediately after adding carbon particles and intense mixing; B) after one minute of relaxation at room temperature; C) after the cryogelation process. In all the photos the left tube contains a NIPA-*co*-allylamine particle suspension/gel and the right tube has NIPA solution/gel (**Paper I**).

3. Cryogels as Stationary Phase

Highly purified large molecules has been achieved faster by using monolith matrices and their binding capacity, which can compete with conventional packed-bed chromatography [69]. Their large pores allow an increase in the flow rate through the column while the breakthrough curve and dynamic binding capacity of the column will not be noticeably affected [122]. Reductions in processing time and in-process control of separation of biopolymers such as proteins and polynucleotides have been reported using monolith columns [123].

Cryogels as a member of the monolith family have been used as a chromatographic stationary phase media for bioseparation of nano- and microparticles such as plasmid DNA, proteins and cells [78,124,125]. Cryogels can be cast directly inside the separation column and used directly after thawing and washing. The low hydraulic and mass transfer resistances and large pores in cryogel columns make this stationary phase a suitable candidate for chromatographic applications. The high throughput of particulate-containing fluids with effective separation and purification makes it possible to isolate the target directly from fermentation broths or unclarified feed in the downstream process. The flexibility in production of cryogels gives the opportunity to modify and functionalize the matrix with desired functional groups, ligands and adsorbent beads during the cryogelation process or after casting the gel in order to adjust the matrix for chromatography purposes [114]. The composition of the initial reaction mixture and freezing conditions influence the structure of the cryogels, therefore they need to be controlled properly in order to form well-ordered matrices [114].

Using cryogels as an HPLC stationary phase has been reported elsewhere [126] by casting highly crosslinked macroporous cryogels in a stainless steel chromatographic cartridge for separations of lyzoyme from a protein mixture and nanoparticles (>200 nm) from a polymerization mixture. Polyethylene glycol diacrylate (PEGDA) as crosslinker and methacrylic acid (MAA) as functional monomer were chosen to form the gel [126]. The preliminary result of this study indicated the potential of cryogels as a

medium for separations of proteins and nanoparticles with wide diameters, ranging from several to hundreds of nanometres. Due to the simplicity of cryogel production, the low cost of polymers or monomers and their high permeability towards liquids and gases, cryogels became an interesting alternative to other available stationary phases for (bio)separation applications such as water and waste water treatment (**Papers I, III and IV**), protein purification from crude extract [127,128] and cell separation [129] by using ion-exchange [64] and affinity chromatography (**Papers II and III**).

3.1 Protein Chromatography

Two well-known types of chromatography for protein purification are ion exchange and affinity chromatography. Based on IUPAC recommendations, ion-exchange and affinity chromatography are defined as liquid chromatographic techniques in which "the differences in the ion-exchange affinities of the sample components" and "unique biological specificity of the analyte and ligand interaction" are utilized for the separation process, respectively [130]. These techniques are normally used as intermediate steps in a purification process [131]. Functional groups for ion exchange and ligands with appropriate affinities can be coupled to a chromatographic matrix before or after the cryogelation procedure. Ion exchange monolith columns based on polymeric materials have attracted attention due to their stability and tolerance to a wide range of pH [64]. Incorporation of functional ion-exchange groups by co-polymerization or post-polymerization on the surface of the monoliths is possible by grafting. Ion-exchange columns can be classified into weak and strong ion-exchange. Functional groups such as diethyl amine and tertiary amine on CIM monoliths and polyacrylamide cryogel form weak ion-exchange while quaternary amine on the same matrices shows strong ion-exchange [64]. Carboxylic [132] and tertiary amino [133] groups were applied for the preparation of cation- and anion-exchange cryogel columns, respectively. Acrylamide has been used as a backbone monomer for both cation- and anion-exchange chromatography, while dimethylacrylamide has been reported for preparation of cation cryogel. Carboxyl groups have also been introduced on a cryogel matrix via graft polymerization for lyzozyme purification [134]. *N*,*N*´-dimethylaminoethyl methacrylate (DMAEMA) was grafted on an acrylamide cryogel in order to form an anion-exchange column for evaluation of BSA purification [135]. Purification of adenosine triphosphate (ATP) [133] and cytidine triphosphate (CTP) [136] from fermentation broth using

cryogels are good examples of applications of macroporous structures as ion-exchange matrices for direct capture of targets without facing clogging problems.

In order to determine the strength of the interaction between the target and the ligand, the dissociation constant (K_d) has been used, which is defined as the rate of dissociation (k_{-1}) divided by the rate of association (k_1). By this definition, ion-exchange chromatography has the highest K_d, around 10^{-3}, and the avidin-biotin interaction has lowest, 10^{-15}. Affinity chromatography with biological ligands often have K_d less than 10^{-7} and with synthetic ligands the dissociation constant increases up to 10^{-5} [137]. Therefore higher affinity towards the target can be expected from biological ligands than ions.

Lectin affinity chromatography, boronate affinity chromatography, immunoaffinity chromatography and IMAC are common types of affinity chromatography, named based on their ligands and their interactions with the product. Despite the attraction of biological ligands due to their high selectivity, most of them suffer from high cost and sensitivity to degradation. Boronate has the ability to interact with diols at high pH [138]. In comparison with lectin it has lower specificity but its stability, low cost and higher flexibility make boronate an interesting ligand for affinity purposes. Specific interactions occurring between immobilized metal ions and histidine groups in different products such as amino acids, peptides, proteins, and nucleic acids is the main concept behind IMAC. The metal ions are immobilized within a column through the use of chelating agents like iminodiacetic acid, nitrilotriacetic acid, carboxymethylated-aspartic acid, and l-glutamic acid (L-GLU) [139]. Cryogel matrices as IMAC for protein purification have also been well studied. A macroporous polyacrylamide cryogel having iminodiacetic acid functionality was characterized for IMAC application [127].

Acrylamide and HEMA have been widely used as backbone structures for bioaffinity or IMAC cryogel matrices. Purification of IgG from plasma using Con A as ligand on a cryogel matrix was studied and showed up to 85% puritification [140]. Using cryogel as an affinity matrix by immobilizing synthetic or biological ligands on the cryogel has been reported for different biological compounds by Denizli's group [141-143]. It was shown that the capacity of cryogel is higher in plasma than in water solution in different experiments, while interference by other proteins in the plasma with the target molecules has never been reported in these studies [141]. Immobilization of functional groups via epoxy groups or copolymerization on the matrix showed that the capacity of a cryogel functionalized by copolymerization was

higher than one with epoxy groups. Lysozyme binding capacity on the copolymerized cryogel and the gel with epoxy groups was 1.34 and 0.12 (mg/mL), respectively [144]. Direct capture of His-tagged recombinant lactate dehydrogenase [127] and organophosphate hydrolase [145] from crude cell homogenate have been reported with enzyme purifications of 99%. Adsorption/desorption of DNA from solution using a metal-chelated polyHEMA cryogel has been studied and the maximum capacity of the matrix was reported to be more than 30 (mg/g) [146]. Table 3.1 summarizes some of the other applications of cryogels with different chromatographic functional groups for separation of biological compounds.

Table 3.1. Applications of cryogel in different chromatography types.

Chromatography type	Ligand/Functional group	Target	Reference
Ion-exchange	Carboxylic group	Lactoferrin/lactoperoxidase	[132]
	Tertiary amine group	cytidine triphosphate/ Nucleotides	[133,136]
	Sulfo functional group	Lysozyme	[147]
Affinity	Con A	Invertase/HRP	[125], **Paper II**
	N-methacryloyl-(l)-histidine methyl ester (MAH)	Plasmid DNA	[148]
	Antibodies	Inclusion bodies	[149]
IMAC	Iminodiacetic acid (IDA)/Cu^{2+}	(His)6-LDH/Cyotchrome C	[127,150,151]
	Cu^{2+}-attached sporopollenin	HSA	[152]

Composite cryogels with affinity interactions have been prepared by embedding different nanoparticles [153,154] and MIP particles [141,155] (**Papers I, III and IV**). Selective removal of bilirubin by using MIP particles embedded in a polyHEMA cryogel was studied. The binding capacity of the composite cryogel was reported to be more than 10 mg/g of polymer while the polyHEMA cryogel had a capacity around 0.2 mg/g of polymer [156]. Recently, fabrication of a composite cryogel from agarose/chitosan was reported for capturing ovalbumin and ovotransferrin. Aminophenylboronic acid was covalently bound to the matrix via epoxy groups as synthetic ligand [155]. The

binding capacity of ovalbumin in solution was reported to be around 55 mg/g of adsorbent and the matrix was reused for more than 10 cycles without significant loss in its capacity. The same affinity matrix composition was also evaluated for capturing IgG and its maximum binding capacity was reportedly more than 70 mg/g of adsorbent [157].

In **paper II**, a PVA cryogel was prepared by crosslinking PVA particles at sub-zero temperature. Porous polymer beads were added to the mixture before cryogelation. These particles had an average diameter of 11 μm and a porous structure with free amino functional groups on their surface. Concanavalin A was immobilized on the composite cryogels via epoxy groups and binding/elution of HRP was studied in batch and continuous systems. The results showed that by increasing the concentration of polymeric adsorbent beads inside the composite cryogels, the concentration of immobilized Con A increased and therefore the capacity of the gel for binding HRP (Table 3.2). Purification of IgG from plasma solution by immobilized Con A on a composite cryogel was also reported using magnetic beads based on poly(glycidyl methacrylate) (mPGMA) [143]. Embedded magnetic nanoparticles of Fe_3O_4 in composite cryogels were also evaluated for capturing BSA [124].

Table 3.2. The amounts of bound enzyme on PVA composite cryogels in chromatographic and batch systems (**Paper II**).

		Chromatographic system		Batch system	
Gel	Polymer beads (mg/mL gel)	Bound enzyme (mg HRP/mL gel)*	Dynamic binding capacities (mg HRP/mL gel)	Bound enzyme (mg HRP/mL gel)*	Specific activity (Unit/mg)
1	0	0.98±0.004	0.09±0.01	0.28±0.02	0.52±0.03
2	10	1.41±0.004	0.14±0.03	0.48±0.01	0.47±0.05
3	20	2.00±0.009	0.21±0.02	0.72±0.03	0.45±0.02
4	30	2.91±0.008	0.30±0.06	1.25±0.05	0.35±0.03

Gel 1 is Plain PVA cryogel; gels 2, 3 and 4 are PVA composite cryogels with different concentrations of particle adsorbent. * The amount of HRP solution passed through the column was higher than what was used in batch systems.

3.2 Cell Chromatography

Developing an efficient and inexpensive method for separating and isolating a desired cell type from a defined cell population is essential for bioanalysis, molecular genetics, cancer research, immunology and stem cell research [158]. The existing technologies for cell separation are divided into two classes: a) based on physicochemical properties of cells such as size, shape and density; b) based on affinity interactions and cell-surface chemistry [159]. Cell filtration and centrifugation are techniques that are used mostly for bulk separation based on physical criteria of cells. Micro- and ultra-filtration have low specificities and yields due to loss of cells during the operation. Centrifugation is a more common method for cell isolation due to its simplicity and high yield [160]. Other methods such as fractionating in ATPE, magnetic bead separation and fluorescence-activated cell sorting are based on the affinity characteristics of cells for each approach. The biggest disadvantages of these techniques can be summarized as either their high cost or long processing time [129].

Cell affinity chromatography operates based on interactions of cell surface molecules with a ligand immobilized on the affinity matrix [159]. The separation process can be based on either a negative or positive selection. In negative selection, the unwanted cells bind to the adsorbent and the desired cells pass through the column, while in positive selection the target cells are captured by the adsorbent and can then be eluted in enriched form [158]. The principal of cell chromatography is similar to protein affinity chromatography but cell affinity chromatography is far more difficult due to the nature of cells. They are quite large, fragile, have complex surface chemistries and are sensitive to any stresses. These features of cells make existing matrices less suitable for cell affinity chromatography [161]. Expanded bed chromatography was reported for cell separation using agarose or silica particles, but non-specific interactions are a major drawback for those materials. Long processing times, limited flow rates and operational velocities and high shear stress are other disadvantages of expanded bed chromatography which can affect the viability of cells [112].

As a backbone formed from hydrophilic polymers, cryogels can be a suitable candidate as a stationary phase in cell affinity chromatography. Large interconnected channels, high elasticity, convective flow and flexibility for immobilization of different ligands are other properties cryogels provide for cell separation. By immobilizing special groups and ligands such as charges,

hydrophobic parts, antibodies etc., cells can be bound to the cryogels [129]. Cells are attached evenly throughout the whole matrix. Approximately similar growth of the cells was observed in different parts of the cryogel after exposure to *E. coli* and overnight incubation, which was an indication the cells bound inside the whole volume of the cryogel [158]. Cryogels can be used for both negative and positive cell separation. Protein A-cryogels were prepared for separation of IgG-labeled B cells from T lymphocytes via negative selection [112]; as for positive selection, isolation of antiCD34-labeled CD34+ human acute myeloid leukemia cells has been reported in the literature [162].

Unlike other cell affinity chromatography, harsh elution conditions are not required when using cryogel matrices. The elasticity and spongy structure of the cryogels make it possible to use mechanical force for elution. Chemo-mechanical elution conditions (mechanical compression combined with mild elution condition) were used for detachment of the cells from cryogel. Therefore the viability of cells recovered from the column is up to 80% [163]. Isolation of yeast cells from *E. coli* was also studied on a cryogel matrix by immobilization of Con A as an affinity ligand on the gel; the recovery was around 95% [159].

3.3 Water and Wastewater Treatment

Wastewater can be considered as another example of a complex media that contains particles and contaminants in a wide range of sizes. The presence of various types of harmful and toxic compounds in wastewater needs to be treated in a proper way before it is discharged to nature. There are conventional methods for treating wastewater by using chemical or biological techniques [164] but the presence of some pharmaceutical products such as β–blockers (**paper III**), endocrine disrupting contaminants (EDCs)[165] , heavy metals ions [166] or by-products which are produced during the treating water process (**paper IV**) is an indication that the conventional methods are not always sufficient. The challenge is to develop a robust adsorption media in order to capture the target selectively. Its macroporous structure will allow transportation of particulate-containing fluid through the gels and it can be functionalized and reused in the process. Composite cryogels can be formed in order to increase the capacity of the cryogels in water and wastewater treatment applications. Hydroxyapatite-PVA composite cryogels were reported for cadmium ion removal [166]. Hydroxyapatite is able to interact with

other heavy metals ions as well, due to its ion-exchange interactions through OH^- and PO_4^{3-} [167]. Releasing non-treated heavy metals ions into the environment creates a huge concern. They are non-biodegradable and their accumulation causes diseases for animals and humans. Therefore finding efficient and cheap adsorbents is always attractive. Adding a chelating group onto the cryogel matrix is another way to remove heavy metals ions [168]. Recently, the binding capacity of composite cryogels containing aluminum oxide or MIP particles and a cryogel having thiol functionalized groups on their surface for removing arsenic(V) from water was studied. The capacity and efficiency of the cryogels containing thiol groups was less than that of the composite cryogels, therefore making them less efficient in the separation process [169].

Composite cryogels containing MIP adsorbents have been used for water and wastewater treatment. The selectivity of MIP particles and the macroporous structure of cryogels make a unique combination for preparation of composite cryogels. The adsorption isotherm and kinetics of the MIP adsorbent were evaluated for bromate removal from water. Bromate is formed as an ozonation by-product in bromide-containing water. The systemic toxicity of bromate (administered as the potassium salt) has been reported as has the carcinogenicity of bromate in the long term; therefore the concentration of this anion in the United States of America (USA) and European Union (EU) countries in drinking water must not exceed a level of 10 μg/L [170]. For selective removal of bromate from water, MIP particles were chosen as a suitable adsorbent. In order to assess desorption of bromate from MIP adsorbent and regeneration of the adsorbent, a composite polyacrylamide cryogel was prepared (**paper IV**). To confer greater mechanical strength, cryogels were formed inside AnoxKaldnes™, plastic carriers (Figure 3.1). Two different types of adsorbent were evaluated for bromate removal, MIP particles for specific adsorption and iron-aluminum double hydrous oxide as an ion-exchange adsorbent for non-specific interaction. Although the ion-exchange adsorbent had faster kinetics and higher capacity towards bromate anions (Table 3.3), the selectivity of MIP adsorbents in the presence of a compatible anion, nitrate anions, was higher (**paper IV**). Composite cryogels with MIP particles act as affinity matrices and have also been used for capturing organic compounds such as EDCs [165] and L-GLU[171]. A composite cryogel formed of MIP particles for removing 17 β-estradiol was used as a column for SPE or in plastic carriers in moving-bed

reactors. In both systems the real wastewater was spiked by the target and high recoveries by the composite cryogels were reported [38,165].

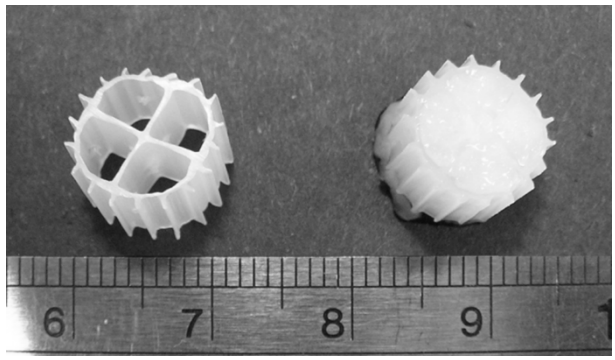

Figure 3.1. Digital photo of (left) the AnoxKaldnes™ carrier as a protective shell and (right) the carrier with polyacrylamide-MIP composite cryogels prepared inside (Scale bar: cm) (**Paper IV**).

Table 3.3. Adsorption isotherm and kinetic parameters for bromate sorption onto the MIP and ion-exchange adsorbent beads (**Paper IV**).

	Pseudo -second-order kinetic			Langmuir isotherm		
Adsorbent	q_e (mg/g adsorbent)	K_2 (mg/g min)	R^2	q_{max} (mg/g adsorbent)	K_l (L/mg)	R^2
MIP	0.19±0.005	0.33±0.01	0.99	0.2±0.02	91.1±0.06	0.99
Ion-exchange	0.29±0.010	12.3±0.11	0.99	0.8±0.04	42.1±0.09	0.99

Immobilization of particles makes it easier to handle them in suspension without any losses or leakage of the adsorbents, which would otherwise lead to lower efficiency after running several cycles. On the other hand, by embedding particles in a matrix, the adsorption/desorption operation can be carried out in continuous mode, which shortens the operation time. In **paper III**, MIP-based composite cryogels were constructed by chemically crosslinking MIP nanoparticles via the amino groups on their shells. MIP nanoparticles were mixed with PVA particles, where they acted as bridges between the adsorbents (Figure 3.2).

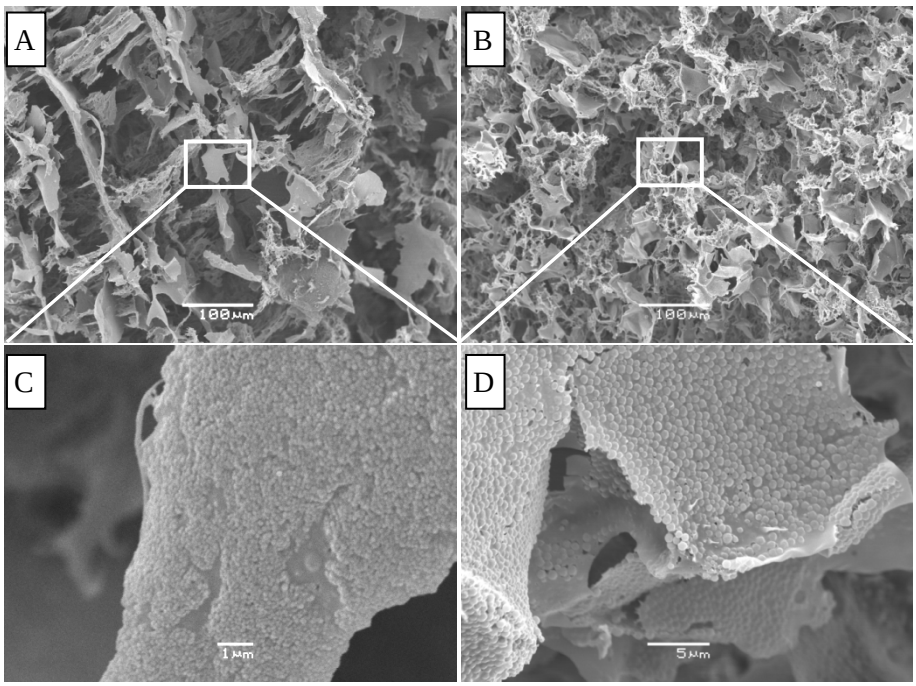

Figure 3.2. Scanning electron microscopy images of PVA composite cryogel. A) Containing MIP particles; B) containing NIP particles; C and D) are high resolution images of (A) and (B), respectively (**Paper III**).

The composite cryogels were used for SPE for a therapeutic compound from complex samples. This composite cryogel was used as an affinity matrix for adsorption of propranolol from water or plasma solution. Beta-blockers in high concentration are toxic compounds that can produce clinical manifestations such as hypotension and arrhythmias. They have been used in different drugs for more than 50 years for treating hypertension and other cardiovascular disorders, migraine headaches and anxiety [172]. As a result of their expanded use they are likely to be found in wastewater systems. The adsorption by the free MIP adsorbent beads in comparison with the MIP composite cryogel was evaluated under the same conditions and it was shown that despite immobilization of the MIP particles inside the network, almost all their binding sites were exposed to the adsorbate and maintained their selectivity (Figure 3.3A). The results also indicated that the MIP composite cryogel can remove more than 80% of propranolol while removal of other similar β-blockers is less than 50% (Figure 3.3B)(**paper III**).

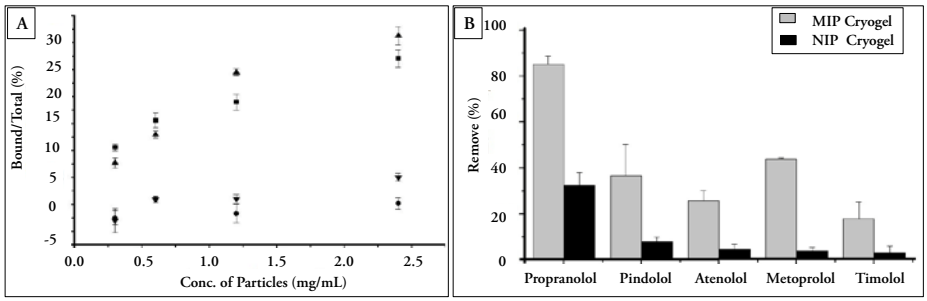

Figure 3.3. A) Uptake of [^{3}H]-(S)-Propranolol (246 pM) by the composite cryogel and free adsorbents (■: MIP particle; ●: NIP particles; ▼: NIP composite cryogel; ▲: MIP composite cryogel). B) Recovery of propranolol by MIP/NIP composite cryogels in the presence of analogue compounds.

Another well known adsorbent that is widely used for water/wastewater treatment is activated carbon. The formation of composite cryogels containing activated carbon and its properties were investigated in this work in two different ways: using a monomer solution and a particle suspension. In **paper I,** carbon particles were mixed with a particle suspension of NIPA-*co*-allylamine and formed composite cryogels by chemical crosslinking polymerization, or they were added to NIPA solution followed by radical polymerization. These two composite gels were then used to study phenol adsorption from water solution and milk, as a complex medium model system. It was shown that the composite cryogel formed from suspension had a higher capacity towards phenol than the gel prepared from a monomer solution (Figure 3.4), due to the greater accessibility of the activated carbon particles to the surface. The unique macroporous structure of the gel in combination with the retained porosity of the carbon particles makes this material suitable for separation and environmental applications.

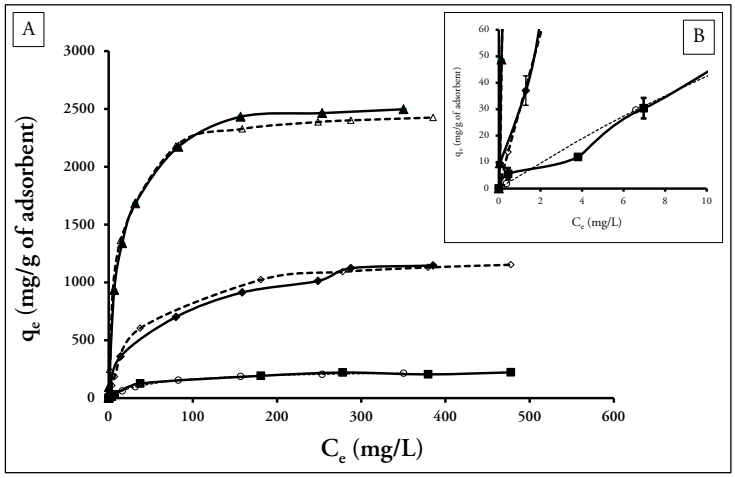

Figure 3.4. A) Adsorption of phenol from 50 mL of solutions with different phenol concentrations, 22 °C for 24 h. (—♦—: Carbon composite cryogel from particle suspension; —■—: Carbon composite cryogel from monomer solution; and —▲—: carbon particles. Fitting the Langmuir equation with the data; …Δ...: carbon particles; …◊…: Carbon composite cryogel from particle suspension and …o…: Carbon composite cryogel from monomer solution. B) The insert shows the magnification of 0 to 10 mg/L in figure A (**Paper I**).

3.4 Other Applications of Cryogel

Phages can also be used as cheaper affinity ligands for screening molecules such as peptides and proteins with high affinity towards the targets. Many examples can be found in the literature using bio-panning methods for screening different targets [173]. Recently a new technique, so called chromato-panning, was developed for identifying binders from a phage display library. The main concept of chromato-panning is similar to that of bio-panning, with some modification in order to improve the method. In the technique developed, the target is immobilized on the cryogel matrix. The advantages of cryogel for bio-panning are: a) pore size makes it possible to directly infect cells with phages bound to the target on the matrix surface; b) mass transfer in cryogel is based on convection while diffusion is the driving force in the traditional method when using other types of plastic materials; c) there is no need for post-coating due to the hydrophilic character of the cryogel; d) it is possible to bind ligands on the matrix via available functional groups; e) dramatic reduction in incubation time; f) high yield of purity in the first cycle in comparison with conventional methods [112,174]. In comparison with affinity

ligands such as antibodies, phages are cheaper and after selection for a specific target they can act as an affinity ligand with high selectivity [175]. Immobilization of phages on cryogels in order to form an affinity matrix has been studied elsewhere for purification and separation of human lactoferrin from milk and von Willebrand factor from blood [176].

Cryogel matrices have been also used in other biotechnological and biomedical applications. Cell scaffolds in tissue engineering, cell carriers and catalyst matrix are some other applications of cryogels (Table 3.4). The properties and characterization of cryogels, such as its porous structure with a bioactive surface and elasticity, provide essential requirements for cell cultivation. Cultivation of cells in three dimensional networks based on natural polymers has shown better results than two dimensional systems [106]. The high mass transfer of the reaction mixture through cryogels is the reason behind its application as a support for enzyme immobilization applications.

Table 3.4. Some of the application of cryogel in biomedical and biotechnology.

Application	Cryogel material	Active part	Target/Use	Reference
Chromato-panning	AAm	Human lactoferrin	Selection of phages from phage library	174
Scaffold/ Carrier	Gelatin	-	Fibroblast cells; adipose tissue; blood vessel; bone cartilage; nerves etc.	85,93
	Dextran	-		
	Chitosan	-		
	Agarose	-		
	Cellulose derivatives	-		
	HEMA	-		
Catalyst support	PVA	*Acetogenium kivui*	CO_2 reduction	177
		Pseudomonas sp.	L- Proline biosensor	
		lactate oxidase	lactate sensor	178
		glucose oxidase	Glucose sensor	179

4. Conclusion and Future Remarks

The manufacture of biotechnological products strongly depends on downstream processing. In recent years, introducing new and complex bioproducts to the market requires a strategy that emphasizes the importance of separation as a major section of the bioprocess. Limitations of conventional separation techniques (packed-bed chromatography) for direct purification from particulate-containing fluids are a challenge in downstream processing. Therefore the development of robust, economical and integrated systems for selective separation of solid and liquid mixtures is needed.

Macroporous cryogels, polymeric gel networks, have been introduced as a monolithic stationary phase for industrial and environmental biotechnology applications. The unique properties of the gels, such as their elasticity, mechanical robustness, and macroporous structure, make this material a great candidate for different applications. High mass transfer and efficient flow of particulate-containing fluids are expected through the porous structure. Flexibility of formation of different formats of cryogel is another interesting feature of this type of material, which can be designed based on processing needs. The low binding capacity of cryogels as a result of their highly porous structure in comparison with packed-bed column needs to be improved. This issue can be addressed by formation of composite cryogels, adding various types of particles or adsorbents, which has been studied in this thesis. Embedded adsorbents increase the surface area as well as the binding capacity of the gel. Applying free particles can be a challenge regarding their handling, continuous operation and applying them directly on complex media. Therefore using the adsorbents in a network will minimize the risk of their leakage as well as enhancing the efficient mass transfer of the adsorbate.

Molecularly imprinted polymer particles are used as synthetic ligands for selective removal of different compounds (organic/inorganic). These particles can be trapped inside the cryogel network during the cryogelation process or chemically crosslinked via covalent bonds with each other. The high selectivity of these adsorbents in combination with the properties of cryogel will form a unique stationary phase that can be used for any type of complex

media such as water/wastewater or biological samples. The high selectivity of the MIP adsorbents towards binding the targets from solutions even in the presence of analogue molecules was a good indication that immobilization does not influence their adsorption and selectivity properties. The possibility of regenerating and reusing the materials for several cycles makes them economically interesting, especially for industrial applications. Developing the new protocol for forming composite cryogels by adsorbent beads is an ongoing project. Adding particles onto preformed cryogels via covalent interaction or using click chemistry are some examples of these new approaches. The accessibility of the adsorbate to the particles will be higher as compared with immobilizing them inside the polymeric layers of the cryogel.

Using porous adsorbents has become more popular due to their high surface area and adsorption capacity. Adding these types of particles to the formation of composite cryogel needs extra attention for preserving the maximum surface area and the capacity of the adsorbent during the process. This cannot happen unless great improvements in the conventional preparation protocol of composite cryogels are made. One way to form an effective and practical matrix with porous adsorbents is to replace the monomer solution with a particle suspension as a polymerization mixture. This will help to avoid blocking the pores of the adsorbent by any polymer/monomer solution during the procedure so most of the pores will remain accessible to the adsorbate. This method was assessed on activated carbon composite cryogels. The results from the adsorption experiment and characterization of the gel by mercury porosimetry confirmed that the carbon composite cryogel formed by particle suspension had higher binding capacity even when tested on complex media. This one step polymerization is straightforward, easy and inexpensive in comparison with other available techniques reported in the literature. This new approach opens the possibility of forming more efficient stationary phases for industrial purposes. Forming activated carbon composite cryogels from particle suspension is only the starting point of using porous adsorbents in more efficient way inside cryogels. Optimization and modification of this method needs to be carried out for other materials such as CNT. The unique characteristics of CNT in combination with cryogel might form a great matrix for different biological applications such as stationary phases for chromatographic systems or (bio)sensors. The chemistry on the surface of CNTs and the backbone of the cryogel needs to be modified for the one step polymerization procedure.

Removing toxic organic compounds from solutions, especially wastewater, by composite cryogels has been studied in recent years. Biodegradation of these compounds once they have been adsorbed on the network can be another perspective for composite cryogels application. Forming composite cryogels based on suitable microorganisms and adsorbent beads might be a future challenge in the separation area. Integrating degradation as well as separation of harmful organic compounds by adsorbent-microorganism composite cryogels might be achieved by the cryogelation process. The mild conditions of production offer the possibility of adding microorganisms in the cross-linking polymerization mixture before cryogelation. Therefore the adsorbents capture and make the target accessible to the microorganisms while the microorganisms have an opportunity to degrade the pollutants to smaller and less harmful compounds. Using photocatalyst particles to form composite cryogels might also be interesting from the degradation point of view.

Composite cryogels formed from monomer solution or particle suspension are not limited to water and wastewater treatment. Preparation of matrices for affinity chromatography is their other application in downstream processing. Purification of bioproducts by immobilizing bioligands on composite cryogels was investigated in this work. The capacity of the composite cryogels was comparable to those of commercial matrices available in the market. There are many reports available on composite cryogels due to their unique properties, flexibility in formation from natural or synthetic materials, high capacity and hydrophilic structure. The potential of composite cryogel applications is not limited to only these examples. Further development of the chemistry of composite cryogels and modification of their materials must be continued in order to make them more suitable and efficient for different applications in the future.

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Paper I

Characterization of macroporous carbon-cryostructured particle gel, an adsorbent for small organic molecules

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Preparation of a new type of cryostructured carbon particle macroporous gel with one-step sub-zero cross-linking was studied in this work. Aggregation of nano- and micro-particles of poly(*N*-isopropylacrylamide-*co*-allylamine) was used to form a polymeric network to integrate activated carbon particles. The cryostructured particle gel was compared with the conventional composite cryogel. The unique macroporous structure of the gel in combination with retained porosity of the carbon particles makes it suitable in separation and environmental applications. The adsorption isotherm of phenol was studied and the advantages of cryostructured particle gel over conventional composite structures were compared in this study. The developed new gels were characterized by scanning electron microscopy, mercury porosimetry and texture analysis.

Introduction

Macroporous hydrogels have been developed for a variety of applications such as biomedical materials or for environmental separation.[1–4] One approach of preparing macroporous materials has been the cryogelation technique where a solution of monomers or polymers is frozen and the reaction proceeds in an apparently frozen state.[5] A similar approach can be used by structuring particles into a gel by freezing a homogenous suspension of particles. If the particles are aggregated in this apparently frozen state and thus form a stable network, only a simple thawing of the sample is required to obtain a structured gel built from particles.[6] Another approach to structure particles through freezing requires freeze-drying of the sample and thereafter in this state stabilization of the structure.[7,8] All these techniques are based on the fact that when water freezes, solutes or particles get expelled from the growing ice crystal front since it is not energetically favourable to entrap the substances in the ice lattice.[9,10]

One method of introducing different functionalities in macroporous hydrogels is to incorporate particles into the network and thus create composite gels. This has been used in a number of different cases such as environmental applications for capturing of endocrine-disrupting compounds and inorganic compounds and purification of immunoglobulin G and living cells.[1,11–13] However making composite cryogels by mixing porous particles with a solution of monomers can result in the monomeric mixture filling the pores of the particles. During the freezing stage of the process the monomer solution in nano-pores will stay in the liquid state[14,15] where it may polymerize and thus the pores can be partly filled with polymer. Polymerization can also occur at the surface of the particles and thus form a skin layer which may restrict access to the pores.

This can be a problem especially when trying to incorporate particles of activated carbon in a composite gel due to the good adsorption properties of activated carbon. In this study efforts were made to produce macroporous gels which incorporate carbon particles without blocking the pores of the carbon particles. By using the technique of structuring particles in an apparently frozen state combined with crosslinking of particles, the problem of pore blocking was addressed. The carbon particles were mixed with a suspension of poly(*N*-isopropylacrylamide-*co*-allylamine) poly(NIPA-*co*-allylamine) particles to prepare the particle structured material. Since the sizes of the used poly(NIPA-*co*-allylamine) particles were larger than the pores of the carbon particles it was thought that the pores could remain accessible since the particles could not fill the pores. The performance of the formed structured particle gels was compared with that of conventional composite cryogels where the carbon particles were mixed with monomers which were then polymerized.

The prepared materials were characterized using scanning electron microscopy (SEM) to study the structure of the gels and mercury porosimetry to study how accessible the pores of the carbon particles were in the prepared composite systems. The application of these composite systems for the removal of toxic compounds from water was studied with phenol as a model system.

Experimental

Materials

Activated carbon (45–63 μm) with nanoporous structure was provided by MAST Carbon International Ltd (Surrey, UK). Allylamine, *N*,*N*′-methylene-bisacrylamide (MBAAm), ammonium persulfate (APS), potassium persulfate (KPS), *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED), potassium ferricyanide, 4-aminoantipyrine and phenol were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium dodecyl sulfate (SDS) was from Sigma (Saint Louis, MO, USA), glutaraldehyde from Merck (Hoenbrunn, Germany) and *N*-isopropylacrylamide (NIPA) was from Acros (Geel, Belgium). Water solution of ammonia (25%) was from Fluka (Steinheim, Germany). Low fat milk (0.1%) was purchased from a local supermarket.

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Preparation of poly(NIPA-*co*-allylamine) nano- and micro-particles

Poly(NIPA-*co*-allylamine) nanoparticles were synthesized as described earlier with some modification.[6] Briefly, different ratios of NIPA:MBAAm (40, 10 and 5 : 1 mol/mol) and NIPA:allylamine (10 : 1 mol/mol) were chosen to synthesize nanoparticles by precipitation polymerization technique. NIPA (1.92 g) was dissolved together with proper amounts of MBAAm and allylamine in 115 mL of distilled water. SDS (0.037 g) was added to the solution of monomers which was heated in an oil bath for 40 min at 70 °C under nitrogen bubbling. KPS (0.077 g) was dissolved in 10 mL distilled water and added as a polymerization initiator to the mixture. The polymerization was run under the same conditions for 2–3 hours till the mixture become milky and white. The suspension was dialyzed against water for 7 days and then freeze-dried.

Fabrication of microparticles from NIPA and allylamine was done according to a procedure described elsewhere[16] except that allylamine was used in the preparation procedure and the molar ratio between NIPA and allylamine was (10 : 1). The particles were sieved with 25 μm mesh and the particle fraction with diameters less than 25 μm was collected.

Preparation of cryostructured particle gels

Different concentrations of synthesized nano- or micro-poly-(NIPA-*co*-allylamine) particles (1, 3 and 5% w/v) were chosen for plain macroporous gel preparation. Dried particles were suspended in 2 mL distilled water. Glutaraldehyde solution 50% (5–20 μL/mL suspension) was added as a crosslinker. The mixture (0.5 mL) was transferred to glass tubes (I.D. 7 mm) and frozen at −12 °C in an ethanol cooled cryostat for 30 min. The tubes were kept at −12 °C overnight, then defrosted at room temperature and rinsed with distilled water to remove any non-reacted chemicals.

Cryostructured particle gel with activated carbon was prepared under the same conditions as mentioned above. Carbon was added to the suspension before the crosslinker. Different concentrations of carbon (0.5, 1 and 5% w/v of suspension) were chosen in order to compare the effect of varying content of carbon on the mechanical stability of the formed macroporous gels.

Preparation of composite cryogel

The plain and carbon composite cryogel with monomers, NIPA and allylamine, were synthesized with total monomer concentration (1, 3 and 5% w/v solution). The molar ratio between NIPA:allylamine and NIPA:MBAAm was chosen (10 : 1). Cryogels were prepared according to the following protocol. The monomers were dissolved in distilled water. The solution was then purged with N_2 to remove dissolved oxygen from the mixture which otherwise inhibits the free radical polymerization. An activator, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) (1% of total monomer weight) was added and the mixture was kept on ice. Finally ammonium persulfate, APS, (1% of total monomer weight) was dissolved in distilled water (500 μL) and added to the monomeric mixture in order to initiate the reaction. The freezing condition and the size of the gels were the same as for the preparation of cryostructured particle gel which was mentioned earlier. For carbon composite cryogel, activated carbon (0.5, 1 and 5% w/v of suspension) was added to the solution before APS.

Characterization of particles and gels

The mean size and particle size distribution of poly(NIPA-*co*-allylamine) particles were measured at 20 and 40 °C by Nano-ZS 3600 (Malvern Instrument, Malvern, UK). Scanning electron microscopy (SEM) was carried out for evaluation of the macroporous gel structures. The samples were dehydrated in solutions of increasing ethanol concentrations and then critical-point dried. The dried samples were coated with gold/palladium (40 : 60) and studied by using a JEOL JSM-5600 LV scanning electron microscope (Tokyo, Japan).

In order to study porosity of the carbon particles entrapped in the cryogel, or in the structure made by aggregation of particles, a PoreMaster mercury porosimeter (Quantachrome Instrument, UK) was used. To remove all the water in the samples and dry them, the gels were freeze-dried and kept in a vacuum oven at 40 °C over night before analysis.

The mechanical stability of the different types of prepared matrices with or without carbon was analyzed by a texture analyzer (XT2i, Stable Micro Systems, Godalming, England) using a 5 kg load cell and a cylindrical probe (25 mm diameter). The measuring points were the centre parts of the gels set on the side position. The gels were placed on a metal plate and a load was transmitted vertically to the gel. The test speed was 1 mm/s. All the mechanical measurements were conducted at room temperature. The samples were compressed 10% of the total height. The applied force was recorded and calculated by the Texture Expert Exceed, v.2.03 software. Each sample was analyzed three times to compare properties with regard to mechanical strength and elasticity.

Batch experiment for phenol adsorption

Batch adsorption experiments were carried out at room temperature on a rocking table for the different synthesized matrices in 50 mL phenol solution. All the experiments were run on plugs of 0.5 mL of cryostructured particle gel (5% (w/v) nanoparticle NIPA:MBAAm (10 : 1 mol/mol) +1% (w/v) carbon, which has 16% of total solid materials weight) and composite cryogel (5% total monomer concentration + 1% (w/v) carbon) and same amount of carbon particle (5 mg). For the kinetic sorption experiments, 5 mg/L of phenol solution was chosen. The sorption isotherm experiments were studied with the initial phenol concentration from 1 to 500 mg/L for 24 h. In order to simulate a more complex solution for batch binding studies, distilled water was replaced by low fat milk (0.1%) and the experiments were run under the same conditions. Phenol concentration was determined by a colorimetric method[17] using 4-aminoantipyrine as the chromogenic reagent. The absorbance of the samples was monitored at 500 nm[18] using a UV/Visible spectrophotometer Ultraspec 1000 from Pharmacia Biotech (Huddinge, Sweden) ($\varepsilon_{500} = 1.45 \times 10^4\ M^{-1}\ cm^{-1}$).

The initial phenol concentration in milk suspension was determined before adding the reagents. Samples were centrifuged

and only supernatant was used for analysis, and the amount of phenol was read at 500 nm ($\varepsilon_{500} = 7.2 \times 10^3\ M^{-1}\ cm^{-1}$).

Results and discussion

Characterization of particles

The goal of the study was to generate a superstructure built of smaller particles that had been aggregated in the interstitial space between ice crystals formed when the suspension was frozen. By mixing particles of different composition it was possible to form composite superstructures. A prerequisite for such a configuration to maintain its structure is that it is stabilized *via* bonds. Through covalent bonds formed directly between particles or using crosslinkers to link the particles.[19] However, the bonds could also be based on non-covalent interactions such as hydrophobic interactions used for self assembly of peptide structures.[20]

Poly *N*-isopropylacrylamide (pNIPA) is an interesting molecule with stimuli-responsive properties.[21] Cryostructurization has been used for soluble polymers and even for monomers which have polymerized in the interstitial phase. A range of different applications have been studied.[22,23] In this case nano- or microparticles of poly (NIPA-*co*-allyamine) were mixed with activated carbon. Glutaraldehyde was used as crosslinker.

Different nano- and micro- poly (NIPA-*co*-allylamine) particles were synthesized in this study, to evaluate them in the preparation and characterization of the new type of cryogel. The mean hydrodynamic diameter of the poly (NIPA-*co*-allylamine) particles is reported in Table 1. The particle size distribution for the nanoparticles was narrow while the microparticles showed a wide range of different sizes. Due to the thermosensitivity of pNIPA, increasing the temperature causes shrinking of the particles which is also reported in Table 1. Although this phenomenon was not used for this work, it is worth mentioning that pNIPA nanoparticles have faster response to external stimuli than what conventional pNIPA gels have and can be used in different applications such as carriers in drug delivery systems.[24]

It was recently reported by Kirsebom *et al.* that the sizes of particles which are used for formation of macroporous gel structures are important factors for the properties of the gel formed. The presence of smaller particles improved gel construction.[6] When preparing gels by cryostructuring particles of all three sizes of nanoparticle with different NIPA:MBAAm molar ratios it was confirmed that smaller particles with NIPA:MBAAm (10 : 1 mol/mol) improved compressibility of the gel (Table 1).

Characterization of cryostructured particle gel

There are some reports on cryostructuring of nanoparticles from carbon[25–29] or other materials[30,31] which were synthesized by the sol–gel method. Although this is a well known technique for fabrication of cryogels, it has the drawback to require a two steps drying procedure (liquid nitrogen and freeze dryer) or supercritical drying and using catalysts in the process thereby making it costly and complicated. In a new method developed in our group, the nano or micro poly (NIPA-*co*-allylamine) particles for macroporous gel formation with or without carbon can be applied within a one-step polymerization procedure at subzero temperature for the preparation of macroporous gels. It was reported earlier that traditional cryogel[22,32] made of different types of monomers by covalent crosslinking or radical polymerization, can be used in various applications.[22,33] The novel cryostructured particle gel with elastic and spongy structure can be introduced as a promising material in several areas in biotechnology.

In order to optimize the characteristics and properties of the cryostructured particle gel, different types of gel were synthesized with varying molar ratio of NIPA to MBAAm from 5 : 1 to 40 : 1, different carbon particle concentrations (0, 0.5, 1 and 5% (w/v)) and different glutaraldehyde concentration (5, 10 and 20 μL/mL suspension) (Table 1) while keeping the allylamine ratio constant.

From the physical observation of the gels, as the concentration of the carbon increases from 0.5 to 5%, the cryogel becomes more rigid. This may be due to entrapment of more carbon particles in the particle matrix and formation of thicker walls in the presence of more carbon particles (Fig. 1).

It was mentioned earlier that the poly (NIPA-*co*-allylamine) particles with NIPA:allylamine and NIPA:MBAAm (10 : 1 mol/mol) formed a better cryogel in comparison with other nanoparticles.

Table 1 Particle size of different poly(NIPA-*co*-allylamine) particles. The results reported for cryostructured particle gel with 5% (w/v) particles with 1% carbon

		Diameter (μm)		Cryogel		
Particle	NIPA:MBAAm	25 °C	40 °C	Glutaraldehyde (μL/mL)	Hardness (N)	Compressibility (N mm)
Nano particle	40 : 1	0.229	0.220	5	0.124	0.011
				10	0.092	0.017
				20	—	—
	10 : 1	0.125	0.103	5	—	—
				10	0.078	0.021
				20	0.081	0.013
	5 : 1	0.176	0.163	5	—	—
				10	0.082	0.015
				20	—	—
Micro particle	10 : 1	≤25	≤25	5	—	—
				10	0.08	0.013
				20	—	—

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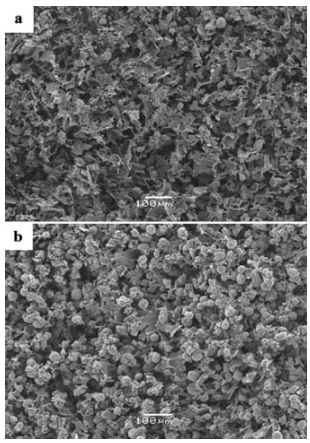

Fig. 1 SEM images of cryostructured nanoparticle gel of 5% (w/v) nanoparticles: a) 1% (w/v) carbon; b) 5% (w/v) carbon.

The mechanical stability of the cryogels was examined and is reported in Table 1. From the resulting force-time curve mechanical parameters such as hardness and compressibility may be derived.[34] Mechanical stability can play a major role in industrial and academic applications of gels made from different sources. Therefore, one goal of this study was to build elastic cryostructured particle gels which will have better pore accessibility than what is found in conventional composite cryogels. In order to achieve this goal, the amount of glutaraldehyde as a crosslinking agent in macroporous gel preparation was chosen based on the mechanical stability test of the gels. The mechanism for formation of cryostructured particle gels can be explained in a similar way to that for production of polyacrylamide cryogel. When the fixation of the suspension is set to proceed at sub-zero temperature, particles were forced into and packed in the unfrozen phase where they link and glue chemically to each other with a crosslinker.

Fig. 2 shows SEM images of cryostructured particle gels and cryogel. Carbon, as an adsorbent material, was entrapped in the network of polymers or integrated in the cryostructured particle gel during the fixation at sub-zero temperature. Fig. 2a presents SEM image of the composite cryogel without carbon particles. Fig. 2b and 2c demonstrate the carbon cryostructured particle gel with poly (NIPA-*co*-allylamine) nano and micro particles, respectively. As mentioned earlier the cryostructured microparticle gel is composed of particles of different sizes (Fig. 2c), smaller particles act as a connection between larger ones and help to attach and fix them in the matrix. The SEM images show that the preparation of particle-based macroporous gels can be made from a wide range of particle sizes. Fig. 2d is showing the pore

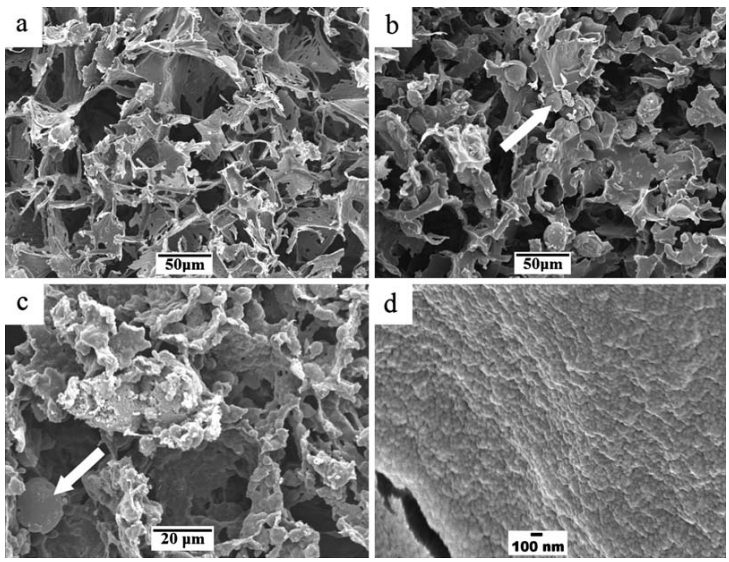

Fig. 2 a) PolyNIPA plain cryogel; b) cryostructured nanoparticle gel with 5% (w/v) NIPA:MBAAm (10 : 1) nanoparticles + 1% (w/v) carbon particles; c) cryostructured microparticle gel with 5% (w/v) NIPA:MBAAm (10 : 1) microparticles + 1% (w/v) carbon particles; d) poly (NIPA-*co*-allylamine) nanoparticles wall in the cryostructured nanoparticle gel which is a magnification of a wall structure section of image (b). The white arrows show the carbon particles trapped on the wall of the gels.

wall structure of cryostructured nanoparticle gel at high magnification of Fig. 2b, which shows how densely the nanoparticles attached to each other in a polymeric network. Even when using carbon powder in the cryogel matrix, macroporous structure can be seen in the SEM images. Due to a highly porous structure and sufficiently large pore size of cryostructured particle gel, the non-hindered diffusion of all solutes including macromolecules are expected to and from the gels which makes them interesting for separation processes.

Porosity of embedded carbon particles

The porosity, pore size distribution and surface area for pores of diameters more than 10 nm of a porous rigid material in dry state are usually determined by mercury intrusion porosimetry.[35–37] The sample must be dried since mercury cannot intrude into the sample when pores are filled with another liquid.[38] During analysis, high pressure is applied to force mercury into the small pores which may compress the sample and damage the pores.[39,40] In addition, the compression of sample causes temperature rise[41] which might affect the pNIPA particles. However, these disturbances can be eliminated with the use of hydraulic oil as a medium for transferring pressure.[42] However, no damage or sample compression of carbon black particles has been reported.[43] In this study, mercury porosimetry was used for calculation of the pores structure in the carbon particles before and after entrapment of them in the particle network.

Mercury porosimetry measurement indicates that there are pores with diameters above 10 μm both in the composite cryogel and in the cryostructured particle gel (Fig. 3).

Although one cannot refer to this result quantitatively due to the mentioned reason of damaging and deforming the sample during analysis, it is however clear that these macro-pores belong to the gel structure and their presence is a prerequisite for efficient mass transfer and good flow-through properties.

Pores with sizes in a range of 100 nm (Fig. 3) represent the pore size distribution inside the carbon particles. Cryogels prepared by the conventional way might show more mechanical stability[44,45] in comparison to that of cryostructured particle gels, but the polymer chains in the cryogel also cover and block the inner porosity of activated carbon since the pores might be filled with monomers which polymerized during the gel formation procedure (Fig. 3). This is a good indicator to show that with the newly developed gel preparation protocol, the surface area of the trapped particles and adsorbents with nano- and microporosity structure in the macroporous gel will remain available while the conventional composite cryogel does not provide this feature. Fig. 3 shows that even in the presence of low concentration of carbon particles in the cryostructured particle gel the porosity of the activated carbon is apparent.

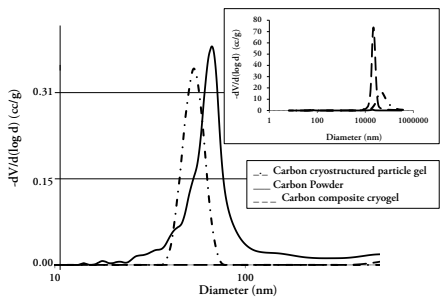

Fig. 3 a) A mercury pore size distribution for carbon particles (0.05 g), cryostructured nanoparticle gel 5% (w/v) nanoparticle + 1% (w/v) carbon and composite cryogel (5% total monomer concentration + 0.5% (w/v) carbon).

During cryopolymerization, the monomers can penetrate into the nano-pores because of their small size and high diffusivity and become polymerized in the capillary channels inside the carbon particle since these channels will act as non-frozen microdomains during the cryopolymerization. The formed polymer will thus reduce the porosity. Cryostructured particle gel, on the other hand, not only can give a macroporous gel-structure but also maintain the carbon porosity as can be expected since the particles which are aggregated have a larger diameter as compared to those of the carbon pores and can therefore not move into the pores.

The cryostructured particle gel has an even distribution of structured particles after preparation, but the composite cryogel has a heterogeneous distribution of carbon (Fig. 4).

The viscosity of the monomer mixture is close to that of water and since carbon particles have higher density, they sediment faster, before the mixture has time to freeze. On the other hand, the particle suspension to be aggregated is more viscous than water and it does not allow the carbon to sediment so fast.

Phenol removal

The Langmuir adsorption isotherm theory has found successful applications for many real sorption processes and it can be used to explain the sorption of small organic molecules such as phenol into carbon. The Langmuir isotherm was applied for adsorption equilibrium,[46]

$$\frac{C_e}{q_e} = \frac{1}{q_{\max} k_l} + \frac{C_e}{q_{\max}} \quad (1)$$

where C_e is the equilibrium concentration (mg L^{-1}), q_e is the amount of phenol adsorbed at equilibrium (mg g^{-1}) and $q_{\max}$ and k_l are Langmuir constants related to adsorption capacity and

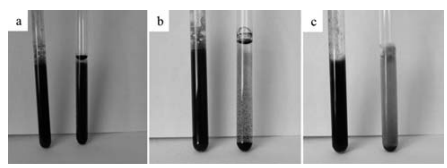

Fig. 4 Photos of two different types of mixture and gel a) immediately after adding carbon particles and intense mixing, b) after one minute relaxation at room temperature, c) after stirring with glutaraldehyde and freezing the tubes at −12 °C. In all the photos the left tube is cryostructured nanoparticle gel (5% (w/v) poly (NIPA-*co*-allylamine nanoparticles) + 1% (w/v) carbon) and the right tube represent composite cryogel (5% total monomer concentration + 1% (w/v) carbon).

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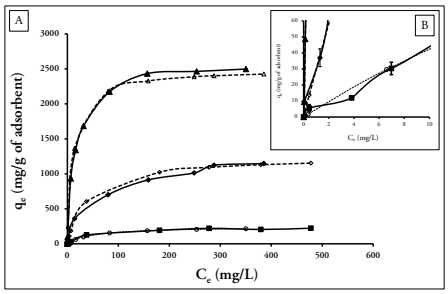

Fig. 5 A) Adsorption of phenol solution in 50 mL of different phenol solution concentrations, 22 °C for 24 h. (—♦—: carbon cryostructured particle gel; —■—: carbon composite cryogel; and —▲—: 5 mg carbon particle. Fitting the Langmuir equation with data of …△…: carbon particle; …◊…: carbon cryostructured particle gel and …□…: carbon composite cryogel). B) The insert shows the magnification of 0 to 10 mg/L in A.

energy of adsorption, respectively. The linear plot of C_e/q_e versus C_e shows that the adsorption follows Langmuir isotherm model.

The binding performance of adsorbents for carbon cryostructured particle gel, carbon composite cryogel and carbon particles was investigated. Their binding isotherms were determined in the 1–500 mg/L range of initial concentration of phenol solution (Fig. 5).

Clearly, the plain carbon particle has better adsorption than the carbon trapped in the cryogels. This difference between the isotherms can be explained by phenol diffusion into the cryogel. In the case of suspended carbon particles the diameter of the particles are in the range 45–63 μm and when the carbon particles are embedded in a gel matrix the sample is rod shaped 7 × 10 mm in size. This significant difference in size which contributes to slower diffusion and lower accessibility for the phenol to adsorb, however the cryostructured gel and the composite cryogel are of same size and thus comparable. Therefore all the carbon particles do not participate in adsorption sufficiently. Instead, the batch binding experiment with carbon was more successful since all the particles had equal access to the phenol solution. There is an obvious difference in adsorption isotherms between the cryostructured particle gel and a carbon composite cryogel in a wide range of phenol concentration (Fig. 5 and Table 2). This was interpreted as if the traditional cryopolymerization protocol for cryogel production is not suitable for this purpose because of the blocked carbon pores.

The batch binding adsorption isotherm experiment was run under same conditions but with phenol dissolved in a complex medium, low fat milk (0.1% fat). Fig. 6 shows the isotherm adsorption for phenol in milk.

Table 2 summarizes the calculation of the maximum amount adsorbed by each adsorbent in milk and water solution. The capacity of both carbon and cryogel decreased noticeably, when the experiment was run with phenol dissolved in milk since the other components in milk can be taken up by the carbon and thus compete with phenol molecules. Even in a complex medium like milk, the cryostructured particle gel shows higher capacity for phenol adsorption than what the composite cryogel does.

The carbon particle curve can be divided into two sections; the first and very sharp slope adsorbing phenol takes less than 15 min and the second part follows at a lower speed till complete saturation. The total time for saturation for carbon powder to adsorb phenol was around 120 min. This difference between the two parts can be explained by having different nano- and micropores in the carbon particles. The first and fast part belongs to the microporous tunnels where mass transfer and diffusion

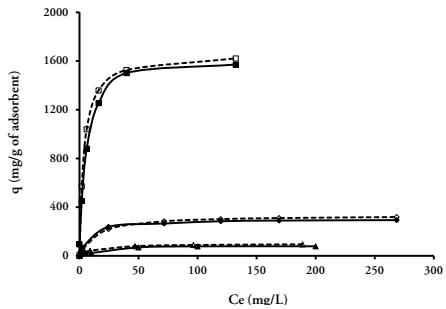

Fig. 6 Adsorption of phenol at 22 °C for 24 h from 50 mL low fat milk supplemented with different phenol concentration. (—♦—: carbon cryostructured particle gel; —▲—: carbon composite cryogel; and —■—: 5 mg carbon particle. Fitting the Langmuir equation with data of …□…: carbon particle; …◊…: carbon cryostructured particle gel and …△…: carbon composite cryogel).

Table 2 The isotherm parameters for phenol adsorption on carbon powder, carbon cryostructured nanoparticle gel and carbon composite cryogel in water solution and milk

	Langmuir isotherm					
	Phenol in water solution			Phenol in milk		
Adsorbent	q_{max} (mg/g adsorbent[a])	K_1 (L/mg)	R^2	q_{max} (mg/g adsorbent)	K_1 (L/mg)	R^2
Carbon particle	2500	0.085	0.99	1666	0.18	0.99
Cryostructured nanoparticle gel	1250	0.025	0.98	322	0.08	0.99
Composite cryogel	244	0.021	0.98	83	0.096	0.99

[a] Adsorbed phenol per gram of carbon.

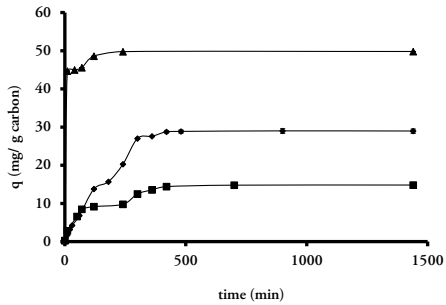

Fig. 7 Adsorption kinetics of phenol. Sorption conditions: 5 mg/L phenol solution (50 mL). (—♦—: carbon cryostructured particle gel; —■—: carbon composite cryogel; and —▲—: 5 mg carbon particle).

coefficient of phenol molecule are higher than in the nanoporous channels. Unlike for carbon powder, the adsorption by the carbon inside the gel was slow due to the mass transfer restrictions of phenol from the bulk solution into the macroporous gel.

The kinetics of phenol adsorption on macroporous cryogel system for a known weight of carbon is depicted in Fig. 7.

The saturation time for the cryostructured nanoparticle gel was approximately 4 times longer than that for the carbon particles. In comparison, the carbon composite cryogel has shown lower capacity than the cryostructured nanoparticle gel which was expected from the mercury porosimetry results. Time consumption can be considered as an important factor in the batch binding experiments. However, working with free carbon particles is not as easy as with carbon trapped within a matrix. This is one the advantages of using cryostructured particle gels. By using the composite cryogel, time of saturation as well as the adsorption capacity was changed and decreased. As was mentioned earlier, by modifying the cryo-structuration of nano- or micro-particles, it is possible to avoid to seriously affect the pores of the carbon.

Conclusion

When forming composites with adsorbent particles introduced into a larger structure, it is important to try to retain as much of the adsorption capacity as possible. Since much of the capacity is ascribed to the pores of many adsorbents it is important that the formation of the superstructure does not involve steps where reagents are blocking the pore mouths or even filling the pores. One way to address this is to avoid using reagents that have the ability to markedly influence the porous structure of the adsorbent. The cryo-structuring of particles is a procedure that fulfils all these demands and as is seen in the report this method yields superstructures with well retained adsorption capacities.

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Paper II

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Research Article

Composite cryogel with immobilized concanavalin A for affinity chromatography of glycoproteins

Composite cryogels containing porous adsorbent particles were prepared under cryogelation conditions. The composites with immobilized concanavalin A (Con A) were used for capturing glycoproteins. Adsorbent particles were introduced into the structure in order to improve the capacity and to facilitate the handling of the particles. The monolithic composite cryogels were produced from suspensions of polyvinyl alcohol particles and porous adsorbent particles and cross-linked under acidic conditions at sub-zero temperature. The cryogels were epoxy activated and Con A was immobilized as an affinity ligand. Binding and elution of horseradish peroxidase (HRP) was studied in batch experiment and in a chromatographic setup. Increasing adsorbent concentration in composite cryogels will increase ligand density, which therefore enhances the amount of bound HRP from 0.98 till 2.9 (milligram enzyme per milliliter of gel) in the chromatographic system. The material was evaluated in 10 cycles for binding and elution of HRP.

Keywords: Adsorbent polymer / Affinity chromatography / Composite / Con A / Cryogel / Glycoprotein
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1 Introduction

A trend in downstream processing is toward the reduction of unit operations when purifying biomolecules. Then it is attractive to combine capturing of the target molecule with concomitant removal of particulate matter. Expanded bed chromatography [1] as well as utilization of superporous adsorbents, e.g. cryogels [2], are two initiatives in that direction.

Cryogels are three-dimensional polymer networks formed from monomer/polymer solutions at sub-zero temperatures both physically [3] and chemically cross-linked [4]. The highlight properties of cryogels are their macroporous structure (pore sizes up to 100 μm) and interconnected channel system that allows efficient mass transfer and diffusion of solutes in the gel. Cryogels are produced by freezing a solution of monomers or polymers, and during freezing exclusion of all dissolved/suspended materials takes place, a gel is formed in the interstitial spaces between the ice crystals. After thawing, a network of interconnected pores remains, where ice was present earlier. Easy production and functionalization of the gels are two reasons why applications in bioseparation and purification seem attractive [5, 6]. There are other methods and techniques reported in the literature, such as sol-gel and freeze-drying, which are used in order to form cryogels from solutions or suspensions [7–10]. These latter methods are either expensive or require special equipments. It was reported recently that cryogels can also be produced from a suspension of particles at sub-zero temperature within one step polymerization/cross-linking [11, 12]. This method not only has advantages over other complicated techniques regarding simplicity of preparation but also provides more available surface area than cryogels synthesized from solutions [13].

In the present study, composite cryogels were prepared from suspensions of polyvinyl alcohol (PVA) particles combined with rigid adsorbent polymer beads. PVA, known as a nontoxic and biocompatible polymer [14], was used to form a polymeric network and Con A was covalently immobilized on the matrix. Horseradish peroxidase (HRP) was chosen as a target molecule for these model studies. This enzyme was selected since it is a well-known glycoprotein that is simple to follow both by its enzymatic activity and also by the absorbance of the porphyrin ring [15]. The focus of this work was on the characterization and modification of the PVA cryogel and evaluation of its capacity to capture high molecular weight biomolecules through affinity interactions in both batch and continuous systems.

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Abbreviations: HRP, horseradish peroxidase; **PVA**, polyvinyl alcohol; **PA**, porous adsorbent polymer beads

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2 Material and methods

2.1 Materials

Glutaraldehyde (50%), HRP, Con A, phenol, 4-aminoantipyrine, hydrochloric acid (37%), sodium borohydride, sodium chloride, potassium phosphate, potassium hydrogen phosphate, and kit for protein assay (bicinchoninic acid assay) were purchased from Sigma-Aldrich (Steinheim, Germany). Acetone, hydrogen peroxide solution (30 wt%), calcium chloride, magnesium chloride were supplied by Merck (Darmstadt, Germany). Epichlorohydrin and sodium carbonate were from Fluka (Steinheim, Germany). Sodium hydroxide and paraffin oil were purchased from Honeywell (Seelze, Germany) and VWR International (Fontenay-Sous-Bois, France), respectively. PVA, Mowiol (4–98), with molecular weight ~27 000 g/mol was from Clariant GmbH (Frankfurt, Germany). Porous adsorbent polymer beads (PA) from divinylbenzene-*co*-vinylimidazole, surface modified with polyetherdiamine, having an average particle diameter of 11 μm, a specific surface of 442 m^2/g, and containing 0.26 mmol/g of free amino groups were kindly provided by Polymerics (Berlin, Germany).

2.2 PVA particles preparation

PVA particles were synthesized as described elsewhere with some modifications [16]. Briefly, 0.5 g Mowiol (4–98) was dissolved in 5 mL distilled water for 30 min at 80°C. After cooling to room temperature, the PVA solution was added drop wise to 200 mL of paraffin oil while stirring at 8000 rpm with a homogenizer (IKA-WERKE, Staufen, Germany). The water in oil emulsion was stirred for five more minutes and then frozen for 24 h at –20°C. The emulsion was then allowed to thaw for 6 h at room temperature. This freezing-thawing cycle was repeated four times. The suspension was centrifuged for 10 min at 15 000 × *g* to separate the PVA particles from the oil. The particles were washed several times with acetone to remove the residual oil and as a final wash with distilled water. They were dried by freeze-drying and kept in a dry place at room temperature.

2.3 Preparation of PVA cryogels

A suspension 3% (w/v), corresponding to 0.03 g/mL, of PVA particles was prepared by dispersing dried PVA particles in distilled water and the pH of the suspension was adjusted to 1 using 5 M hydrochloric acid. The suspension was kept on ice for 30 min while stirring. Different concentrations of PA adsorbents (1, 2, and 3% (w/v)) were added to the suspension. Glutaraldehyde (5 μL/mL) was added as a cross-linker to the well-mixed suspension. The PVA-PA suspension (1 mL) was poured into 2-mL plastic syringes (I.D. 9 mm) and kept at –12°C overnight. The frozen samples were defrosted at room temperature and rinsed with distilled water to remove any nonreacted chemicals. Plain PVA cryogels were prepared as mentioned above under the same conditions but without adding PA adsorbents. For all the experiments, the gels that were used had a rod shape (approximately 8 mm diameter and 13 mm height) and approximately 1 mL volume. There is a significant shrinking of the cryogel when using chemical cross-linking polymerization that can be explained by interpolymerization inside the gel [17].

2.4 Coupling of epoxy groups on PVA cryogels

Before modifying the gels with epoxy groups, they were washed with 0.1 M sodium borohydride in carbonate buffer (pH 9) in order to reduce aldehyde groups. Then they were washed with plenty of water and sodium hydroxide (0.1 M). Epichlorohydrin (2% v/v) was added to 0.5 M sodium hydroxide and stirred to obtain a well-mixed emulsion. Gels were added to the emulsion and kept stirring for 48 h at room temperature. In order to not destroy the gels during the modification step, they were placed inside a porous nylon support that was immersed in the reaction mixture. The modified gels were then washed with distilled water until the used water became neutral (tested with pH indicator papers) [18].

2.5 Immobilization of Con A on epoxy-modified PVA cryogels

The immobilization of Con A on epoxy modified gels was described elsewhere [19]. Briefly, a Con A solution (4 mg/mL) was prepared by dissolving the protein in 50 mM sodium carbonate buffer pH 9, containing 1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Gels were incubated in the Con A solution on a rocking table for 24 h at room temperature. Ethanolamine (0.1 M) in the same buffer was used in order to block the nonreacted epoxy groups. The amount of unbound Con A washed out from the gels was measured by bicinchoninic acid assay [20] using a plate reader, ASYA, Biochrom (Cambridge, England) and then subtracted from the initial concentration in order to evaluate the amount of immobilized Con A in the gels.

2.6 Affinity capture of HRP on Con A-modified PVA cryogels in batch system

HRP (100 μg/mL) was prepared by dissolving the enzyme in 0.1 M potassium phosphate buffer pH 7.5 containing 0.1 M NaCl. Con A-modified gels, prewashed with phosphate buffer, were incubated in 5 mL of HRP solution overnight under gentle stirring at 6–7°C. The gels were washed with buffer and the amount of enzyme bound on the gels was calculated by subtracting washed out enzyme from the initial amount of enzyme added. Unbound enzyme was measured by using a spectrophotometer, BioWave II (Cambridge, England) [21] at 404 nm.

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The activity of the bound enzyme on the gel was also monitored [22, http://www.worthington-biochem.com/HPO/assay.html]. The gel was placed in substrate solution (4-aminoantipyrine-phenol) and the absorbance at 510 nm was read every 60 s for 4 min. The gel and substrate were shaken between each step to improve mass transfer inside the gel.

2.7 Binding and elution of HRP in a chromatographic test

Binding tests of HRP were carried out in a chromatographic column (I.D. 6.6 mm) with flow adaptors at both ends. All solutions were pumped through the column using a peristaltic pump (Alitea, Sweden) with a flow rate of 1 mL/min. Gels with immobilized Con A were placed inside the column and the flow adaptors were adjusted so that there was no empty space on the top and bottom of the gel. The gels were washed with 0.2 M phosphate buffer pH 7.5 containing 0.1 M NaCl until a stable baseline was obtained. A solution of HRP (100 μg/mL, 8 mL) was pumped through the column in order to study the breakthrough curve. The dynamic binding capacity (DBC) of the modified gels was calculated at 10% HRP breakthrough. The absorbance of the eluate was monitored continuously at 404 nm.

The gels were then washed with the 0.2 M phosphate buffer and HRP was eluted from the column by using 1 M glucose solution in the buffer. Regeneration of the affinity column was done by passing 0.2 M phosphate buffer pH 7.5 containing 0.1 M NaCl and 0.2 M carbonate buffer pH 9 through the gels.

2.8 Characterization and properties of particles and gels

The mean size and particle size distribution of PVA particles were measured at 25°C using a Nano-ZS 3600 particle sizer (Malvern Instruments, Worcestershire, UK).

SEM was carried out for evaluation of the structure of the macroporous gels. The samples were cut into thin discs (1–2 mm) and gradually dehydrated in different concentrations of ethanol solutions (25, 50 75, 95, and 99%) and then critical-point dried. The dried samples were coated with gold/palladium (40:60) and studied using JEOL JSM-80 5600 LV and JEOL JSM-6700F scanning electron microscopes (Tokyo, Japan) for low and high magnifications, respectively.

The mechanical stability and elasticity of the gels with or without adsorbent particles was analyzed using a texture analyzer (XT2i, Stable Micro Systems, Godalming, England) by using a 2 kg load cell and a cylindrical probe (25 mm diameter). The gels were placed on a metal plate from the bottom side and a load was transmitted vertically to the gel. The test speed was 0.5 mm/s. All the mechanical measurements were conducted at room temperature. The samples were compressed up to 50% of the total height. The applied force was recorded and calculated by the Exponent, v.5.0.9.0 software (Stable Micro Systems, Cambridge, England). Each sample was analyzed twice to compare properties with regard to mechanical strength and elasticity modulus.

Water flow resistance was determined as described by Plieva et al. [23]. Briefly, gels were inserted into a glass column (id 7 mm). The water flow through the gels was measured while a constant hydrostatic pressure equal to 1 m of water column was maintained for the setup. Data presented here are averages of triplicate measurements.

Table 1. Particle size of PVA particles under different conditions

Conditions	PVA particle size (nm)
Directly after formation	400 ± 50
Freeze–thawing cycle at pH 1	420 ± 25
Freeze–thawing cycle at pH 7	425 ± 10
Glutaraldehyde solution (5 μL/mL) at RT	380 ± 40

3 Result and discussion

3.1 Characteristics of PVA cryogel

The PVA particles produced for this study were synthesized through a freeze–thawing cycle method in order to minimize usage of chemical cross-linkers [24]. The average size of the PVA particles was determined to 400 ± 50 nm by particle size analyzer at room temperature in aqueous suspension (Table 1, Supporting Information Fig. S1).

The PVA suspension was subjected to sub-zero temperature with and without adjusting the pH to 1 in absence of any cross-linking agent for several freeze-thawing cycles. No gel was formed under these conditions and the size of the PVA particles was analyzed again after thawing in order to evaluate effects of low pH and freezing on the particle size (Table 1). The effect of cross-linking agent on the PVA suspension was evaluated by adding glutaraldehyde (5 μL/mL) to a dilute suspension of PVA particles and kept overnight at room temperature. The suspension was dialyzed against water to remove the cross-linker and then analyzed (Table 1). The results demonstrate that neither acidic condition combined with freezing nor the presence of cross-linker affect the size distribution of PVA particles.

To the best of our knowledge, there is no report available in the literature on the formation of physically cross-linked cryogels from suspensions of PVA particles. However, cryogel formation through freeze-thawing cycles of a PVA solution is well known [25–29]. Cryogels were synthesized by chemical cross-linking of PVA particles for this study. Physical cross-linking for preparing cryogel from PVA suspension was not appropriate due to the mechanism of cryogel formation from PVA solution. The result of the cryotropic gelation process through freeze-thawing cycles of a PVA solution is the formation of a porous structure with a highly interconnected network consisting of crystalline regions of PVA

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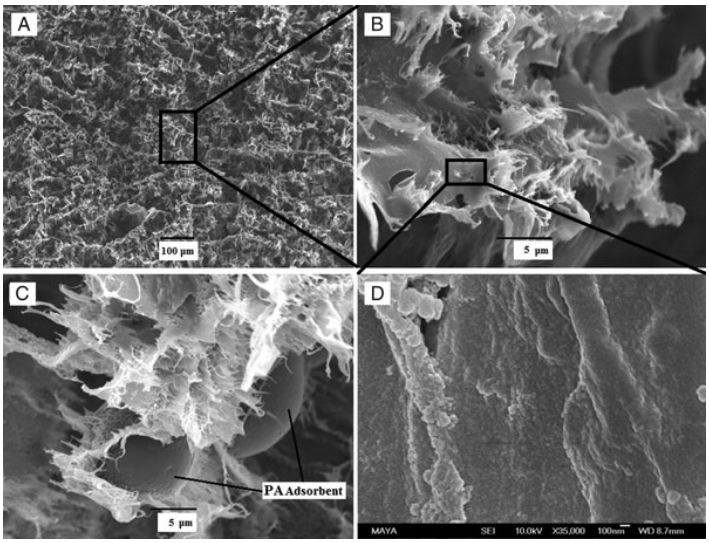

Figure 1. SEM images of 3% (w/v) PVA cryogel. (A) Dehydrated with ethanol and critical point dried gel; (B) high magnification of image (A); (C) freeze-dried gel; (D) high resolution of image (B)

chains. These chains act as a glue in the presence of water and the end result will be a physically cross-linked PVA gel [30]. The size of the crystalline regions in PVA gels as well as the number of freeze-thawing cycles has important influence on the physical properties of the gels and affects stability and their tolerance to stresses [30, 31]. Therefore, PVA particles produced by freeze-thawing techniques already form stable particles and dispersing them in suspension will not change their structure due to their mechanical stability and physical properties [32]. In other words, there are no free PVA chains in a PVA particle suspension that could form crystalline regions of PVA and connect the particles together and form a three-dimensional network, even through freeze-thawing cycles. Therefore, a cross-linking agent, glutaraldehyde, was used to form a cryogel from the suspension of PVA particles.

The porous structure of the gel is shown in Fig. 1A where the pores sizes have a broad distribution range (from few micrometers up to 100 μm). At higher resolution, the structure of the wall can be studied (Fig. 1B and C). The uneven and rough surface of the wall can be observed at high magnification (Fig. 1D) and shows how cross-linked PVA particles attached tightly in dry form.

The morphology and structure of the pores as well as the wall surface of the gel can be studied on SEM images. The size of a dried PVA particle on SEM image (Fig. 1D) is smaller than the value obtained in the size distribution analysis carried out on particles in wet form. This observation can be due to different factors such as particle compression during the cryogelation process and pretreatment of the cryogels by gradual dehydration with ethanol and then critical point drying, which influenced the particle size. It was mentioned earlier that low pH at frozen condition does not affect particle size distribution (Table 1).

In order to evaluate effects of ethanol and critical point drying of the particles, the gels were freeze dried instead as a pretreatment for SEM. Any differences in the structure of gels might then be evaluated by comparing images. As it appeared on the SEM images (Fig. 1B and 1C) the gels have very similar structure. It was reported that PVA cryogel formed by freeze-thawing has a porous structure [32]. Removing water and drying the gel completely can affect the wall surface and PVA particles size particularly. In order to check the shrinking of particles when removing water completely, a PVA suspension was prepared and flash frozen with liquid nitrogen and then freeze dried. SEM image from the dried PVA particle confirmed that a reduction in particle size occurred (Supporting Information Fig. S2). PVA particle shrinkage during the drying step might be due to their porous structure. Another possibility and explanation is the effect of formation of ice crystals in the presence of a cross-linker during the gelation procedure of the PVA particles. Aggregation and chemical cross-linking of PVA particles by glutaraldehyde during the cryogelation step might result in size reduction in the final product. The formed macroporous structure was a result of the ice crystals formed during the freezing process. The cross-linking took place in the interstitial space, and the structure formed may not be allowed to return to its original particle size after defrosting.

Mechanical stability and flow properties of monoliths of PVA cryogels were studied. The plain PVA cryogel allowed higher flow through and had better mechanical stability than the gels containing PA adsorbent (Table 2). The lower

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Table 2. Flow path and elasticity modulus of 3% (w/v) PVA cryogel with and without PA adsorbents

Gel code	PA concentration (w/v%)	Flow (mL/min)	Stress at 50% strain (kPa)
G	0	3.80 ± 1.07	14.3 ± 0.42
GPA1	1	1.04 ± 0.40	4.82 ± 0.39
GPA2	2	1.06 ± 0.20	5.91 ± 0.43
GPA3	3	1.16 ± 0.50	6.83 ± 0.20

mechanical stability of the composite monolith might be explained by the presence of the adsorbent beads. The beads have an average diameter of 11 μm and in comparison with PVA particles they are 25 times larger (Fig. 1C). These beads can hamper efficient cross-linking between PVA particles during the cryogelation procedure. Elasticity modulus of the gels with PA adsorbents increases slightly by increasing the concentration of adsorbents. The hardness of PA beads can be the reason for small mechanical improvement but still it was not as good as that of the plain gel.

3.2 Immobilization of Con A on modified gels

At physiological pH, Con A has four identical active sites that can bind separately with sugar or glycoconjugates. Due to this characteristic, Con A has been used as a suitable ligand for capturing many proteins and substances [33,34]. The amount of Con A immobilized on the different gels is shown in Fig. 2. The results show that Con A content on the PVA cryogel is 9 mg/mL and increases to 25 mg/mL when PA adsorbent is introduced in the monolith. The capacity of the PVA cryogel is comparable with those of commercial matrices available in the market (Con A Sepharose 4B; GE Healthcare, Buckinghamshire, United Kingdom), which contain 10–16 mg/mL. The Con A is covalently immobilized via the epoxy

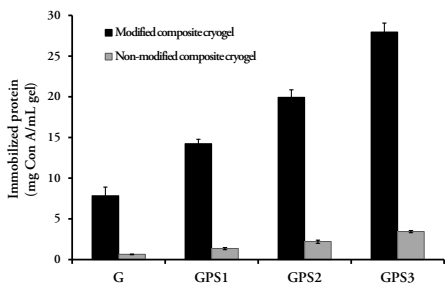

Figure 2. Comparison of the amount of immobilized Con A on the PVA cryogel with and without embedded PA particles before and after epoxy modification. G: 3% (w/v) PVA; GPA1: 3% (w/v) PVA + 1% (w/v) PA; GPA2: 3% (w/v) PVA + 2% (w/v) PA; GPA3: 3% (w/v) PVA + 3% (w/v) PA cryogel.

groups on the gel. Nonspecific interactions between the gel matrix and Con A take place to some extent. In Fig. 2, the nonspecific interactions between Con A and cryogels are illustrated with gray bars. The plain gel that is cross-linked with glutaraldehyde and has been treated with $NaBH_4$ in order to remove remaining free aldehdye groups still bound Con A nonspecifically. Nonspecific adsorption of Con A can be attributed both to the PVA and to the PA adsorbent. The capacities of the gels with or without PA adsorbents increase markedly after introduction of epoxy groups and subsequent immobilization of Con A.

Free PA particles (20 mg) were also modified with epoxy group by using ECH/NaOH emulsion and Con A was immobilized under the same conditions as mentioned earlier. The aim of using free PA particles was to compare the immobilization of Con A on free and embedded particles in the cryogels. The results of the free particles were compared with the gel (GPA2) that has an equal amount of PA as was used of the free particles. Clearly, the free PA particles have more immobilized Con A (1250 mg Con A/g PA particle) than the PA particles embedded in the gel (600 mg Con A/g PA particle). This difference between the amounts of immobilized Con A can be explained by more available and accessible epoxy groups on free particles that can react with Con A. The other reason that can affect immobilization is diffusion into the gel. The differences between particle size in suspension (11 μm) and the embedded particle in the gel (id 8 mm, height 10 mm) will significantly affect the diffusion of Con A. Free particles show more capacity to bind Con A but using them as free particles in a suspension is a challenging matter. The particles are slightly hydrophobic and due to this physical characteristic it is difficult to handle them in aqueous media in free form. Embedding the adsorbent in a cryogel will minimize the adsorbent lost during each step of the process and make them more suitable for bioseparation applications.

In this study, HRP was chosen as a target molecule as a representative of macromolecular glycoconjugates. HRP is a glycoprotein that has mannose residues within its carbohydrate chains and therefore is capable of binding to Con A. This has earlier been utilized for isolation and purification of the enzyme [35].

The size of the interconnected channels in the gel is much larger than the dimensions of the ligand and the target molecules (Con A (4.0 × 4.2 × 3.9 nm) [36] and HRP (4.0 × 4.4 × 6.2 nm) [37]), therefore the mobile phase can flow through the gel easily with efficient mass transfer thereby allowing efficient immobilization and affinity capture, respectively.

3.3 Capture of HRP

3.3.1 Batch experiment

The HRP concentration was monitored by a spectrophotometer at 404 nm ($\varepsilon_{404} = 5.4 \times 10^4\ M^{-1}\ cm^{-1}$). After incubating the gels in HRP solution and washing with 0.2 M phosphate

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Table 3. The amount and specific activity of bound HRP on different cryogels in batch systems

Gels	Batch system		
	PA (mg/mL gel)	Bound enzyme (mg HRP/mL gel)	Specific activity (Unit/mg)
G	0	0.28 ± 0.02	0.52 ± 0.03
GPA1	10	0.48 ± 0.01	0.47 ± 0.05
GPA2	20	0.72 ± 0.03	0.45 ± 0.02
GPA3	30	1.25 ± 0.05	0.35 ± 0.03

buffer containing 0.1 M NaCl to remove the nonspecifically bound enzymes, the amount of captured enzyme was calculated and the results are presented in Table 3. The amount of the HRP bound to the ligand (Con A) increases by increasing the concentration of PA adsorbent in the gel, i.e. it increases with the amount of ligand present on the support (Fig. 2).

It was also shown that captured enzyme on the gel retained its activity. The specific enzyme activity reported in Table 3 was measured by treating the gel itself with substrate solution.

The decrease in specific activity that is observed with increasing amount of bound enzyme might be attributed to diffusion limitations. Materials containing a higher amount of bound enzyme will be more affected by diffusion limitations of both the substrate and product moving in and out of the gel.

3.3.2 Affinity capture in a chromatographic system

Capture of HRP has also been evaluated in a chromatographic system. In order to determine the dynamic capacity of the gel, a total volume of 8 mL HRP solution was pumped through the monolith during the loading step. The chromatograms obtained are shown in Fig. 3. The presence of the PA adsorbent increased the capacity. It was shown that PVA cryogel with PA under the same conditions required more time (= more feed) to reach its saturation point (Fig. 3, peak a) than what the plain gel needed (Fig. 3, peak a′). This confirms the higher capacity of the PVA cryogel with adsorbent than plain PVA cryogel. The elution peaks are representing the amount of the HRP bound to the column (Table 4). By comparing the peak height and area of the elution peaks in Fig. 3 (b and b′), it is clear that the amount of bound HRP on the plain PVA cryogel with Con A was lower than that from the gel containing PA adsorbent.

In order to regenerate and reuse the gels, 50 mM carbonate buffer pH 9 was used. As it is shown in Fig. 3, two peaks appeared during the regeneration process after washing with phosphate buffer and carbonate buffer (peaks c, c′). HRP with its porphyrin group has absorbance at 404 nm while glucose does not. The additional peaks in the chromatogram can be explained by refractive index effects inside the flow cell when effluents of different compositions pass [38]. As a control, the system was operated with a full cycle of steps, except that

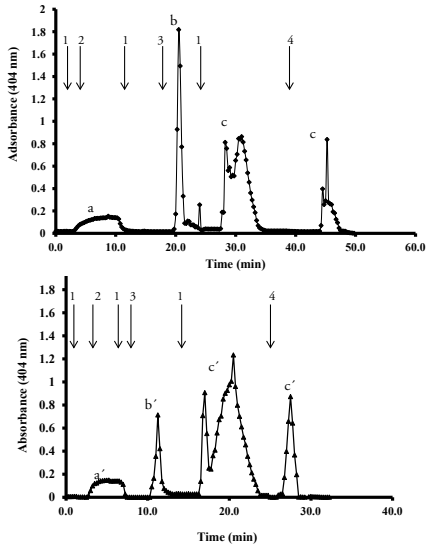

Figure 3. Chromatograms of binding/elution of HRP. -◆-: 3% (w/v) PVA cryogel + 3% (w/v) PA adsorbent; -▲-: 3% (w/v) PVA cryogel. Arrows with numbers represent the solutions during the process: (1) 0.2 M phosphate buffer pH 7.5 containing 0.1 M NaCl, (2) 100 μg/mL HRP in buffer 1, (3) 1 M glucose solution in buffer 1, (4) 0.05 M carbonate buffer pH 9. Order of peaks in chromatogram: (a, a′) Loading; (b, b′) Elution; (c, c′) Regeneration.

Table 4. The amount of bound HRP and DBC for different cryogels in affinity chromatographic system

Gels	Chromatographic system		
	PA (mg/mL gel)	Bound enzyme (mg HRP/mL gel)	Dynamic binding capacities (mg HRP/mL gel)
G	0	0.98 ± 0.004	0.09 ± 0.01
GPA1	10	1.41 ± 0.004	0.14 ± 0.03
GPA2	20	2.00 ± 0.009	0.21 ± 0.02
GPA3	30	2.91 ± 0.008	0.30 ± 0.06

no enzyme was loaded. A similar chromatogram (except for the enzyme related ones) was obtained from the control experiment, which shows that the peaks appearing during the regeneration step do not contain any peroxidase (Supporting Information Fig. S3).

In order to confirm that the peaks (c, c′) in Fig. 3 are free from peroxidase, enzyme activity as well as SDS-PAGE of the peaks were also tested. The only peaks that contain peroxidase were loading peak (a, a′) and the elution peak (b, b′). The other peaks that appear during the regeneration step have no enzyme activity and no band was observed on SDS-PAGE.

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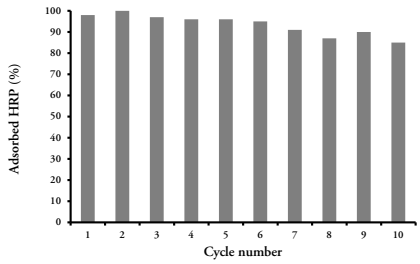

Figure 4. Percentage of bound enzyme on 3% (w/v) PVA cryogel in continuous system for 10 adsorption/regeneration cycles.

DBC of the gels was calculated when they reached to 10% of their breakthrough curves (Table 4). The calculated DBC from loading curves was in agreement with the amount of enzyme eluted from the gels.

Binding and elution on 3% (w/v) PVA cryogels were repeated in 10 continuous cycles and the amount of HRP eluted from the gels decreased 8% after six cycles (Fig. 4). The matrix begins to lose its capacity after being used six times because of decreasing activity of Con A. The regeneration process was carried out by phosphate and carbonate buffers (pH 9). The gels were washed with phosphate buffer (pH 7.5) afterwards to recondition the Con A preparation. It was reported that the Con A on affinity matrices loses its activity after five or 10 cycles and in some cases even earlier depending on the experimental conditions [39].

4 Concluding remarks

Macroporous monoliths seem to overcome a problem of conventional columns with respect to hydrodynamic properties since they have a lower mass transfer resistance and lower pressure drop. On the other hand, their capacity is not comparable with conventional columns due to smaller surface area. Embedded porous adsorbents for selective adsorption of target molecules in monolithic matrices are one way to improve and develop their capacities. It was shown in this work that composite cryogels containing porous adsorbents can be produced within one step polymerization to obtain an adsorbent with high ligand density. Therefore they can be a good candidate as affinity chromatography matrices for bioseparation applications.

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The authors have declared no conflict of interest.

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Supplementary information

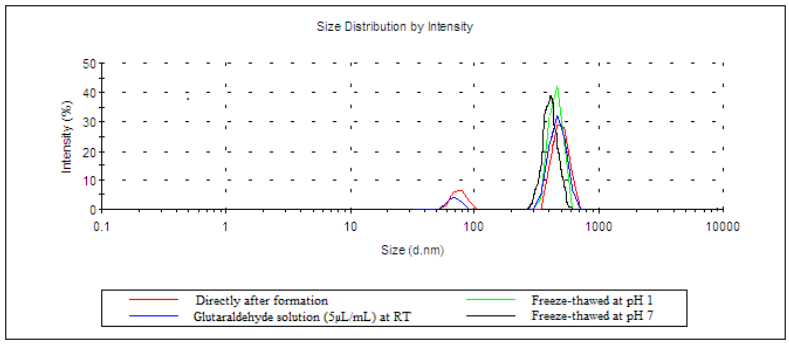

Figure S. 1. Size distribution of PVA particles in aqueous solution.

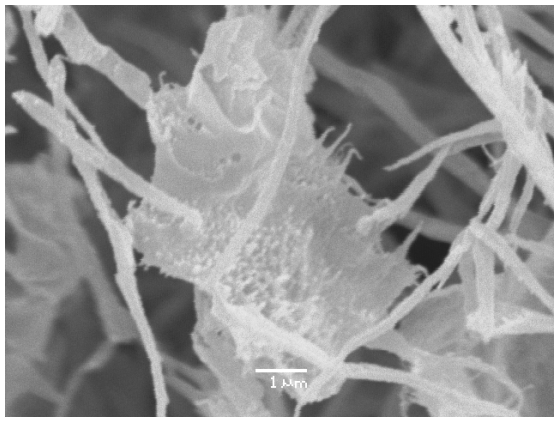

Figure S. 2. SEM image of dried particles PVA in absence of crosslinker.

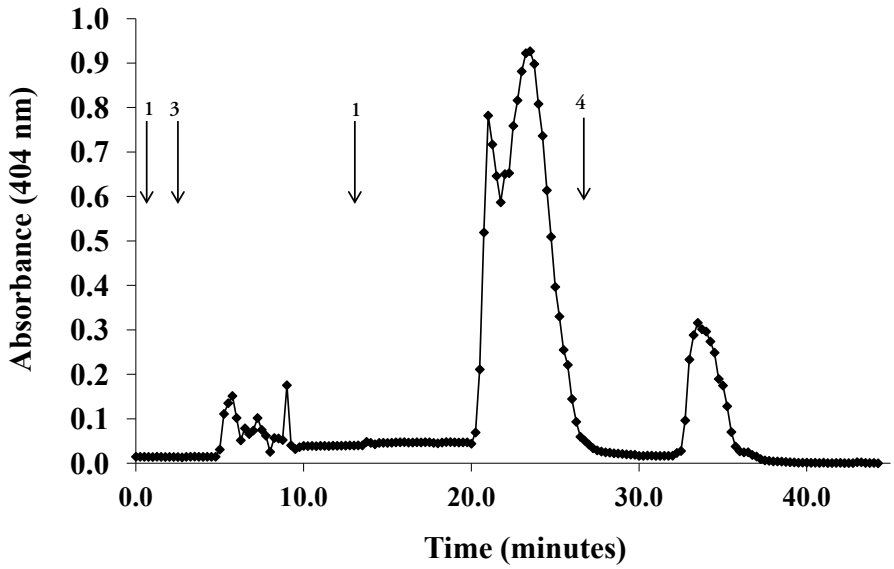

Figure S. 3. Chromatograms of the control test on 3% (w/v) PVA cryogel without loading peroxidase. Arrows: 1) 0.2 M phosphate buffer pH 7.5 containing 0.1 M NaCl, 3) 1M glucose solution in buffer 1, 4) 0.05 M carbonate buffer pH 9.

Paper III

Cryogelation of Molecularly Imprinted Nanoparticles: A Macroporous Structure as Affinity Chromatography Column for Removal of β-Blockers from Complex Samples

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Abstract
In this work, a new macroporous molecularly imprinted cryogel (MIP composite cryogel) was synthesized by glutaraldehyde cross-linking reaction of poly(vinyl alcohol) particles and amino-modified molecularly imprinted core-shell nanoparticles. The MIP core-shell nanoparticles were prepared using propranolol as a template by one-pot precipitation polymerization with sequential monomer addition. The characteristics of the MIP composite cryogel were studied by SEM and texture analyzer. The macroporous structure of the composite (with the pore size varying from a few micrometers to 100 μm) enabled high mass transfer of particulate-containing fluids. In a solid phase extraction (SPE) process, the efficiency and selectivity of the MIP composite cryogel was investigated, where the cryogel was used as an affinity matrix to remove propranolol from aqueous solution as well as from complex plasma sample without prior protein precipitation. The MIP composite cryogel maintained high selectivity and stability and could be used repeatedly after regeneration.

Keywords: molecularly imprinted polymer, composite cryogel, affinity chromatography, complex media

1. Introduction

Presence of trace concentrations of drugs and biological compounds causes contamination in the environment. The application of highly selective adsorbents, molecularly imprinted polymer (MIP) for capturing different organic [1,2] and inorganic [3,4] chemicals has increased in recent years [5]. Molecular imprinting is a well known technique for the preparation of artificial synthetic materials with specific binding sites for target compound. This technique involves the use of suitable functional monomer and a template to form complementary covalent [6] or non-covalent [7] interactions in a pre-polymerization solution. After the cross-linking polymerization is finished, the template can be removed to leave behind a “memory site” in the polymer, which has a size, shape and three-dimensional structure defined by the template. Molecularly imprinted polymers have been used in different biotechnology areas such as bioanalysis [8], chromatographic separation and solid phase extraction [9-11], food analysis [12], and environmental analysis [13,14].

Composite porous monoliths have major applications in solid phase extraction, chromatography systems and pre-concentration in analytical sample preparation due to their unique capabilities to minimize the back pressure and to increase binding capacities [15]. Composite cryogels are classified as porous polymeric monolith which is synthesized under semi-frozen conditions, where a solution/suspension mixture is polymerized around ice crystals. The result of this cryogelation polymerization is a three dimensional polymeric network with continuous interconnected pores. The pores are up to 100 μm wide which can facilitate mass transfer and provide good flow-through properties even for particulate-containing fluids [16]. Recently, cryogel composite containing embedded MIP microspheres has been developed to remove 17 β-estradiol, 4-nonylphenol and atrazine from water [17,18]. In these studies, the MIP adsorbents were physically entrapped inside the polymeric network.

The purpose of this work was to develop a new method to construct MIP-based macroporous cryogel by chemically cross-linking MIP nanoparticles, and to achieve simplified SPE for a model therapeutic compound from complex samples. It remains challenging to develop practical technique/material which can offer simple but effective removal of low concentration chemicals. Although MIP nanoparticles have shown great potential in this respect, handling the free adsorbents for adsorption/desorption experiments are not easy, especially for the most commonly used hydrophobic particles. In this work, we introduced amino functional groups on the surface of propranolol-imprinted nanoparticles, and used PVA/glutarladehyde to cross-link the MIP nanoparticles under cryogelation conditions to achieve macroporous composite cryogel. The cross-linked MIP particles form stable composite cryogel and reduce the leakage of nanoparticles, allowing the structure to be used as stable affinity column to realize efficient separation of the propranolol from complex samples.

2. Materials and method

2.1. Materials

Polyvinyl alcohol (PVA), Mowiol (4-98), with molecular weight ~27,000 was from Clariant GmbH (Frankfurt, Germany). Glutaraldehyde (50%), allylamine (98%), bovine plasma and hydrochloric acid were purchased

from Sigma-Aldrich (Steinheim, Germany). Trimethylolpropane trimethacrylate (TRIM, technical grade), atenolol (≥98%) metoprolol (+)-tartrate salt (97%), timolol maleate salt (98%) and pindolol (98%) were purchased from Sigma-Aldrich (Dorset, UK). Acetone, acetic acid (glacial, 100%), acetonitrile (99.7%) and azobisisobutyronitrile (AIBN, 98%) were purchased from Merck (Darmstadt, Germany) and re-crystallized from methanol before use. Methacrylic acid (MAA, 98.5%) was purchased from ACROS (Geel, Belgium). *N*-isopropylacrylamide (NIPA) and *N,N'*-methylene-bis-acrylamide (MBAAm) were purchased from Monomer-Polymer Laboratories (Windham, USA) and ICN Biomedicals Inc. (Warrendale, USA). Acrylamide, (R,S)-propranolol hydrochloride (99%) and (S)-propranolol hydrochloride (99%) were supplied by Fluka (Dorset, UK). (R,S)-propranolol hydrochloride and (S)-propranolol hydrochloride were converted into free base form before use. (S)-[4-^{3}H]-Propranolol (specific activity 555 GBq mmol^{-1}, 66.7μM solution in ethanol) was purchased from NEN Life Science Products Inc. (Boston, USA). Scintillation liquid, Ecoscint A was from National Diagnostics (Atlanta, USA). Paraffin oil was supplied from VWR International (Fontenay-Sous-Bois, France).

2.2. MIP preparation

Propranolol imprinted core nanoparticles were synthesized following a procedure described by Yoshimatsu *et al.* [19]. Briefly, the template molecule, (R,S)-propranolol (137 mg, 0.53 mmol) was dissolved in 40 mL of acetonitrile in a (150 × 25 mm) borosilicate glass tube equipped with a screw cap. MAA (113 mg, 1.31 mmol), TRIM (648 mg, 2.02 mmol) and AIBN (28 mg) were then added to the solution under a gentle flow of nitrogen for 5 min and then sealed. Polymerization was carried out in a Stovall HO-10 Hybridization Oven (Greensboro, NC, USA), and rotated at a speed of 20 rpm, at 60 °C for 24 h (note: the glass reactor was introduced into the preheated oven at 60 °C). This polymerization step led to propranolol imprinted core particles. After preparation of imprinted core particles, 0.5 mL of core particles was withdrawn and stored in the fridge. To obtain imprinted core-shell particles, a mixture of NIPA (566 mg, 5 mmol), MBAAm (77.2 mg, 0.5 mmol), allylamine (188 μL, 2.5 mmol) and AIBN (24 mg) was added into the reaction tube and sonicated

for 3 min. The mixture was then purged with nitrogen for 5 min before the polymerization was continued for another 48 h under the same gentle rotation in the oven. After polymerization, polymer particles were collected by centrifugation at 11300 g for 15 min. The template was removed by washing with methanol containing 10% acetic acid (v/v), until no template could be detected from the washing solvent by using UV spectrometric measurement at 290 nm wavelength. The polymer particles were finally washed with acetone and dried in a vacuum chamber. For comparison, non-imprinted polymer (NIP) nanoparticles were synthesized under the same conditions except that no template was added.

2.3. FT-IR analysis

The presence of amino groups in the core-shell nanoparticles was confirmed by FT-IR analysis. Attenuated total reflection (ATR) infrared spectra were recorded on a Thermo Fisher-Scientific FT-IR instrument (Thermo Fisher-Scientific Inc., Waltham, USA). All spectra were collected in the 4000-375 cm^{-1} region with a resolution of 4 cm^{-1}, with 32 scans, and at 25 ^{o}C.

2.4. Preparation of PVA particles

Preparation of PVA particles was done as Li *et al.* described through freeze-thawing cycles [20]. Mowiol (4-98) (0.5 g) was dissolved in distilled water (5 mL) at 80 ^{o}C. When PVA solution was cooled to room temperature, it was added drop wise to 200 mL paraffin oil while stirring at 8000 rpm with a homogenizer (IKA-WERKE, Staufen, Germany). The emulsion was stirred for 5 minutes and then frozen for 24 h at -20 ^{o}C. Then it was defrosted for 6 h at room temperature. This freezing-thawing cycle was repeated 4 times. The suspension was centrifuged for 10 min at 15000 g to separate the PVA particles from oil. Acetone was used to wash the particles and remove the residual oil. Final wash was done by water to remove acetone. The particles were dried by freeze-drying and kept in a dry place at room temperature.

2.5. Size determination of MIP and PVA particles

Polymer core-shell particles (2 mg) were mixed with DDi water (1 mL), sonicated in a bench top ultrasonic cleaner for 20 min until no particle aggregate could be observed. The colloidal sample was diluted with DDi

water to a final concentration of 20 μg mL^{-1} prior to particle size measurement. Dynamic light scattering measurement (DLS) was performed at 25 °C by a Zetasizer Nano ZS instrument equipped with a software package DTS Ver. 4.10 (Malvern Instruments Ltd., Worcestershire, UK). The average particle size of PVA particles was measured at 25 °C in aqueous phase using the same equipment. Each measurement for the MIP and PVA particles was carried out in triplicate.

2.6. Preparation of MIP composite cryogel

A suspension of MIP particles 3% (w/v), corresponding to 0.03 g mL^{-1}, was prepared by sonicating MIP particles in distilled water. Dried PVA particles 2% (w/v) were added to the suspension and the pH of the mixture was adjusted to 1 by using hydrochloric acid (5 M). The mixture was mixed and kept on ice for 30 minutes while stirring. Glutaraldehyde (0.5% (v/v)) as a crosslinker was added to the well mixed suspension. The PVA-MIP suspension (1 mL) was poured into 2 mL plastic syringes (I.D. 9 mm) and kept at -12 °C for 16 hours. The frozen sample was thawed at room temperature and rinsed with distilled water to remove any non-reacted chemicals. Composite cryogel containing the NIP particles was prepared in exactly the same way as mentioned above, except that the MIP particles were replaced with NIP particles. In order to block the free aldehyde groups, the gels were washed with sodium borohydride (0.1 M) in carbonate buffer followed by distilled water.

2.7. Radioligand binding analysis

In a series of microcentrifuge tubes, dried composite cryogels and core-shell particles were suspended in 1 mL of 25 mM citrate buffer (pH 6.0): acetonitrile (v/v, 1:1). After addition of (S)-[4-^{3}H]-propranolol (246 fmol), the mixture was stirred on a rocking table at room temperature overnight. The cryogels and particles were settled by centrifugation at 11300 g for 10 min. Supernatant (500 μL) was withdrawn and mixed with 10 mL of scintillation liquid (Ecoscint A). The radioactivity of the supernatant was measured with a Tri-Carb 2810TR liquid scintillation analyzer (Perkin-Elmer, Waltham, USA). The amount of radioligand bound to the materials was calculated by subtracting the free radioligand from the total radioligand added. The data were mean values of measurements obtained

from three independent samples. Competitive radioligand binding analysis was also carried out under the same conditions except that non-labelled competing compound was added.

2.8. Characterization and properties of gels

Scanning electron microscopy (SEM) was used to study the structure of macroporous gels. The wet samples were cut into thin discs (1-2 mm) and freeze-dried. The dried samples were coated with gold:palladium (40:60) and studied using a JEOL JSM-80 5600 LV microscope (Tokyo, Japan).

Polymerization yield and swelling degree for both gels containing MIP and NIP were calculated based on equations 1 and 2, respectively:

$$Y\,(\%) = W_1/W_0 \times 100 \qquad (1)$$

$$S_w = (W_2 - W_1)/W_1 \qquad (2)$$

where W_0 and W_1 are theoretical weight and completely dried weight of the gels, respectively. The gels were dried in oven at 60 ºC. W_2 is the weight of the wet gel.

The mechanical stability of the gels was analyzed using a texture analyzer (XT2i, Stable Micro Systems, Godalming, England) by applying a 2 kg load cell and a cylindrical probe (25 mm diameter). The gels were placed vertically on a horizontal metal plate and the load applied on the same direction. The test speed was adjusted at 0.5 mm/s. All the mechanical measurements were conducted at room temperature. The samples were compressed up to 60% of the original height. The applied force was recorded and calculated by the Exponent, v.5.0.9.0 software (Godalming, England). Texture analyzing test was done in triplicate and the average of them are reported here. The elasticity modules of cryogel in linear range can be calculated by Young's modulus [21]

$$E = \sigma/\varepsilon = (F/A_0)/(\Delta l/L_0) \qquad (3)$$

where E is the Young's elasticity modulus (Pa), F is the force applied to the object (N), A_0 cross-sectional area where the force is applied (m^2), Δl is the change in length under the compression (m) and L_0 is the object's original height (m).

In order to check the water permeability of the gels, a method described by Plieva *et al.* [22] was applied. Gel was inserted into a glass column (I.D. 7 mm). Constant hydrostatic pressure equal to 1 m of water column was applied on the column and the water flow through the gel was measured.

There was no air present in the column during the experiments. Data presented here are result of average from triplicate measurements.

2.9. Solid phase extraction (SPE)

To investigate the selectivity of the MIP composite cryogel, a cryogel-based SPE process was performed to extract (R,S)-propranolol and its structural analogues. Before the SPE process, the imprinted and non-imprinted cryogel columns were washed repeatedly with a washing solvent composed of water:methanol:formic acid (5:3:2) using a peristaltic pump (Alitea C-4V; Stockholm, Sweden), until no propranolol could be detected by HPLC/MS/MS analysis. In order to remove the washing solvent, the columns were then washed with double distilled (DDi) water using the peristaltic pump for 6 h (20 mL/h). In the SPE experiment, 2 mL of a solution containing propranolol and its structurally related compounds, namely atenolol, metoprolol (+)-tartrate, timolol maleate and pindolol, each at 340 nM in distilled water, was pumped into the MIP/NIP composite cryogel columns in 2 h, and the effluent was collected in 2 mL centrifuge tubes. Acetonitrile containing 20% (v/v) of 10 mM ammonium acetate (pH 6.6) (2 mL) was then pumped through the column in 2 h. Finally the bound analytes were eluted from the column twice, each time by pumping 2 mL of water: methanol: formic acid (5:3:2) through the column in 2 h. The amount of the eluted analytes was quantified by HPLC-MS/MS.

2.10. HPLC–MS/MS analysis

HPLC–MS/MS analyses were carried out on a SunFireTM C18 (3.5 μm, 2.1 mm × 50 mm) column mounted on a Waters 2695 Separation Module. Solvent A: formic acid in water (0.1%), Solvent B: methanol. Flow rate: 0.3 mL min^{-1}. The analytes were separated in 3 min under isocratic elution (A:B = 65:35, v/v), using the following multiple reaction monitoring (MRM) for quantification of individual compounds: ES+ 260 > 116 (propranolol), ES+ 317 > 130 (timolol), ES+ 267 > 116 (atenolol), ES+ 268 > 116 (metoprolol), ES+ 249 > 116 (pindolol). A Waters Quattro micro API mass spectrometer (triple quadruple) was used for the quantification. Samples collected in the SPE procedure were injected directly into the HPLC–MS/MS modules [23,24].

2.11. Extraction of β-blockers from plasma

Bovine plasma (1.3 ml) spiked with atenolol, metoprolol (+)-tartrate, timolol maleate, pindolol and (R,S)-propranolol hydrochloride (each at 340 nmol L^{-1}) was pumped through the MIP/NIP composite cryogel columns in 1.3 h using the peristaltic pump. The columns were then washed with 1.3 mL of a mixture of acetonitrile containing 20% (v/v) of 10 mM ammonium acetate (pH 6.6) (the solvent was kept in the column for 1.3 h before it was pumped out), and finally eluted in two steps, each time by pumping 1.3 mL of water:methanol:formic acid (5:3:2) through the column in 1.3 h. The amount of the eluted analytes was quantified by HPLC-MS/MS.

3. Results and Discussions

3.1. Synthesis of MIP core-shell nanoparticles

Propranolol imprinted core-shell nanoparticles were synthesized by one-pot precipitation polymerization method. The core nanoparticles were first prepared following a procedure described by Yoshimatsu *et. al.*[19]. The amine groups were then introduced through copolymerization of allylamine and NIPA monomers with the residual C=C double bonds of the core particles, which resulted in the formation of hydrophilic shell layer on the core nanoparticles. The presence of the hydrophilic amino shell layer was confirmed by FT-IR analysis of the core and the core-shell nanoparticles (Fig. 1). The N-H stretching band and bending vibration of allylamine primary amines at 3300 and 1648 cm^{-1} were clearly observed in the core-shell nanoparticles. The infrared spectrum of the core nanoparticles displayed a strong peak at 1730 cm^{-1} that was attributed to the C=O stretching vibration of MAA carboxylic acid, whereas the relative intensity of this C=O bond in the core-shell particles decreased due to the reduced relative amount of MAA. Moreover, the DLS further proved the existence of a hydrophilic shell layer. The imprinted core-shell nanoparticles had a mean hydrodynamic diameter of 830 nm and were significantly larger than the core particles (151 nm) (Fig. S. 1). The swelling of the hydrophilic shell and the repulsion of the amine groups in water may result in the markedly large size difference between these two particles. Like the imprinted core particles, the core-shell particles also had a very narrow size distribution, indicating that all the core particles were

converted into the intended core-shell structure, and no new particles were generated in the second polymerization step.

3.2. Preparation and characterization of the composite cryogel

Size distribution of PVA particles produced by freeze-thawing cycles was measured 400 ± 50 nm in aqueous phase at 25 °C. Freeze-thawing method does not require any chemicals as a crosslinker. The end product of cryotropic gelation process through freeze-thawing cycles of PVA solution is a porous structure with a highly interconnected network formed between hydroxyl groups of PVA chains by hydrogen bonds interactions [25-27]. Effect of different factors such as low pH, low temperature and presence of crosslinker was studied separately on particle size distribution. Results showed that formed PVA particles are mechanically stable and changes on temperature or pH will not affect on their size [28].

Composite cryogel were formed by chemical crosslinking of PVA and MIP/NIP particles. Using a crosslinker allows the MIP/NIP particles to crosslink together due to amino functional groups on their surface and PVA particles will act as bridge between them. The gels have a rod shape (approximately 8 mm (diameter), 13 mm (height)), and were packed into a 2 mL glass column (I.D. 7 mm) to be used as affinity matrices. The shrinking of the cryogel can be explained by inter-polymerization inside the gel [29]. The structure and size of pores of the dried composite cryogel were studied by using SEM. The size of pores varied from a few micrometers to 100 μm for both gels containing MIP and NIP (Figs. 2A, 2C). This macroporous structure allows efficient mass transfer and diffusion of solutes in the gel even for mobile phase containing small particles. The structure of the walls of the composite cryogels formed by MIP or NIP particles can be studied at higher resolution (Figs. 2B, 2D). PVA particles are not visible at higher magnification since it was reported that PVA cryogel formed by freeze-thawing has porous structure [30] therefore removing water completely for SEM preparation might affect on their appearance and cause reduction of their size. Removing the water also affects the MIP and NIP particles sizes and causes shrinking. Size reductions of MIP and NIP particles are not as noticeable as that of PVA

particles due to their more firm and rigid structure.(Figs. 2B, 2D; S. 1A, 1B).

Both composite cryogels showed quite similar physical properties with regards to polymerization yield and flow resistance which can be expected due to the same production conditions. Small difference on MIP and NIP size did not noticeably influence on the physical properties of the composite cryogel (Table 1).

Elasticity of the gels was also studied by texture analyzer and the results confirmed that the both types of the gels containing MIP or NIP have comparable mechanical strength (Fig. 3). The linear changes under mechanical compression for both gels up to 30% makes it possible to apply Young's modulus calculation for the gels (Table 1). It was reported that the main contribution of water in swelling of the gels is ascribed to pores larger than 10 μm. Water bound to the polymer does not have considerable effect [31]. Therefore similar swelling /de-swelling behaviour of both composite cryogels is another proof for their almost similar structure. The polymerization yield achieved in this study was around 93% for both MIP and NIP composite cryogels (Table 1) and was in the same range as it was reported for other composite cryogels prepared by using MIP/NIP particles [32].

3.3. Molecular recognition property of the MIP composite cryogel

To investigate if the composite cryogels still have high molecular selectivity to propranolol, radioligand binding analysis was carried out using [^{3}H]-(S)-propranolol as a tracer in a mixture of citrate buffer (25 mM, pH 6.0):acetonitrile (1:1). Fig. 4 shows that propranolol radioligand was absorbed by different amounts of the imprinted and non-imprinted materials. Both the imprinted cryogel and the core-shell nanoparticles displayed significantly higher binding for [^{3}H]-(S)-propranolol than the non-imprinted materials did. Compared to the core-shell nanoparticles, the amount of radioligand bound to the corresponding composite cryogel only increased less than 5%, which indicates that the PVA particles in the composite cryogel only contribute little to the total propranolol binding. The selectivity of the MIP composite cryogel was also further confirmed by competitive radioligand binding experiment. As shown in Fig. 5, binding of the labeled propranolol could be much more easily inhibited by

(S)-propranolol than by the structurally-related atenolol. All the above results indicate that the binding sites in the composite material are still highly exposed to the propranolol template, and the composite cryogel still maintains its high selectivity.

3.4. Extraction of propranolol by MIP composite cryogel-based SPE

The MIP composite cryogel is a novel material that not only possesses high porosity but also high specificity for the targets. According to these attractive properties, the composite cryogel can be used to extract propranolol from water and even more complex samples such as plasma. To demonstrate this, a composite cryogel as stationary phase was applied to a SPE procedure where the propranolol and its analogs were extracted from water and then were analyzed by HPLC/MS/MS. In this procedure, 2 ml of test solution (containing propranolol and its analogs, each at 340 nM) was first loaded to the cryogel column. Based on our previous work [23,24], acetonitrile containing 20% (v/v) of 10 mM ammonium acetate (pH 6.6) was then used to remove the non-specifically bound analytes from the column, and all the analytes were eluted twice from the column with a mixture of water: methanol: formic acid (v/v/v, 5:3:2). As shown in Fig. 6a, the majority of analytes (57-97%) were removed at the washing step from the NIP composite cryogel absorbent, which was much more than that (4-47%) from the MIP composite cryogel. Quite significantly, only 4% propranolol was removed by the washing solvent, which indicates that the washing solvent could efficiently remove the non-specific binding from the absorbents. In Fig. 6 (b) is seen that 86.3% of propranolol could be recovered from the MIP composite cryogel column at the elution step, even in the presence of all the structural analogs, which is significantly higher than that recovered from the NIP composite cryogel column. This result indicates that the MIP composite cryogel have great potential to be applied in the treatment of water samples. Moreover, the amount of the propranolol analogs recovered from the imprinted column was also higher than that recovered from the non-imprinted column, which was in agreement with our previous reports [23,24]. Therefore, the MIP composite cryogel column not only specifically extracted propranolol, but also its structurally related analogs. The different recoveries obtained at the

elution step for the propranolol analogs can be attributed to their structural similarity to propranolol.

To further verify the stability, application and selectivity of the prepared composite cryogel material in a complex environment, the MIP composite cryogel-based SPE process was applied to extract propranolol directly from plasma. To compare with the extraction results from water sample, the same conditions were used for extraction of the plasma. Plasma sample (1.3 mL, each analyte conc.: 340 nM) was loaded to the cryogel column, then washed with a mixture of acetonitrile containing 20% (v/v) of 10 mM ammonium acetate (pH 6.6), and finally eluted twice with a 1.3 mL mixture of (water:methanol:formic acid) (5:3:2). This propranolol concentration (340 nM, namely 100 ng mL^{-1}) is relevant to that found in human plasma following a therapeutic treatment (from 5.3 to 300 ng mL^{-1}) [33]. As shown in Fig. 7, the MIP/cryogel displayed high propranolol recovery (94%) from the plasma sample, which indicates that the material maintained high selectivity. The high stability of cryogels under running conditions allows regeneration and reuse them in more than six cycles of continuous adsorption/desorption of propranolol. It is worthwhile to note that the recoveries of some analytes in plasma from the MIP and NIP/cryogel columns were slightly higher than that obtained from water sample. This phenomenon may be caused by two factors: firstly, the washing condition used to remove the non-specific binding may not be efficient enough in the presence of large amount of plasma proteins. Secondly, the plasma proteins are likely to interact with the test analytes [33], which may cause the analytes to be firmly bound in the column and escape the washing step.

4. Conclusion

Using MIP particles as affinity adsorbent for selective removal of target molecules has been applied from aqueous or organic media. In this work, we developed a novel MIP composite cryogel material by chemically crosslinking PVA and MIP nanoparticles. The composite cryogel showed high porosity and mechanical stability, and can be used as chromatography matrix for efficient separation of target analytes. The combination of high selectivity of the MIP particles with the highly porous interconnected composite cryogel structure form a unique affinity

matrix, providing a new type of SPE adsorbent for efficient removal of the target analyte from not only aqueous solution but also complex plasma sample. The easy regeneration and the reusability of the MIP composite cryogels is another attractive feature for more practical applications.

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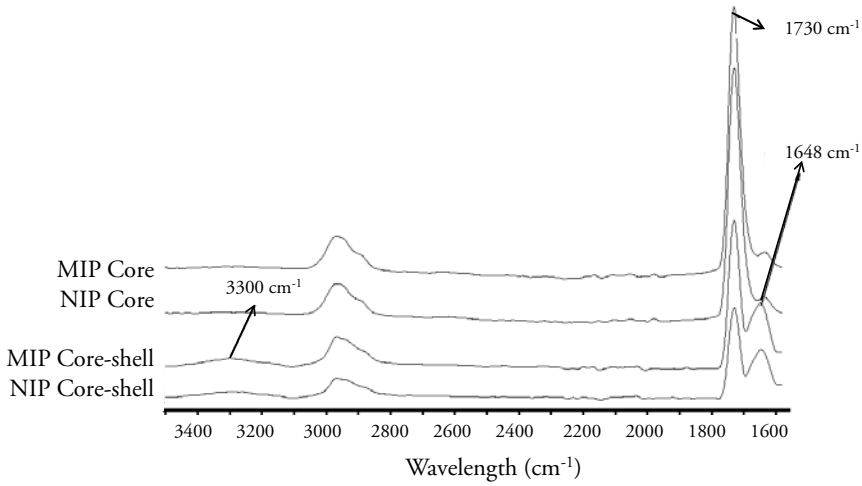

Fig. 1. FT-IR analysis of propranolol-imprinted MIP and NIP nanoparticles. Characteristic IR absorption bands for the primary amine groups are at 3300 and 1648 cm^{-1}.

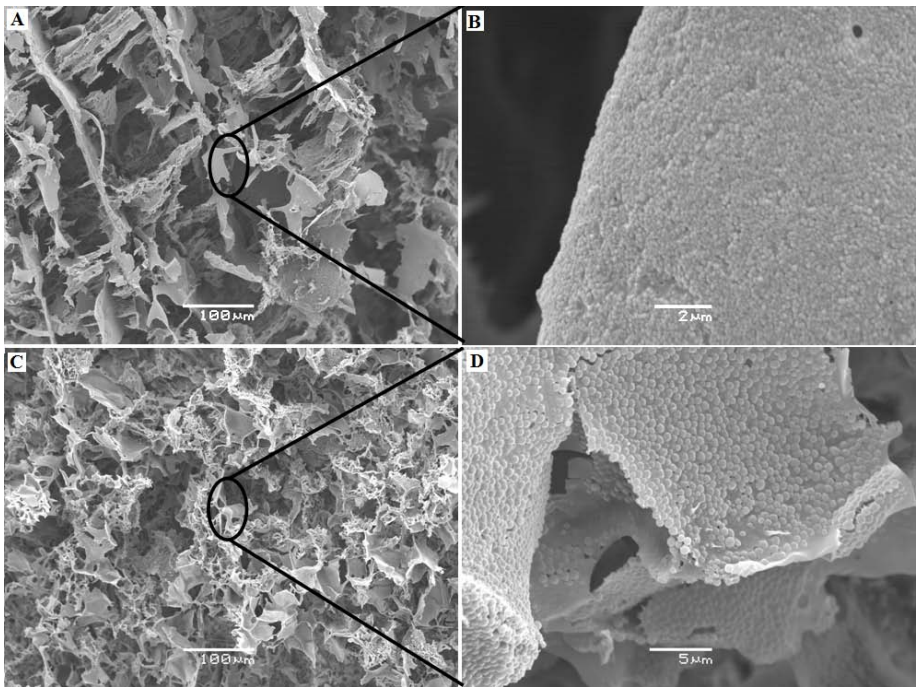

Fig. 2. SEM images of composite cryogel of 2% (w/v) PVA. A) containing 3% (w/v) MIP particles; B) high magnification of image (A); C) containing 3% (w/v) NIP particles; D) high resolution of image (C).

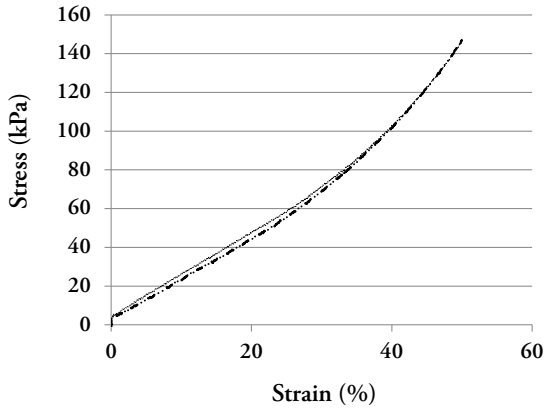

Fig. 3. Stress curve versus strain for MIP and NIP composite cryogel for 50% compression. (_ .. NIP composite cryogel; ….. MIP composite cryogel).

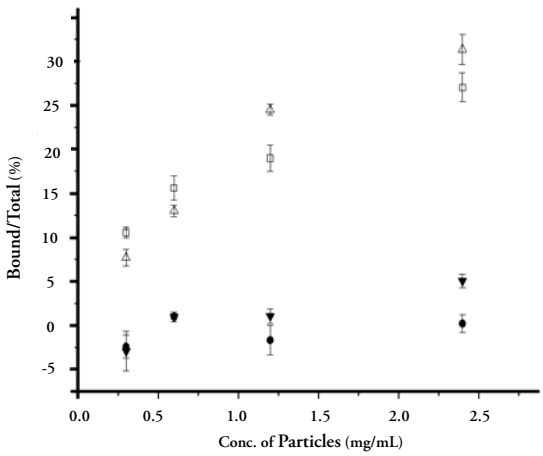

Fig. 4. Uptake of [^{3}H]-(S)-propranolol (246 pM) by the composites cryogel in 1mL of citrate buffer (25 mM, pH 6.0):acetonitrile (1:1, v/v). ● NIP core-shell particles; □ MIP core-shell particles; Δ MIP composite cryogel; ▼ NIP compiste cryogel.

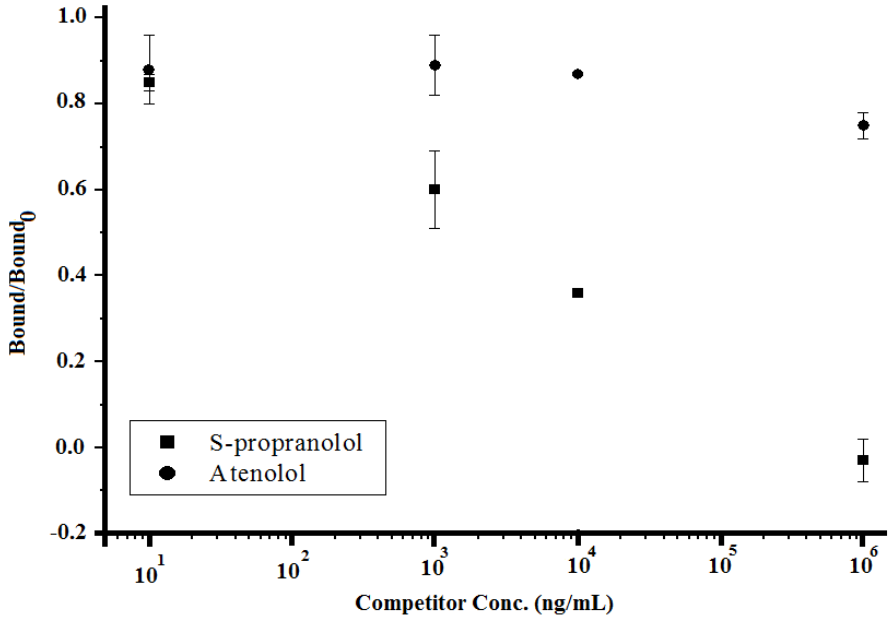

Fig. 5. Competitive radioligand binding analysis for 1 mg/mL MIP composite cryogel in acetonitrile:citrate buffer (1:1). $Bound_0$ and Bound were the amount of (S)-[4-^{3}H]-propranolol bound in the absence and presence of the competing compounds, respectively.

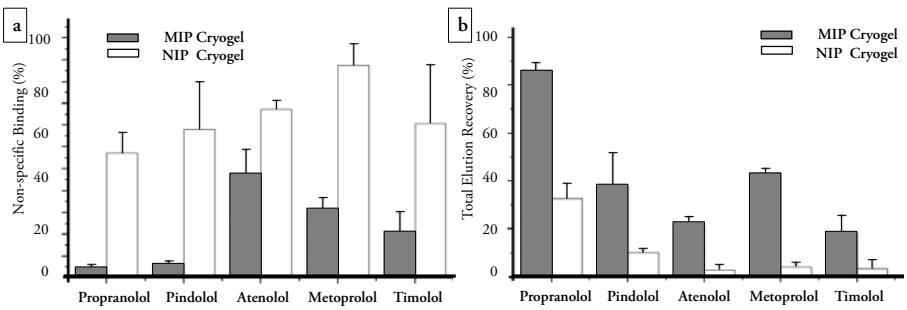

Fig. 6. Recovery of the analytes at the washing (a) and elution steps (b).

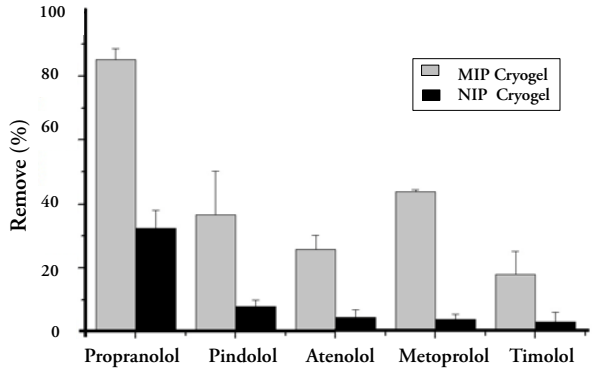

Fig. 7. Total elution recovery of β-antagonists from bovine plasma at the two elution steps in the cryogel-based SPE procedure.

Table 1 Flow-through property, polymerization yield, degree of swelling and elasticity of the MIP/NIP composite cryogels.

Composite cryogel	Flow* (mL/min)	Polymerization Yield (%)	E+ at 30% (KPa)	S_w*+ (g/g)
MIP 3% (w/v)	3.5±0.15	92±3	3.8±0.2	26.5±1.3
NIP 3% (w/v)	3.6±0.11	94±2	3.9±0.3	28.4±1.5

*Flow-through the composite cryogel under constant hydrostatic pressure; +Elasticity of the composite cryogels based on Young's modulus; *+Swelling degree of the composite cryogels

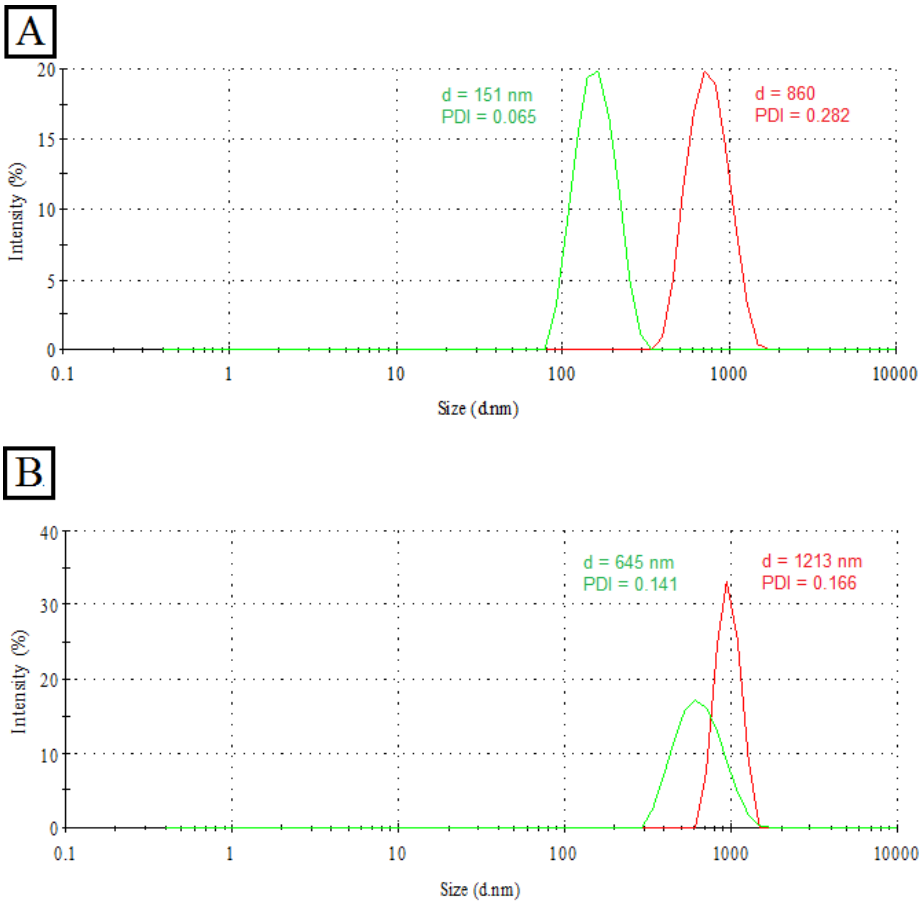

Fig S. 1. Hydrodynamic size of propranolol-imprinted (a) and non-imprinted (b) nanoparticles measured by DLS in water. Core particle (green), core-shell particle (red).

Paper IV

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Research Article

Evaluation of selective composite cryogel for bromate removal from drinking water

Bromate, which is a potential carcinogen, should be removed from drinking water to levels of less than 10 μg/L. A chitosan-based molecularly imprinted polymer (MIP) and a sol–gel ion-exchange double hydrous oxide ($Fe_2O_3 \cdot Al_2O_3 \cdot xH_2O$) adsorbent (inorganic adsorbent) were prepared for this purpose. The sorption behavior of each adsorbent including sorption kinetics, isotherms, effect of pH and selective sorption were investigated in detail. Sorption experimental results showed that the MIP adsorbents had better selectivity for bromate, even in the presence of high concentrations of nitrate, as compared to the inorganic adsorbent. It was found that pH does not affect the adsorption of bromate when using the inorganic adsorbent. Additionally, both adsorbents were immobilized in a polymeric cryogel inside plastic carriers to make them more practical for using in larger scale. Regeneration of the cryogels either containing MIP or inorganic adsorbents were carried out by 0.1 M NaOH and 0.1 M NaCl, respectively. It was found that the regenerated MIP and inorganic adsorbents could be used at least three and five times, respectively, without any loss in their sorption capacity.

Keywords: Composite cryogel / Isotherm study / Molecular imprinting / Selective adsorption
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1 Introduction

A large part of distributed water for human consumption must be effectively and frequently controlled. Water used for the preparation of drinking water is often contaminated with organic and inorganic substances [1]. It is therefore essential to purify and disinfect water source in order to obtain water of drinking quality. Water is one of the good sources of different trace elements which play an important role in the functioning of life on our planet. Some elements can be highly toxic to various life forms, whereas others are considered to be essential, but at higher doses they become toxic [2].

One disinfection processes of drinking water is ozonation, which has emerged as the most promising alternative to chlorination. The treatment of drinking water by ozone has gradually become common because it provides many benefits such as the reduction of taste and odor compounds, inactivation of microbial contaminants and the reduction of halogenated by-products [3].

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Abbreviations: **CE,** capillary electrophoresis; **DEAE,** diethylaminoethyl; **ECH,** epichlorohydrin; **MIP,** molecularly imprinted polymer; **MQ,** Millipore milli-Q; **NIP,** non-imprinted polymer

Bromate is formed as an ozonation by-product in bromide-containing water [4–6]. The rate of formation of bromate during ozonation is affected by temperature, alkalinity and ozone concentration [7–9].

Bromate was shown to be carcinogenic for animals [10]. The systemic toxicity of bromate (administered as the potassium salt) has been reported from long-term studies [11, 12]. Bromate has been shown to induce cancer in the kidneys, peritoneum and thyroid. In testes, it has shown to lower sperm count [10].

The World Health Organisation classified bromate as a group 2B or a ''possible human'' carcinogen and the allowed concentration of that in drinking water was suggested at 3 μg/L. In the USA and EU, the concentration of BrO_3^- in drinking water must not exceed a level of 10 μg/L [13].

Different approaches have been taken to reduce bromate concentration in drinking water. The majority of studies have focused on optimizing ozonation processes to minimize bromate formation. In addition, removal of bromate after its formation has also been investigated, although to a lesser extent [3, 9, 14–22], but these methods are either not efficient enough or require specific conditions and equipments.

Molecular imprinting is a useful technique, exploiting covalent [23] as well as non-covalent [24] interactions, to create specific sites for the target molecule in a molecularly imprinted polymer (MIP). This method allows for the production of a new class of materials that have artificially created receptor structures. Development of MIP involves polymerization of functional monomers in the presence of a template compound. The interactions are preserved during

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the polymerization by co-polymerization with cross-linkers into a highly cross-linked network. Subsequent removal of the template compound from the polymer gives rise to "memory sites" that are sterically and chemically complementary to the imprint species [25]. This technique has been used for bioanalysis [26], food analysis [27] and environmental analysis [28, 29]. The other method which was used in this study to produce adsorbents was sol–gel method. Sol–gel-derived materials have diverse applications in optics, electronics, energy, space, (bio)sensors, medicine and separation technology. The interest in sol–gel inorganic ceramic processing can be traced back in the mid-1880s [30]. An original sol–gel method of synthesis has been developed that allows production of spherically granulated sorbents from inexpensive raw materials [31, 32]. Activated alumina [17], activated carbon [6, 9] and ion-exchange resins [33] are widely used for anion removal, but inorganic ion exchangers have more potential because of their chemical stability and surface chemistry [34]. In order to get more practical advantages from the synthesized adsorbents both in MIP and sol–gel forms, preparing cryogel composite will be investigated.

Cryogels are synthesized in semifrozen aqueous media where ice crystals act as a porogen and form continuous interconnected pores after melting. One of the most attractive features of cryogels is their macroporosity with pores up to 100 μm, which enables efficient mass transfer and good flow through properties [35, 36]. Cryogels have proven to be useful in a wide variety of applications from purification of proteins from crude extracts [35–37] to tissue engineering [38, 39] and removing of endocrine disruptors from wastewater [40].

In this study, different kinds of adsorbents were synthesized and used to decrease the bromate concentration in water samples. The MIP adsorbents using chitosan crosslinked with epichlorohydrin (ECH) were prepared and used to selectively remove bromate from water solution as well as the sol–gel method to synthesize inorganic ion-exchange adsorbents based on a mixed hydrous oxide of iron and aluminum in order to investigate adsorption of bromate ions. The adsorbents were prepared both in the shape of beads and incorporated into a cryogel as composite material. CE technique was used to analyze the concentration of bromate in the samples. The sorption performance of the adsorbents including kinetics, adsorption isotherms and effect of pH were investigated in details. Finally, the adsorption selectivity in the presence of nitrate as well as the regeneration of the inorganic and MIP adsorbents were studied.

2 Materials and methods

2.1 Materials

Chitosan from crab shells with 85% deacetylation degree was from Sigma, Steinheim, Germany. Epichlorohydrin 99% (ECH) was from Aldrich (Rehovot, Israel) and sodium bromate was purchased from Fluka (Rehovot, Israel). Sodium hydroxide was from BDH, Poole, England. Chloride salts of iron and aluminum ($FeCl_3 \cdot 6H_2O$ and $AlCl_3 \cdot 6H_2O$) were obtained from Riedel-deHaen and Sigma-Aldrich, Steinheim, Germany, respectively. Water solution of ammonia (25%) and decane (Fluka, Steinheim Germany), Glutaraldehyde 50% and sodium chloride (Merck, Darmstadt Germany), hydrochloric acid 37% (w/v), TEMED, acrylamide 99+%, ammonium persulfate 98% and Bis 99% (Aldrich, Steinheim, Germany), phosphoric acid solution (87%), methanol and potassium dihydrogen phosphate (Merck, Darmstadt, Germany), DEAE Sepharose CL-6B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and sodium sulfate (Sigma) were used in different experimental stages. All solutions were made up with Millipore Milli-Q (MQ) purified water. The plastic carriers (AnoxKaldnes^{TM}) were supplied by AnoxKaldnes (Lund, Sweden).

2.2 CE apparatus and method

CE was performed on a Beckman P/ACE 2001 (Beckman Instruments, Fullerton, CA, USA) controlled by P/ACE Station and gold system software (Beckman Instruments). A fused silica capillary (Beckman, USA) with a total length of 67 cm, effective length to the detector of 50 cm, and its inner diameter of 50 μm and outer diameter 375 μm was used. The temperature of the capillary was controlled at $25.0 \pm 0.1^{\circ}C$ by circulating temperature-controlled coolant.

BGE was a phosphate buffer with a concentration of 10 Mm, which contains 10 mM sodium sulfate [41]. The buffer was potassium dihydrogen phosphate (10 mM) and the pH was adjusted with phosphoric acid (10 mM) to 2.1. The BGE was filtered through 0.45 μm pore size filters and degassed under vacuum each time before using.

Newly installed capillaries were flushed with methanol (20 min), followed by 0.1 M NaOH (20 min), MQ water (30 min) and finally with the BGE (30 min). To ensure repeatability, capillaries were flushed in between runs with methanol (1 min), followed by 0.1 M NaOH (1 min), MQ water (2 min) and finally with the BGE (2 min).

A sample solution was injected into the capillary from the cathodic end electrokinetically. Electrokinetic injection at 10 kV for 40 s was adopted in this study [41]. A voltage of 20 kV was applied for the electrophoretic separation and the analyte anions were detected photometrically at 200 nm.

2.3 Adsorbents and methods

2.3.1 Chitosan-based MIP adsorbents/non-imprinted polymer adsorbent

The MIP adsorbents using chitosan crosslinked with ECH were prepared and used to selectively remove bromate from water samples. First, the chitosan beads were prepared according to the method reported elsewhere [42]. Briefly,

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0.8% (w/v) chitosan solution was prepared in 2% (v/v) acetic acid and then the solution was added dropwise slowly through the needle syringe into a vessel containing 0.5 M sodium hydroxide and kept for 4 h. The beads were filtered and washed with copious quantities of MQ water till the outlet solution became neutral. Twenty gram of chitosan beads (wet weight) was crosslinked in a total volume of 25 mL with ECH aqueous solution (0.043 M) at 30–40°C for 12 h and rinsed with MQ water. The second step was crosslinking the beads in the presence of the template. Four gram of wet crosslinked chitosan beads was added into a vessel containing the template bromate in 500 mL solution at different concentrations for imprinting (100, 200, 300, 400 mg/L) and incubated under stirring for 12 h. The chitosan beads were filtered and added into 25 mL of ECH aqueous solution (0.24 M) solution for crosslinking at 40°C for 12 h. The beads were washed with 0.5 M sodium hydroxide solution to remove the bromate and finally rinsed with MQ water and freeze-dried. The preparation of the non-imprinted polymer (NIP) adsorbents followed the above procedure except for the added template.

2.3.2 Sol–gel adsorbent (inorganic adsorbents)

The ion-exchange adsorbents were prepared according to the method reported elsewhere [31, 32]. Chloride salts of iron and aluminum were dissolved in water solution of ammonia (25%) in order to have the final concentration 2 M for each salt. The mixture was stirred for 1 h at room temperature. The streams of these precursors (sols of Al–Fe–$(OH)_5Cl$) were added dropwise to a two-phase system of decane and ammonia solution (25%) (1:1 v/v). The small drops formed in decane fell down into an ammonia solution thereby forming spherical granules. The granules were filtered on a filter paper and washed with MQ water. The wet beads were heated in an oil bath at 90–95°C for 6 h and dried at room temperature. The sorbent was produced according to the general reaction scheme: $FeCl_3 + AlCl_3 + 6NH_4OH \rightarrow Fe(OH)_3 \cdot Al(OH)_3 + 6NH_4Cl$.

2.4 Preparing composite cryogels

Monolithic composite cryogels of polyacrylamide and inorganic or MIP adsorbents were prepared as through the following protocol. About 7% (w/v) monomeric mixture of acrylamide and Bis ratio 4:1 (mol/mol) was prepared in MQ water. Dried adsorbent (20% of total monomer weight) was milled to fine powder and suspended in the monomeric mixture and stirred to obtain a homogenous suspension. The suspension was then purged with N_2 to remove dissolved oxygen from the suspension which otherwise inhibits the free radical polymerization. Thereafter an activator, TEMED (1% of total monomer weight), was added and the mixture was kept on ice. Finally ammonium persulfate (1% of total monomer weight) was dissolved in 500 μL MQ water and added to the monomeric mixture in order to initiate the reaction. The suspension was then quickly transferred to glass tubes of 10 mm diameter packed with AnoxKaldnes™, plastic carriers, till the suspension covered the carriers and was frozen at −12°C in an ethanol-cooled cryostat. The samples were kept at −12°C for 16 h before being thawed at room temperature and washed with large amount of water to remove any non-reacted monomers.

2.5 Batch binding experiment

Batch adsorption experiments were carried out at room temperature on a magnetic stirrer (130 rpm) with 0.01 g of the synthesized adsorbent beads in 100 mL bromate solution. In the kinetic sorption experiments, 30 μg/L bromate solution was chosen. The sorption isotherm experiments were conducted with the initial bromate concentration ranging from 30 to 300 μg/L and equilibration time of 24 h. In order to evaluate the pH effect on the adsorption, a range of different pH value was chosen, the initial solution pH varied from 3 to 9 with the initial bromate concentration at 30 μg/L.

2.6 Selectivity test

In order to evaluate the selectivity of the adsorbents, solutions with different nitrate (30, 60, 100 and 1000 μg/L) and bromate (30, 60 and 100 μg/L) concentrations were prepared as follows: 30 μg/L of nitrate and bromate; 60 μg/L of nitrate and bromate; 100 μg/L of nitrate and bromate and 30 μg/L of bromate and 1 mg/L nitrate. The experiments were carried out under the same conditions as mentioned earlier in Section 2.5.

2.7 Regeneration step

In order to evaluate the regeneration rate for the MIP and inorganic adsorbents, the particles were incorporated into a composite cryogel. Using composite cryogels has some more advantages than using particles. The particles were trapped in the polymeric matrix and there is very low chance for leakage or losing particles during the process. This was an important issue, which makes the experimental results reproducible. Batch binding experiment with 30 μg/L bromate solution (100 mL) was carried out under the same conditions. After bromate was adsorbed onto the inorganic composite cryogel, the carriers were placed in 100 mL of 0.1 M HCl or 0.1 M NaCl at room temperature for 24 h and then rinsed with MQ water until a neutral pH was obtained.

Composite cryogels containing MIP adsorbent were washed with 0.5 M sodium hydroxide for 24 h and rinsed with MQ water for regeneration process.

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3 Result and discussion

3.1 Adsorbents and methods

An ideal adsorbent for bromate from potable water should be specific in adsorbing bromate in the presence of other anions (*e.g.* EU and US regulated values for nitrate concentration in drinking water is 11.3 and 10 mg/L, respectively [43]); allow fast processing of large volumes, *i.e.* have fast binding kinetics and low flow resistance; and finally be reusable. To match the first requirement, a new method quite different from the conventional MIP synthesis was used to prepare the MIP adsorbents. Classical preparation of MIPs was not possible to make using bromate as a template since bromate is not soluble in any organic solvents used for the preparation of MIPs. The natural polymer chitosan was used to prepare the MIP through a two-step crosslinking reaction. The size of the particle was 2 ± 0.5 mm. It has been shown that using ECH as a crosslinker has a positive effect on the imprinting procedure as ECH reacts with different chitosan functional group (both amino and hydroxyl groups) contrary to other common crosslinkers [42].

Using the hydroxyl groups of chitosan for the crosslinking reaction will leave the amino groups of chitosan available to interact with the anionic bromate. Bromate was adopted as the template during the MIP adsorbent preparation and the effect of its concentration on the imprinting efficiency was studied. By increasing the bromate concentration from 0 to 400 mg/L, the sorption capacity of the MIP adsorbents increased sharply in the beginning when the bromate concentrations rise from 0 to 200 mg/L and then enhanced slowly. For this study, 200 mg/L template concentration was chosen for imprinting during the MIP procedure (Fig. 1).

Synthesizing ion-exchange adsorbent was also carried out in this study by using iron and aluminum salts. The particle size of inorganic adsorbent was 1 ± 0.5 mm.

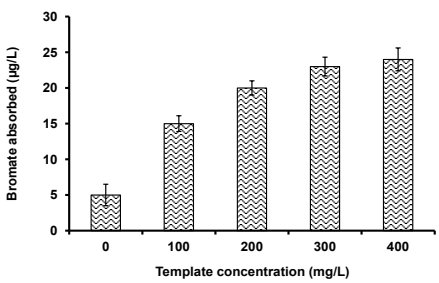

Figure 1. The effect of the template concentration on MIP adsorbent. Sorption conditions: 0.01 g adsorbents in 30 µg/L bromate solution (100 mL), 22°C for 12 h

To match the requirement of low flow resistance, adsorbent particles could be embedded into highly porous system, *e.g.* porous cryogels in protective plastic carriers [44], which is an attractive format for large-scale processes for the removal of unwanted contaminants. It was shown that using plastic carriers does not affect the adsorbents characteristics [45].

3.2 Selectivity of the synthesized adsorbents

Competitive adsorption of bromate and nitrate from their mixtures were studied in a batch system. In order to evaluate the reduction of bromate in the presence of nitrate, the experiments were carried out in a range of different nitrate and bromate concentrations under the same batch binding conditions (Section 2.5). Even in the presence of nitrate in high concentrations in comparison with that of bromate, the adsorption of bromate anion could decrease to the acceptable level of free bromate to less than 10 µg/L (Fig. 2). There is a significant increase in the selectivity for bromate removal between the MIP and the NIP adsorbent.

To investigate the sorption selectivity of the synthesized adsorbents, the binding of bromate to them was compared with that of a commercial anion-exchanger DEAE (diethylaminoethyl)-Sepharose. Figure 3 shows the concentration of adsorbed nitrate and bromate after batch binding experiment. Due to the non-selective binding to DEAE, both nitrate and bromate were adsorbed by this adsorbent from the solution. Moreover, nitrate was preferentially adsorbed even when its concentration used in these experiments was ten times less than the accepted level. Thus, commercial anion-exchange adsorbent removes bromate only *via* ion-exchange adsorption, which is severely compromised by the presence of other anions like nitrate.

MIP adsorbents had better sorption selectivity to bromate in the presence of nitrate than the inorganic

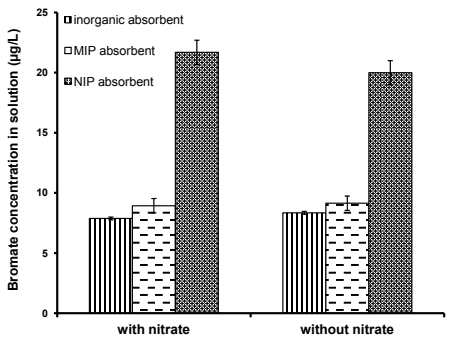

Figure 2. Bromate concentration in solution after batch binding experiment in absence or presence of 1 mg/L nitrate. Sorption conditions: 0.01 g adsorbents in 30 µg/L bromate solution (100 mL), 22°C for 12 h.

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adsorbent and DEAE-Sepharose (Fig. 3). In contrast, DEAE adsorbent has no selectivity to any of these anions. Since drinking water contains different anions in different quantity it is important to apply selective, cheap and reusable adsorbents for removing the target anion bromate.

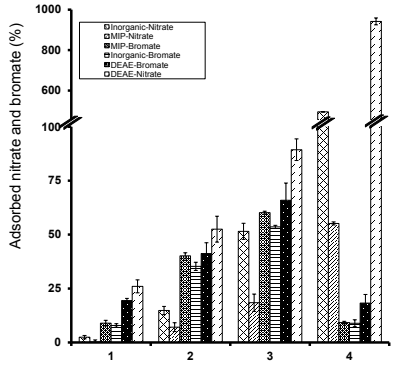

Figure 3. Adsorption of nitrate and bromate by DEAE-Sepharose and by MIP or inorganic adsorbents. Experimental conditions: (1) 30 μg/L bromate and 30 μg/L nitrate; (2) 60 μg/L bromate and 60 μg/L nitrate; (3) 100 μg/L bromate and 100 μg/L nitrate; and (4) 30 μg/L bromate and 1 mg/L nitrate, 22°C for 12 h. Adsorption is presented as the concentration of bromate and nitrate removed by the adsorbent from solution, respectively.

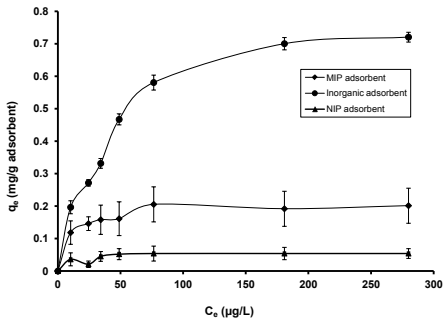

Figure 4. Adsorption of bromate on inorganic, NIP and MIP adsorbents after batch experiment (sorption conditions: 0.01 g adsorbents in 100 mL of different bromate solution concentrations, 22°C for 12 h).

3.3 Adsorption isotherm

Adsorption capacity, q (mg/g adsorbent), was calculated using the following equation [46]:

$$q = \frac{V(C_0 - C_{eq})}{M} \quad (1)$$

where q (mg/g) is the amount of anion adsorbed *per* gram of adsorbent, C_0 (mg/mL) the initial concentration of the anion in solution, C_{eq} (mg/mL) the final (or equilibrium) concentration of the anion in solution, V (mL) the volume of the solution and M (g) the mass of the adsorbent. In order to investigate the binding performance of both adsorbents, MIP and inorganic, their binding isotherms were determined in the 30–300 μg/L range of initial concentration of bromate solution (Fig. 4).

In this range, the binding data obtained were dealt with the linearized form of the Langmuir adsorption isotherm equation [46]

$$\frac{C_e}{q_e} = \frac{C_e}{q_{max}} + \frac{1}{K_l q_{max}} \quad (2)$$

where C_e is the equilibrium or final concentration of bromate (mg/L), q_{max} and q_e are the maximum and equilibrium adsorption capacity (mg/g adsorbent), respectively. K_l is the Langmuir adsorption equilibrium constant (L/mg). Table 1 summarizes the calculated constants for adsorption isotherm and pseudo-second-order kinetics for inorganic, NIP and MIP adsorbents. According to correlation coefficient ($r^2 = 0.99$), the models fit well with the data. Clearly, the inorganic adsorbent has higher capacity than that of the MIP adsorbent.

3.4 Kinetics of adsorption

For MIP adsorbent, sharp increase in amount of adsorbed bromate occurred in the first minutes with only further slight increase (Fig. 5). The adsorption process reached equilibrium after about 10 h. Bromate adsorption for inorganic adsorbent was even faster and the whole process was essentially completed within 2 min (Fig. 6).

The pseudo-second-order adsorption was used to fit the experimental data as the first-order equation did not fit well in the whole range of contact times and it is generally applicable over the initial stage of the adsorption processes. The pseudo-second-order adsorption is more likely to

Table 1. Kinetic parameters of the second-order equations for bromate sorption onto the MIP and inorganic adsorbents

Adsorbent	Pseudo-second-order kinetics			Langmuir isotherm		
	q_e (mg/g adsorbent)	K_2 (mg/g min)	r^2	q_{max} (mg/g adsorbent)	K_l (L/mg)	r^2
Inorganic	0.29 ± 0.010	12.3 ± 0.11	0.99	0.800 ± 0.04	42.10 ± 0.09	0.99
MIP	0.19 ± 0.005	0.33 ± 0.01	0.99	0.200 ± 0.02	91.10 ± 0.06	0.99
NIP	0.078 ± 0.03	8.5×10^{-4} $\pm 5 \times 10^{-5}$	0.98	0.062 ± 0.01	0.042 ± 0.02	0.97

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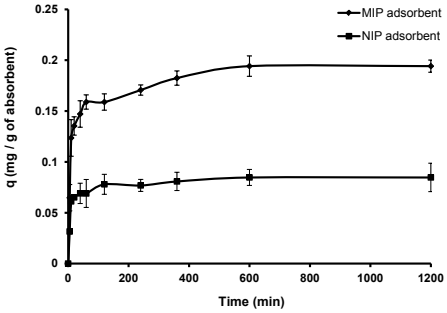

Figure 5. Adsorption kinetics of bromate anion on MIP and NIP beads. Sorption conditions: 0.01 g adsorbents in 30 μg/L bromate solution (100 mL), 22°C for 20 h.

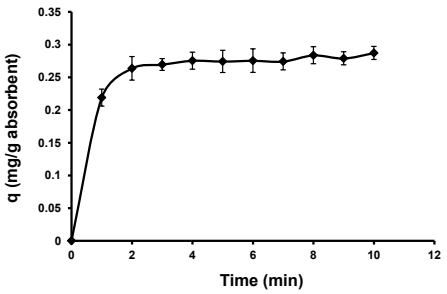

Figure 6. Adsorption kinetics of bromate anion on inorganic beads. Sorption conditions: 0.01 g adsorbents in 30 μg/L bromate solution (100 mL), 22°C for 15 min.

predict the behavior over the whole range of adsorption times [13, 47].

The pseudo-second-order kinetic model [47] is expressed as

$$\frac{t}{q} = \frac{1}{K_2 q_e^2} + \frac{t}{q_e} \quad (3)$$

where K_2 (mg/g min) is the rate constant of pseudo-second-order adsorption and t the time (min). The slopes and intercepts of plots of t/q *versus* t were used to calculate the pseudo-second-order rate constants K_2 and q_e (Table 1).

Ho and McKay described the chemisorption kinetics by this model [47], which can be applied as well to the sorption of bromate onto the inorganic and MIP adsorbents by possible electrostatic interaction between bromate anions and positive sites in both adsorbents.

3.5 pH effect

Figure 7 shows the effect of pH on bromate adsorption onto MIP and inorganic adsorbents. Bromate adsorption by MIP adsorbent has a clear pH optimum around pH 3, whereas pH has almost no effect on the removal of bromate by inorganic adsorbent in a wide pH range of 3–9.

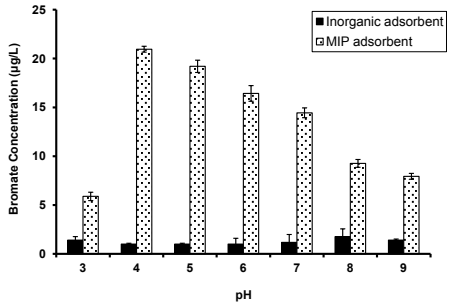

Figure 7. The bromate concentration in solution after batch binding with MIP and inorganic particles at different pH. Initial bromate concentration was 30 μg/L.

The pH effect on bromate removal by the MIP adsorbent is shown in Fig. 7. It indicates that better adsorption was obtained in the lower pH range, where more protons will be available to protonate the amino groups of chitosan molecules to form NH_3^+-groups, thereby increasing electrostatic attractions between bromate ions and adsorption sites and causing the observed increase in bromate adsorption.

Figure 7 indicates that the pH has almost no effect on the removal of bromate with initial concentration of 30 μg/L by the inorganic adsorbent.

3.6 Regeneration and reproducibility

Immobilizing the adsorbent beads into a cryogel matrix in order to make them more suitable for practical application was evaluated. The adsorbent material was incorporated into the walls of the prepared cryogel, this is an effect of freezing the suspension of particles and monomeric precursors. When freezing the suspension, the particles and the monomers will be expelled from the growing ice front and create a non-frozen phase in which the monomers react and thus entrap the particles within the polymeric network. Figure 8(B) presents SEM of the plain cryogel, whereas Figs. 8(C) and (D) present SEM images of the composite cryogels with inorganic and MIP adsorbents, respectively. The adsorbent particles incorporated into the cryogel structure were milled before mixed with the monomeric solution. Thus the particles become small and irregular in size, which makes it difficult to identify individual particles in the final structure. The non-smooth surface of pores of composite cryogel (Figs. 8(C) and (D)) is a good evidence that the inorganic and MIP adsorbent particles were immobilized within the cryogel matrix.

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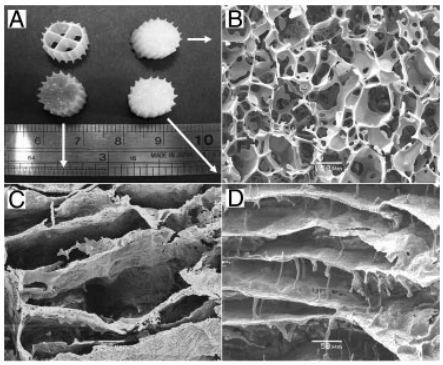

Figure 8. (A) A picture of carriers with different cryogels. The gels were prepared in Kaldnes carriers as protective shells. SEM images of (B) polyacrylamide plain cryogel, (C) inorganic adsorbent in polyacrylamide cryogel, (D) MIP adsorbent in polyacrylamide cryogel. The scale bar shows 50 μm in all three images. The arrows in image (A) show which carrier belongs to which SEM image.

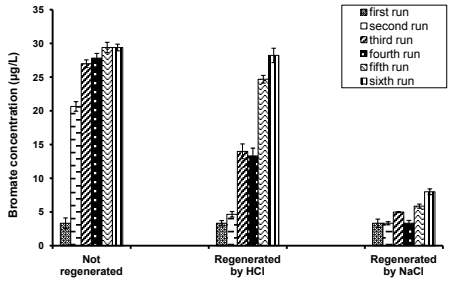

Figure 9. Bromate concentration in solution after batch binding with cryogels containing inorganic adsorbent in six adsorption–regeneration cycles by 0.1 M HCl or 0.1 M NaCl (50 mL). The starting concentration of bromate was 30 μg/L. Not regenerated: Using the same composite containing inorganic adsorbents continuously in batch binding experiments without any regeneration stage. Regenerated: Using the carriers with cryogel composite containing inorganic adsorbents continuously in batch binding experiments with washing with 0.1 M NaCl or 0.1 M HCl between batches.

Producing cryogels within plastic carriers makes them mechanically robust and suitable for large-scale industrial applications.

Composites with inorganic adsorbent were regenerated with 0.1 M HCl or 0.1 M NaCl. Even after five regeneration cycles, NaCl-regenerated composites allow decrease of bromate concentration below the required level of 10 μg/L under the experimental conditions, whereas non-regenerated carrier lost its bromate removal capacity already after the first cycle (Fig. 9). Regeneration with 0.1 M NaCl was much more efficient than regeneration with 0.1 M HCl.

Composite cryogels containing MIP adsorbent were regenerated with 0.1 M NaOH. Non-regenerated composite lost its ability to capture bromate after the second cycle, whereas regenerated composite maintained its performance somewhat longer (Fig. 10). In contrast to traditional adsorbents such as activated carbon [4], both the composite cryogel based on inorganic and MIP adsorbent have advantages of reuse and selectivity for bromate adsorption.

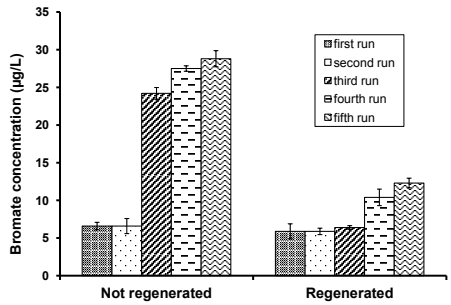

Figure 10. Bromate concentration in solution after batch binding with the cryogels containing MIP adsorbent in five adsorption–desorption cycles by 0.1 M NaOH (50 mL). The starting concentration of bromate was 30 μg/L. Not regenerated: Using the same cryogel composite containing MIP adsorbents continuously in batch binding experiments without any regeneration stage. Regenerated: Using the cryogel composite containing MIP adsorbents continuously in batch binding experiments with washing with 0.1 M NaOH between batches.

4 Concluding remarks

Two different adsorbents, the chitosan-based MIP and ion-exchange double hydrous oxide ($Fe_2O_3 \cdot Al_2O_3 \cdot xH_2O$) were synthesized in this study. Both adsorbents have shown the ability to reduce the bromate concentration in water below the accepted level of 10 μg/L. The MIP adsorbents can be used to selectively remove bromate from solutions in the presence of nitrate and have higher selectivity compared to inorganic adsorbent. Inorganic adsorbent adsorbs bromate faster than the MIP adsorbent does and it also has higher capacity (0.8 mg/g adsorbent) than the MIP (0.2 mg/g adsorbent). The sorption of bromate was pH dependent in a range of 3–9 for MIP adsorbent and has no effect on the inorganic adsorbent.

Inorganic and MIP adsorbents were immobilized within a cryogel matrix inside plastic carriers making them suitable for large-scale applications. Both composite materials could be regenerated up to five times maintaining their ability to remove bromate to reach acceptable concentration levels in water indicating potential application for selective removal of bromate in water treatment.

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Paper V

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Cross-linking cellulose nanofibrils for potential elastic cryo-structured gels

Kristin Syverud[1*], Harald Kirsebom[2], Solmaz Hajizadeh[2] and Gary Chinga-Carrasco[1]

Abstract

Cellulose nanofibrils were produced from *P. radiata* kraft pulp fibers. The nanofibrillation was facilitated by applying 2,2,6,6-tetramethylpiperidinyl-1-oxyl-mediated oxidation as pretreatment. The oxidized nanofibrils were cross-linked with polyethyleneimine and poly *N*-isopropylacrylamide-*co*-allylamine-*co*-methylenebisacrylamide particles and were frozen to form cryo-structured gels. Samples of the gels were critical-point dried, and the corresponding structures were assessed with scanning electron microscopy. It appears that the aldehyde groups in the oxidized nanofibrils are suitable reaction sites for cross-linking. The cryo-structured materials were spongy, elastic, and thus capable of regaining their shape after a given pressure was released, indicating a successful cross-linking. These novel types of gels are considered potential candidates in biomedical and biotechnological applications.

Keywords: cellulose nanofibrils, MFC, cryogelation, cross-linking

Background

Cellulose nanofibrils

The main raw material for the production of microfibrillated cellulose [MFC] is cellulose fibers, produced from wood by chemical pulping. Properly produced MFC contains a major fraction of cellulose nanofibrils [1]. Nanofibrils are composed of bundles of cellulose molecules, arranged in crystalline and amorphous areas. Nanofibrils have threadlike shapes, with diameters in the nanometer scale (< 100 nm), with high aspect ratio and high specific surface area. The fibrillated material retains many of the advantageous properties of cellulose fibers, such as high strength and the ability to self-assemble by making strong inter-fibril bonds. The small dimensions and the large specific surface area open up for applications that may not yet be foreseen. Several recent publications demonstrate how the strength properties of cellulose nanofibrils can be utilized for various purposes, e.g., in nanocomposites [1-6], to improve strength properties of paper [7,8], in thin films with high strength [9] and with added functionality such as antimicrobial activity [10].

Nanofibrils have hydroxyl groups on their surfaces, which can be used as targets for surface modification. Pretreatment of cellulose fibers with 2,2,6,6-tetramethylpiperidinyl-1-oxyl [TEMPO] prior to fibrillation introduces carboxylic acid groups and small amounts of aldehyde groups (0.2 to 0.3 mmol/g) [11], which can react easily with amines [12].

Cryogelation

Subjecting a solution or suspension to temperatures below the freezing point but above the eutectic point of the system leads to the formation of a two-phase system, with one solid and one liquid phase. When ice crystals form, any solutes or particles are expelled into a non-frozen phase, which forms around the crystals. In cryogelation, the gelation process occurs in the non-frozen phase, and hence, a material is formed under apparently frozen conditions [13]. The gelation can either occur through chemical cross-linking, polymerization reactions, or through non-covalent interactions. However, it is crucial that the interactions do not reverse when the sample thaws since that would make it impossible to form a material through cryogelation. Thawing the sample results in melting of the ice crystals while the material, formed through gelation, retains its shape. A macroporous material can thus be formed, in which the pores are a replica of the ice crystals [13].

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Pores in materials formed through cryogelation are interconnected and normally exhibit diameters between 1 and 100 μm, depending on freezing temperatures and composition of the starting mixture. Cryogelation does not require a freeze-drying step in order to produce a macroporous structure. The technique is only based on a freeze-thawing process. Cryogels are highly macroporous and often elastic materials, which can make them suitable in applications where traditional hydrogels would not be applicable. These gels have been used for biotechnological applications such as chromatography materials to process particle-containing fluids or enzyme immobilization [14]. Within biomedical applications, cryogels are being used in scaffolds for the cultivation of mammalian cells in tissue engineering applications [15].

The application of cellulose nanofibrils as a main component, in combination with polymers/particles as cross-linkers to form macroporous hydrogels, has not been investigated yet. It is expected that such gels can have a major potential within, e.g., biomedical applications. This study thus focuses on the ability of cellulose nanofibrils combined with cryogelation to produce cryo-structured gels with elastic properties. Two different routes will be applied for cross-linking, i.e., reactions with polyethyleneimine [PEI] and poly *N*-isopropylacrylamide-*co*-allylamine-*co*-methylenebisacrylamide [pNIPA] particles.

Methods

Production of cellulose nanofibrils

Two series of nanofibril qualities were produced from 100% *P. radiata* kraft pulp fibers. One of the series was chemically pretreated by using TEMPO-mediated oxidation, according to Saito et al. [11]. The other series was homogenized without pretreatment. The fibers were homogenized with a Rannie 15 type 12.56X homogenizer operated at 1,000 bar pressure. The pulp consistency during homogenizing was 0.5%. Samples of the fibrillated materials were collected after five passes through the homogenizer. For details, see the work of Syverud et al. [16].

Cross-linking nanofibrils

The nanofibrillated material had a concentration of approximately 0.5% (*w*/*v*). PEI (0.4% *w*/*v*; molecular weights 600 and 1,800 g/mol) from PolyScience (Niles, IL, USA) was added to this suspension. This mixture was thereafter frozen at -12°C and stored for 16 h; after which, the samples were thawed at room temperature, and the obtained gels were washed with water.

The second route for preparing gels consisted the adding of pNIPA particles (0.04% *w*/*v*) [17] to the nanofibril suspension. Allylamine and *N,N'*-methylene-bisacrylamide were purchased from Sigma-Aldrich (Steinheim, Germany), and *N*-isopropylacrylamide was from Acros (Geel, Belgium). The mixture was thereafter frozen at -12°C and stored for 16 h. The samples were then thawed at room temperature, and the obtained gels were washed with water.

Characterization

The prepared samples were cut into a 2-mm-thin disc and fixed in 2.5% *w*/*v* glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at +4°C. The samples were thereafter stepwise dehydrated in ethanol (0%, 25%, 50%, 75%, 96%, 99.6%) and then critical-point dried. The dried samples were sputter-coated with gold/palladium (40/60) and examined using a JEOL JSM-5000LV scanning electron microscope [SEM] (JEOL Ltd., Akishima, Tokyo, Japan). In addition, the cross-linked nanostructures were freeze-dried and assessed with a Zeiss Ultra field-emission scanning electron microscope [FESEM] (Carl Zeiss AG, Oberkochen, Germany) at various magnifications.

The mechanical stability of the cryo-structured gels was assessed using a texture analyzer (XT2i, Stable Micro Systems, Godalming, England), using a 5-kg load cell and a cylindrical probe (25 mm in diameter).

Results and discussion

Non-oxidized nanofibrils did not form into gels when the nanofibrils were mixed with PEI. The lack of aldehyde groups on these fibrils does not allow any reaction between the fibrils and the PEI; therefore, the obtained results were not unexpected. However, the addition of PEI to the oxidized nanofibrils resulted in the formation of gels (Figure 1). It is likely that the aldehyde groups enabled the reaction with the added PEI, which formed stable inter-fibril bonds. It is worth to mention that from the physical observation of the gels, the addition of 1,800 g/mol PEI produced more stable and spongy gels than the addition of 600 g/mol PEI under compression. PEI acts as a cross-linker between the fibrils, and thus, the length of the cross-linker will influence the properties of the formed material.

The addition of pNIPA particles (size approximately 125 nm) to the oxidized nanofibril suspension also resulted in the formation of stable and spongy gels (Figure 1B). The amino groups on the pNIPA particles, due to the allylamine, made it possible for the particles to react with the nanofibrils. Using pNIPA particles as a cross-linker can introduce temperature-responsive properties of the material [18]. It is well known that pNIPA collapses at temperatures above the lower critical solution temperature [LCST], which is about 32°C [19]. Therefore, at temperatures above the LCST, the cryo-structured material with cellulose nanofibrils will undergo a volumetric shrinking (Figure 2). The cryo-

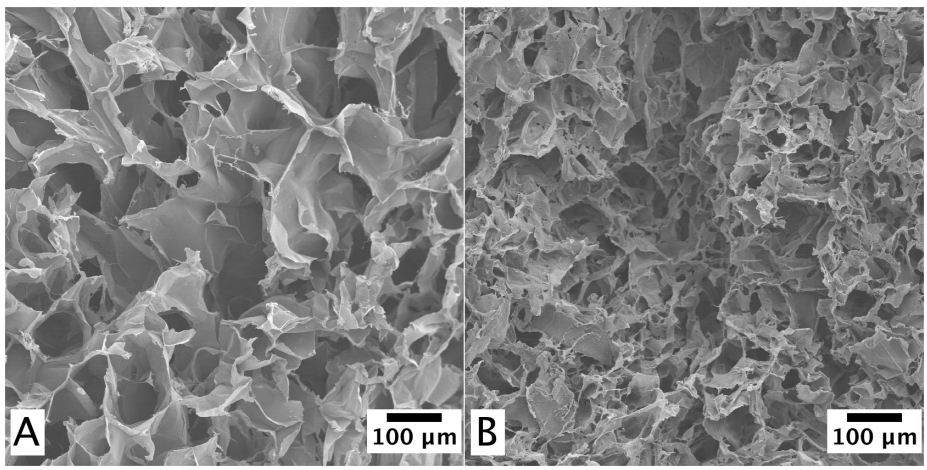

Figure 1 SEM images of gel prepared from nanofibrils. The nanofibrils have been linked with (**A**) 1,800 g/mol PEI and (**B**) pNIPA particles.

structured nanofibril gel shrank in all three dimensions due to the presence of the pNIPA particles in the gel.

High-resolution FESEM images were acquired to reveal the nanofibril structure and assembly in the gels (Figures 3 and 4). Note the relatively thin layers revealed in Figures 3B and 4B. The layers are composed of nanofibrils with diameters < 20 nm, as has been reported recently for this fibrillated material [20]. Such nanofibrils are clearly exposed in a fracture area visualized at high-resolution (Figure 3B).

Figure 5 shows photographs of the gels obtained after cross-linking with 1,800 g/mol PEI. It displays a sponge-like property in which the water can easily be squeezed out by pressing the cryo-structured gel. The gel easily

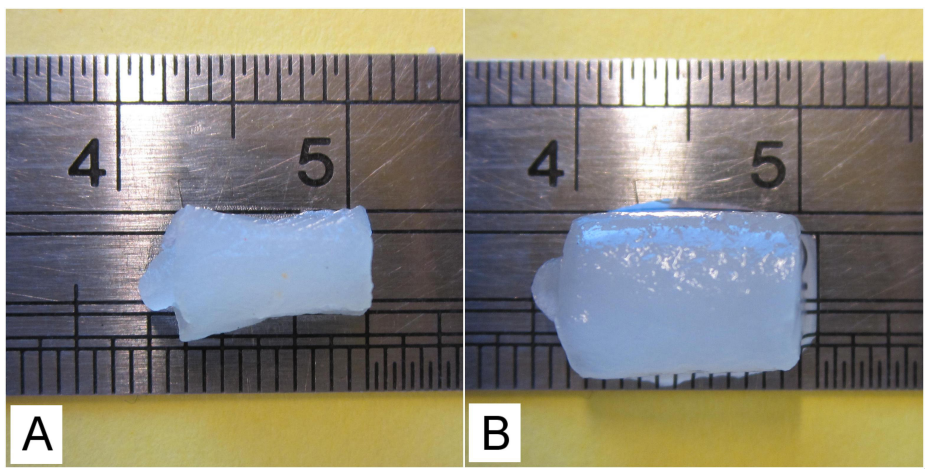

Figure 2 Digital photos of nanofibril pNIPA cryo-structured gel. (**A**) Photo of gel at room temperature. (**B**) Photo of gel at 60°C.

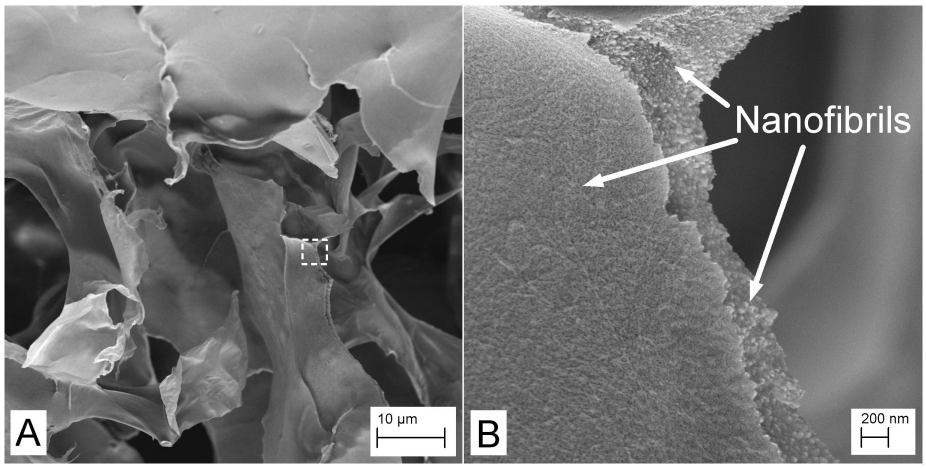

Figure 3 FESEM images of a gel prepared from nanofibrils mixed with pNIPA particles. Images acquired at relatively (**A**) low and (**B**) high magnifications. The image in (B) has been acquired from the area marked with a dotted rectangle in the image in (A). Note the nano-sized fibrils forming the network structure.

regains its shape after the pressure is released. Similar results were obtained when the gel was cross-linked with pNIPA particles. The mechanical stability of the cryo-structured gels were determined by a texture analyzer, and from force-distance curve, mechanical elasticity of the gels can be derived. Data show that even after compression of the gels, they will be expanded to their original form (Figure 5). The gels

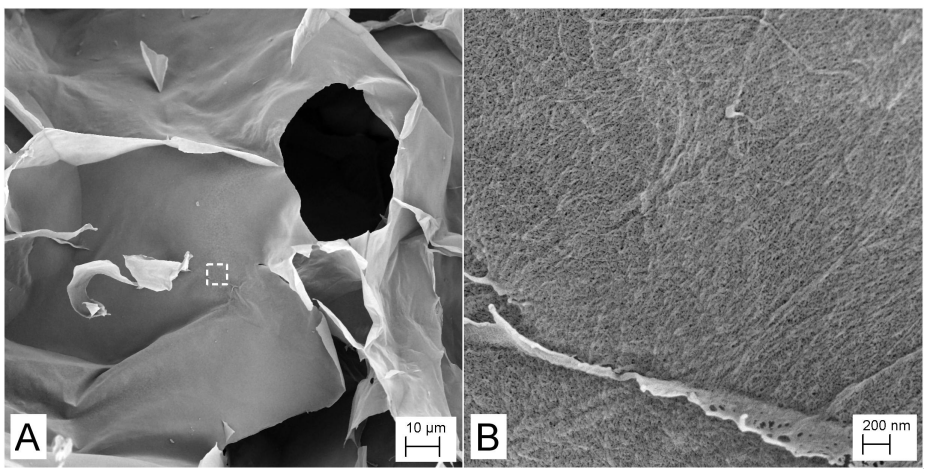

Figure 4 FESEM image of a gel prepared from nanofibrils linked with 1,800 g/mol PEI. Images acquired at relatively (**A**) low and (**B**) high magnifications. The image in (B) has been acquired from the area marked with a dotted rectangle in the image in (A). Note the nano-sized fibrils forming the network structure.

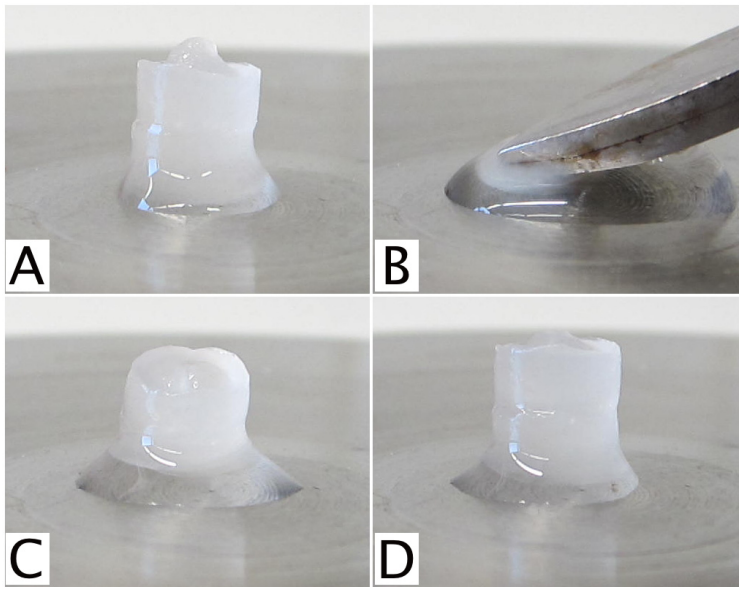

Figure 5 Digital photographs of a gel prepared from cellulose nanofibrils cross-linked with 1,800 g/mol PEI. (**A**) Before compression, (**B**) during compression, and (**C**) after pressure have been released. (**D**) The gel regains its original shape.

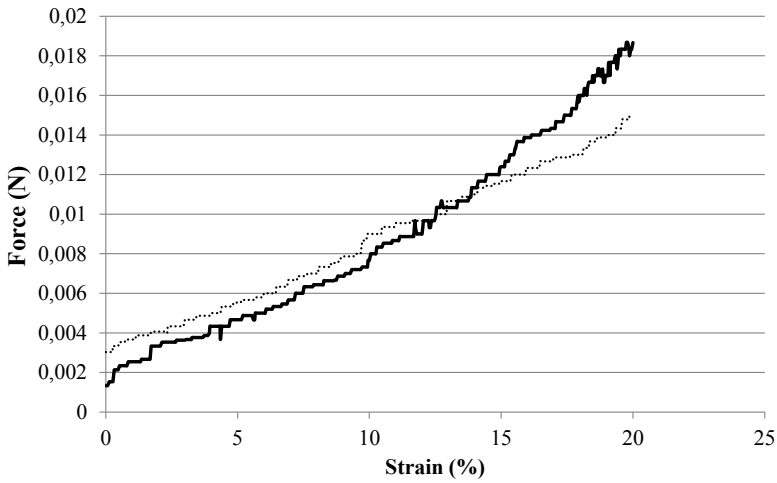

Figure 6 Force plotted against strain. Cellulose nanofibrils cross-linked with PEI (dark black line) and cellulose nanofibrils with pNIPA particle cryo-structured gels (dash line).

were compressed up to 20% of their height for the mechanical testings (Figure 6).

The results presented in this study indicate that the nanofibrils are interesting building blocks to prepare cryo-structured materials. Based on the sponge-like property of these cryo-structured materials, we foresee high-tech applications, such as modified macroporous structures in biomedical and biotechnology areas.

Conclusions

Oxidized nanofibrils, produced from *P. radiata* pulp fibers, were cross-linked with PEI and pNIPA particles in order to form cryo-structured gels. Due to a successful cross-linking, the nanofibrils formed stable 3-D networks. The cryo-structured materials were spongy, elastic, and thus capable of regaining their shape after a given pressure was released. Such characteristics propose the cryo-structured nanomaterials as most promising within biomedicine and biotechnology applications.

Abbreviations

FESEM: field emission scanning electron microscope; LCST: lower critical solution temperature; MFC: microfibrillated cellulose; PEI: polyethyleneimine; pNIPA: poly *N*-isopropylacrylamide-*co*-allylamine-*co*-methylenebisacrylamide; SEM: scanning electron microscope.

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Authors' contributions

HK has been involved in planning and synthesizing the cryo-structured materials and in writing and revising the manuscript. KS has made a substantial contribution to the conception of the experiments, has been involved in the production and characterization of cellulose nanofibrils, and in revising the manuscript critically for important intellectual content. SH has been involved in the production of pNIPA particles and synthesis of cryo-structured gels from cellulose nanofibrils and particles, performed the texture analysis, and contributed in revising the manuscript. GCC has been involved in the production and characterization of cellulose nanofibrils, performed the FESEM analysis of the cryo-structured gels, drafted the manuscript, and performed the corresponding revisions. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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