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PRIMARY RECOVERY OF BIOLOGICALLY ACTIVE COMPOUNDS USING MACROPOROUS MONOLITHIC HYDROGELS

LUNDS TEKNISKA HÖGSKOLA
Lunds universitet

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Lund Institute of Technology
Lund University

2005

Akademisk avhandling som för avläggande av doktorsexamen vid tekniska fakulteten vid Lunds Universitet kommer att offentlingen försvaras Onsdagen den 9th November, kl. 10:30 i hörsal A på Kemicentrum, Sölvegatan 39, Lund

Academic thesis which, by due permission of the Faculty of Engineering at Lund University, will be publicly defended on Wednesday the 9th of November, in Lecture Hall A, in the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy.

Faculty opponent: Associate Professor Timothy John Hobley, BioCentrum, Technical University of Denmark (TUD), Denmark.

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To My Family

Table of Contents

LIST OF PAPERS **5**

ABBREVIATIONS **6**

ABSTRACT **7**

AIM OF THE PRESENT INVESTIGATION **8**

INTRODUCTION **9**

PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS **10**

CELL CULTURE **10**

DOWNSTREAM PROCESS **11**

1. BIOSEPARATION OF LOW-MOLECULAR-WEIGHT SUBSTANCES 12

2. IN SITU RECOVERY OF LOW-MOLECULAR-WEIGHT-SUBSTANCES USING EXPANDED-BED ADSORPTION CHROMATOGRAPHY 13

3. BIOSEPARATION OF HIGH-MOLECULAR-WEIGHT COMPOUNDS 15

3.1. ANALYTICAL BIOSEPARATIONS *15*

3.2. PREPARATIVE BIOSEPARATION: CAPTURE OF PLASMID DNA USING MACROPOROUS CRYOGEL *19*

4. REMOVAL OF CONTAMINANTS 26

CONCLUSIONS **30**

ACKNOWLEDGEMENTS **33**

REFERENCES **34**

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals.

I. **Cryostructuration of polymer systems. XXIV. Poly (vinyl alcohol) cryogels filled with particles of a strong anion exchanger: Properties of the composite materials and potential applications**
Irina N. Savina, Amro Hanora, Fatima M. Plieva, Igor Yu. Galaev, Bo Mattiasson, Vladimir I. Lozinsky
Journal of Applied Polymer Science (2004) Volume 95, Issue 3, p 529-538.

II. **Screening of peptide affinity tags using immobilised metal affinity chromatography (IMAC) in 96-well plate format.**
Amro Hanora, Florent Bernaudat, Fatima M. Plieva, Maria B. Dainiak, Leif Bülow, Igor Yu. Galaev and Bo Mattiasson
Journal of Chromatography A (2005) Volume 1087, Issue 1-2, p 38-44.

III. **Capture of bacterial endotoxins using a supermacroporous monolithic matrix with immobilized polyethyleneimine, lysozyme or polymyxin B.**
Amro Hanora, Fatima M. Plieva, Martin Hedström, Igor Yu. Galaev and Bo Mattiasson
Journal of Biotechnology (2005) Volume 118, Issue 4, p 421-433.

IV. **Direct capture of bacterial plasmid DNA from non-clarified cell lysate using monolith columns from macroporous gel grafted with polycations**
Amro Hanora, Irina Savina, Fatima M. Plieva, Vladimir A. Izumrudov, Bo Mattiasson and Igor Yu. Galaev
Submitted Journal of Biotechnology.

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ABBREVIATIONS

BACs	Biologically active compounds
BE	Bacterial endotoxin
BSA	Bovine serum albumin
CTAB	Cetyltrimethylammonium bromide
DMAEMA	N,N-dimethylaminoethyl methacrylate
DNA	Deoxyribonucleic acid
DSP	Downstream processing
EBAC	Expanded-bed adsorption chromatography
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
GFP	Green fluorescent protein
GFPuv	Green fluorescent protein with improved fluorescence and expression in *E. coli*
HGMF	High gradient magnetic fishing
IDA	Iminodiacetic acid
IMAC	Immobilized metal affinity chromatography
META	(2-[methacryloyloxy]ethyl)-trimethyl ammonium chloride
MIPs	Molecular imprinting polymers
NTA	Nitrilotriacetic acid
RNA	Ribonucleic acid
PECs	Polyelectrolyte complexes
PEI	Polyethyleneimine
PVA	Poly(vinyl)alcohol
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TED	N,N,N´-tris-carboxymethyl ethylene diamine

ABSTRACT

Completion of the sequencing of some mammalian genomes, including the human genome, has opened up a new era of drug development. New approaches have been proposed for tackling diseases, namely the development of small molecule therapeutics based on an understanding of the human genome and proteome, new protein-based therapies, based on inhibiting or modifying specific metabolic pathways and DNA-based therapies involving the delivery of a gene into cells to correct or inhibit imperfect gene function. The potential production of novel therapeutics (small, proteinous and DNA based substances) in biological systems such as mammalian cells or microorganisms such as bacteria or yeast, presents new challenges for the subsequent separation and purification processes. Moreover, new methods for high-throughput screening are needed to identify potentially important lead molecules within large molecular libraries. Indeed, designing the downstream processing (DSP) of biologically active compounds depends on the nature and intended use of such compounds. For example commodity compounds that are produced by the tonne require fast and cheap purification methods. However, high-molecular-weight molecules intended for pharmaceutical applications require DSP to be carefully designed, in order to meet legislative demands, such as the removal of contaminants and toxic substances. This study describes two examples of the design of downstream purification processes applied to low- and high-molecular-weight compounds. Lactic acid was purified from a cell broth using a composite ion exchanger in an expanded-bed chromatographic format. Plasmid DNA was captured directly from the cell lysate using monolithic macroporous hydrogel ('cryogel'), grafted with different polycations. Using cryogels coupled with specific ligands, bacterial endotoxin was successfully removed from protein solutions and captured from disposed cell homogenate. Fast parallel screening of complex samples containing the target substance was demonstrated using affinity mini-cryogel columns in a 96-well plate format.

AIM OF THE PRESENT INVESTIGATION

In this thesis, a new approach for the capture of a low-molecular-weight substance (lactic acid) directly from fermentation broth utilizing expanded-bed adsorption is demonstrated (paper I). The application of monolithic cryogel columns for the removal of bacterial endotoxin from protein solutions (paper III) and for the primary capture of plasmid DNA from non-clarified cell lysate (paper IV) is also presented. In addition, the use of the monolithic cryogels for high-throughput screening of a peptide library was demonstrated (paper II).

The Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt, supported the work presented in the thesis. The work presented in papers II-IV was performed within the Swedish Center for Bioseparation (CBioSep), which is a competence center in which several companies and departments within Lund University collaborate.

INTRODUCTION

Biologically active compounds (BACs) can be classified as either low- or high-molecular-weight substances. The biotechnological production of BACs usually involves fermentation technology using microorganism or mammalian cell culture; alternatively, BACs can be extracted from natural sources such as plants. In both cases, BACs are present in the starting material in low concentrations and in complex media containing a variety of contaminants. Purification of BACs from such complex media requires a series of expensive unit operations. Downstream processing (DSP) of biological products is at least five times more expensive than the processing of small organic drugs, and may represent up to 50% of the final product costs (Geisow 1992). The yield of the purification step plays an important role in the economy of industrial-scale BAC purification. Assuming a yield at each step of 90%, a purification train consisting of 6 steps will result in only a 53% total yield of the target product (Figure 1), hence nearly half the product is lost. In practice, the yield of any individual purification step could be significantly less than 90%, resulting in even greater product losses. Thus, the development of purification processes aimed at reducing the number of purification steps and increasing the yield are urgently needed. As the level of competition between rival product manufacturers increases, yields and DSP costs will become increasingly critical when determining product competitiveness.

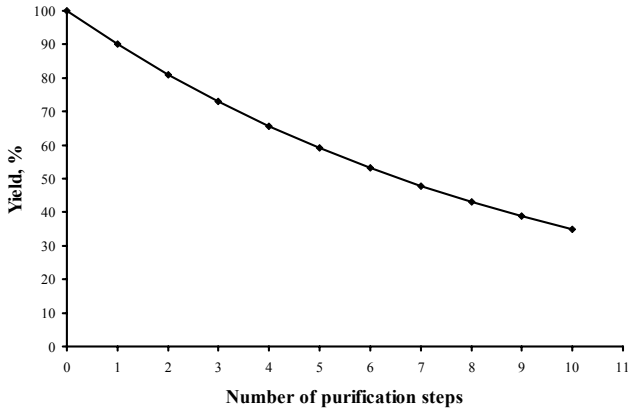


Figure 1. The effect of the number of purification steps on the overall product yield, assuming a 90% yield for each individual step.

PRODUCTION OF BIOLOGICALLY ACTIVE COMPOUNDS

Production of BACs from microbial cells usually involves three main stages; upstream processing, cell culture (fermentation) and downstream processing (Figure 2). When producing BACs via recombinant technology the appropriate selection of plasmid DNA vectors, in terms of plasmid copy number, incompatibility group, selection marker, size, promoter, codon usage and/or incorporation of affinity tags, are important upstream factors for facilitating downstream processes. In addition, the selection of the host strain and its ability to promote high plasmid DNA production are important. Thus, an ideal host strain is one which is able to grow to high cell densities thus promoting a high plasmid copy number with minimal fraction of plasmid free cells and resulting in a minimal number of genetic alterations and which is compatible with subsequent downstream processes (Durland *et al.* 1998).

CELL CULTURE

The fermentation protocol affects not only the content of the target product in the cells, but also the final profile of impurities, thus impacting strongly on the efficacy of DSP. The fermentation process must therefore be developed concomitantly with the purification protocol as both affect each other (Thiry *et al.* 2002). High productivity, reduced culture volume and reduced waste are desired (Prazeres *et al.* 2001). There are a number of factors that significantly affect productivity: 1) the choice of the fermentation mode (either batch, fed batch or continuous); 2) the size and type of the compounds (small or large molecule) and location (intracellular or extracellular); 3) the environmental conditions (such as pH, temperature, aeration and agitation) and 4) metabolic factors (such as product inhibition, expression profile and the choice of suitable media). In some cases the target molecules are produced in small volumes (~ 1 ml). In this study microbial cell culture in 96 well plates was used for the parallel production of BACs for screening studies.

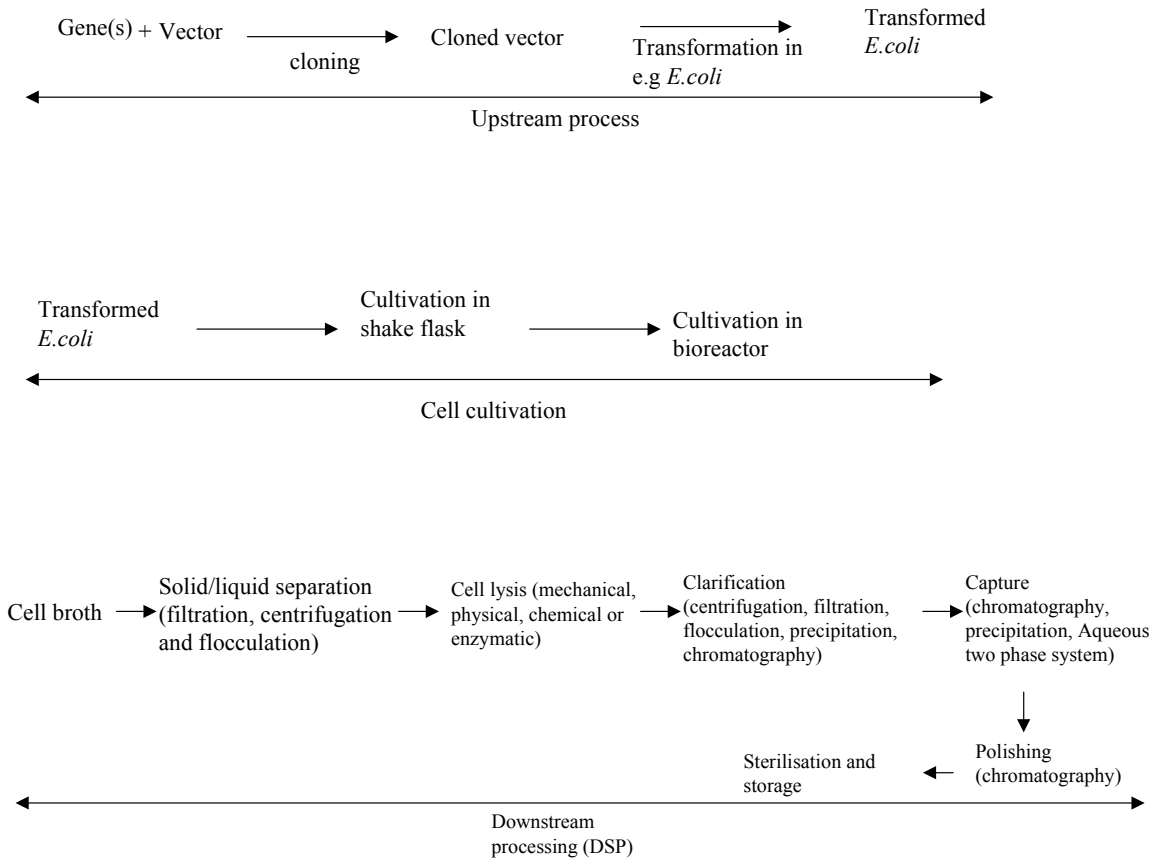


Figure 2. Schematic representation of the three primary steps used during the biotechnological production of biologically active compounds (BACs); upstream processing, cell culture and downstream processing.

DOWNSTREAM PROCESS

At the end of the fermentation phase, large volumes of cell broth with relatively low concentrations of product are obtained. The product arrives at the DSP stage in a relatively dilute form, and is contaminated with numerous, closely related species (Lightfoot *et al.* 2004). DSP usually involves the following steps or unit operations: cell harvest, cell lysis, primary recovery followed by a primary purification step, polishing and formulation. The specific DSP depends largely on whether the product is extracellular or intracellular. In both cases, the first step is to separate the cells from the suspension broth; this can be achieved with one of several typical cell harvesting methods such as centrifugation, flocculation or filtration. If the product is intracellular, including inclusion bodies, soluble proteins and plasmid DNA,

cell disruption is required. Four different methods are predominantly employed for cell disruption: mechanical, physical, chemical or enzymatic. The choice of method depends on the nature of the cell-producer used, the stability and activity of the product, and the nature of the subsequent DSP unit operations. Integration of the cell/cell debris separation with a capture step can be achieved by using expanded-bed adsorption chromatography (EBAC) (Ameskamp *et al.* 1999; Beck *et al.* 1999; Chase 1994; Chase *et al.* 1992; Pai *et al.* 1999; Varley *et al.* 1999) or high gradient magnetic fishing (HGMF) (Hubbuch *et al.* 2001; Meyer *et al.* 2005). The selection of bioseparation techniques is largely dependent on the target product (small or large molecule, its stability, sensitivity to degradation and extracellular or intracellular localization), its intended use (commodity, analytical, biotechnological or pharmaceutical application) and the demand for the final product.

1. BIOSEPARATION OF LOW-MOLECULAR-WEIGHT SUBSTANCES

The ever increasing demand for the production of low-molecular-weight substances places an economic pressure on industrial biotechnology with respect to cost reduction, increasing productivity and reducing processing time (Schügerl *et al.* 2005). Reducing the number of DSP unit operations in the purification train increases the yield and decreases the cost. Lactic acid (α-hydroxypropionic acid) is usually produced in the chemical industry from petroleum sources as a racemic mixture; however, production of pure isomer of D (-) or L (+) lactic acid for food processes and biomedical applications is achieved by microbial fermentation. When producing lactic acid in microbial cells, one faces the problem that accumulating lactic acid inhibits the growth of the cells and subsequently its own formation. (Iyer *et al.* 1999; Schügerl 2000). Traditionally, lactic acid is purified either by precipitation with calcium salt (Atkinson *et al.* 1991), extraction in an aqueous two-phase systems (Planas *et al.* 1999), extraction with organic solvents (Dai Y *et al.* 1996) or by adsorption to ion exchange resins in a batch process (Vaccari *et al.* 1993). To avoid the product inhibiting its own production, *in situ* recovery of lactic acid is favored. Ion exchange chromatography is the method traditionally used in industrial bioprocessing. Packed-bed

chromatography using an anion exchange chromatography has been used for lactic acid purification; however, it is not suitable for processing particulate containing cells feeds due to increased pressure drops and eventual clogging of the column. Hence primary processing of feed is necessary.

2. IN SITU *RECOVERY OF LOW-MOLECULAR-WEIGHT-SUBSTANCES USING EXPANDED-BED ADSORPTION CHROMATOGRAPHY*

Expanded-bed adsorption chromatography (EBAC) has been used for the purification of bio-molecules directly from cell broth containing particulate matter. EBAC allows the integration of solid/liquid separation, volume reduction and partial purification in a one-unit operation at high flow rate (Anspach *et al.* 1999). In EBAC, the beaded particles with heterogeneous bead sizes (typically ranging from 50 to 400 μm) and densities are lifted by the up-flow of the mobile phase. The bed expands, giving rise to large void volumes between the beads. The larger particles with higher densities populate the lower portion of the expanded bed while the smaller particles, with lower densities, populate the upper portion. When non-clarified cell broth is introduced onto an EBAC column, the particulate material and cell debris move freely around the resin beads and eventually exit through the top of the column. The compound of interest specifically binds to the beads while the non-bound and weakly-bound materials are washed out. The expanded bed is allowed to settle, and the flow is reversed, allowing the target compound to be eluted from the beads as in conventional packed-bed chromatography (Figure 3). A variety of functional groups have been used as the 'capture' ligand immobilized on EBAC resins such as affinity, hydrophobic and ion exchange ligands as well as immobilized metal affinity chromatography (IMAC) ligands and dyes. Nevertheless, the feedstock composition may be critical due to the potential interaction of cells and cell debris with adsorbent beads leading to their aggregation and resulting in bed instability and channeling and a subsequently dramatic decrease in column performance (Anspach *et al.* 1999). In order to overcome these limitations, beads have been specifically modified to prevent or reduce binding of the cells or cell debris components without significantly affecting their binding capacity with the target molecule. For example, ion-exchange EBAC beads have been covered with thin, shielding layers of oppositely charged

or non-charged polymers, thus preventing cells and cell debris from binding but with minimal effects on the mass transport of the target, low-molecular-weight compounds through the shielding layer and their adsorption to the EBAC beads (Dainiak *et al.* 2002b; Viloria-Cols *et al.* 2004).

An alternative way of generating a shielding layer is to make composite EBAC beads consisting of adsorbent particles embedded in a cryogel. Cryogels are polymeric gels resulting from cryogenic treatment (freezing and thawing) of low- or high-molecular-weight gel precursors. Cryogels are produced via gelation in a semi-frozen reaction medium at temperatures a few degrees below the crystallization temperature of the solvent used, for example water. Thus, the water in the reaction medium is partially frozen and forms a network of crystals, while the dissolved gel-forming reagents are concentrated in small non-frozen zones between the ice crystals. The gel is formed in the non-frozen medium and the ice crystals act as a porogen. When the ice crystals are melted, the gel - with large continuous interconnected pores, but with dense and essentially non-porous pore walls - is formed. The pore size depends on the initial concentration of reagents in solution and the freezing conditions involved.

In paper I, the ability of poly(vinyl alcohol) (PVA) to form a cryogel upon cycles of freezing and thawing and in the presence of anion exchange particles was exploited for the preparation of a new composite material designed to capture negatively charged, low-molecular-weight compounds such as lactic acid. This composite material has been produced in the form of 0.5- to 1.0-mm round beads with large (0.1- to 1.0-µm) interconnected pores which allow the capture of small molecules while hindering the attachment of negatively charged cells to the positively charged anion exchanger, Amberlite IRA-410. The composite material has been successfully used for lactic acid capture in EBAC mode directly from the fermentation broth with the recovery of 96% has been achieved (paper I). In contrast with plain Amberlite, the behavior of the composite material was not affected by passing the suspension of yeast cells (10 mg/mL) through the EBAC column. However, when the cell suspension was passed through the EBAC column containing plain Amberlite, the result was aggregation of the beads and strong channeling. Also, approximately 5% of the applied yeast cells bound to the fluidized plain Amberlite beads and 2% of

the yeast cells were irreversibly retained by the plain Amberlite resin, even after regeneration with 0.5 M NaOH.

3. BIOSEPARATION OF HIGH-MOLECULAR-WEIGHT COMPOUNDS

3.1. ANALYTICAL BIOSEPARATIONS

The need for analytical methods to characterize new drug candidates has boosted research into novel techniques for the rapid screening of large numbers of biological compounds. Assays that facilitate the detection of biological substances and biological activities are thus highly desirable. Such systems could also be applied to the analysis of biomolecular recognition at cellular membranes, ligand–receptor binding events and other processes of biotechnological significance (Jelinek *et al.* 2001).

Increased demand for the parallel processing of large numbers of biological samples has stimulated new developments in sample handling and processing. Traditionally, samples have been processed and analyzed in 96-well plates. Parallel sample processing involved sample clarification from cells and/or cell debris and capture of target biomolecules followed by their identification and quantification (Galaev *et al.* 2005; Rossi *et al.* 2000). Clarification of large sets of biological samples containing target biomolecules is both time-consuming and laborious. Employing the selectivity of affinity chromatography as a capture step with the integration of a clarification step is thus desirable. An ideal matrix for this purpose should have interconnected macropores with a pore size of 10-100 μm, allowing cells and cell debris to pass through without being retained. On the other hand, the matrix should be capable of efficient capture of the target molecule via strong affinity interactions.

Cryogels produced from polyacrylamide are mechanically strong, elastic matrices with a pore size in the range of 10-100 μm. Besides the advantage of large pore size, the cryogel monoliths are used for processing particulate (cells or cell debris) containing solutions (Arvidsson *et al.* 2002; Arvidsson *et al.* 2003; Dainiak *et al.* 2002a; Dainiak *et al.* 2004; Galaev *et al.* 2005; Kumar *et al.* 2003; Plieva *et al.* 2004, paper numbers II, III and IV). Moreover, cryogel monoliths are elastic, allowing them to be slightly

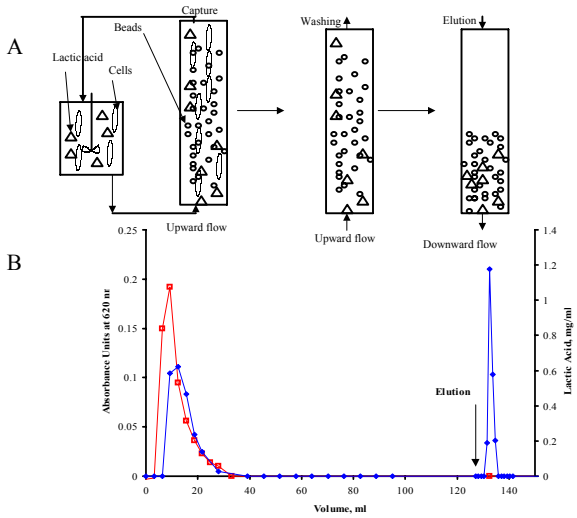


Figure 3. Lactic acid capture from a microbial cell broth by expanded-bed adsorption chromatography.
A) Capture of lactic acid by composite beads in an expanded-bed mode from non-clarified *Lactobacillus delbrueckii* fermentation broth. Non-clarified broth was applied to the column comprising composite beads (4-ml settled volume) in an expanded-bed mode at a linear flow rate of 160 cm/h. The column was washed with de-ionized water in an expanded-bed mode. The flow was interrupted to allow the adsorbent to settle, and the elution was performed with 0.1 M HCl in a packed-bed mode.
B) The chromatographic profile for lactic acid adsorption and elution. Lactic acid was analyzed using HPLC (closed rhombus). The cell content was analyzed by measuring the turbidity at 620 nm (open square).

compressed and easily placed inside a chromatographic column. When expanded, cryogel monoliths fill the column tightly with no risk of leakage between the monolith and the walls of the column. Capillary forces keep the liquid inside the pores of the cryogel monolith, rendering the columns drainage-protected. Applying a certain volume of liquid to the top of a cryogel monolith column results in the displacement of exactly the same volume of liquid from the column (Galaev *et al.* 2005).

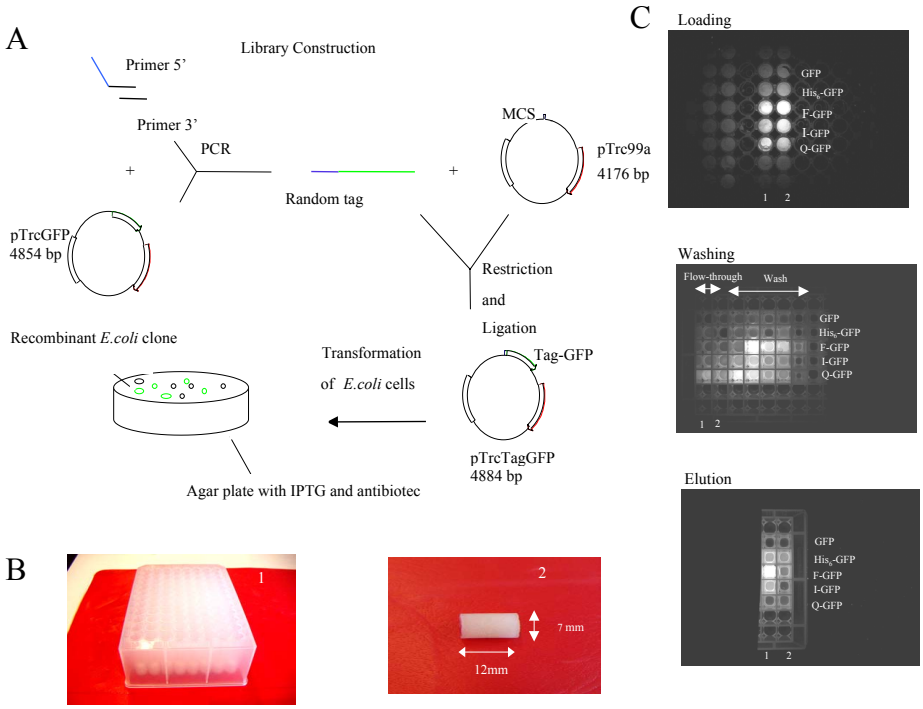
Traditional 96-well plates with mini-columns packed with beaded particles require either vacuum or pressure to drive the flow of the solution through the mini-columns. By contrast, gravity is sufficient for liquid flow in the 96-well plates with mini-columns containing cryogel monoliths. Alternative techniques for parallel processing of numerous samples, such as the use of magnetic beads with immobilized affinity ligands, require special equipment and expensive materials (Ko *et al.* 1992).

Immobilized metal affinity chromatography (IMAC), was first introduced by Porath *et al.* (1975). IMAC involves the formation of a complex between an immobilized chelating agent (e.g., tridentate iminodiacetic acid [IDA]), tetradentate (e.g., nitrilotriacetic acid [NTA]) and pentadentate (e.g., *N,N,N´*-

tris-carboxymethyl ethylene diamine [TED]) and metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+} as well as Cd^{2+}. Proteins bind to IMAC resins via an interaction between an electron donating group on the surface of a bio-molecule such as histidine, tryptophan and cysteine residues, and the metal ion which presents one or more co-ordination sites (Ueda *et al.* 2003).

Green fluorescent protein (GFP) was first isolated from the jellyfish *Aequorea victoria* (Shimomura *et al.* 1962). GFP has an emission maximum at 508 nm upon excitation at 395 nm, which enables easy identification and quantification. Recently, GFP has been widely used as a reporter protein since it provides a fluorescent probe (Misteli *et al.* 1997; Tsien 1998) upon UV excitation. GFP has also been used as fusion partner, at the N- or C-terminus (or both) of a target protein, providing a molecular recognition site for selective interaction with ligands immobilized onto a suitable matrix.

In paper II, we constructed a random peptide library fused to a mutant form of GFP with enhanced fluorescence (GFPuv). The tagged-GFPs were expressed in *E. coli* cells which were cultured in 96-well plates and lysed by either chemical or mechanical (sonication) methods. Using IMAC-cryogel columns in a novel miniature-chromatographic mode, the expressed protein was captured directly from the cell lysate without prior treatment. The fused peptides could be simultaneously screened against different metals immobilized with IDA ligands without applying vacuum or pressure. It was evident from this study that the use of these novel cryogel composite matrices may facilitate the screening of large peptide libraries (Figure 4). The ability to immobilize a large variety of ligands (peptide, protein and nucleic acids) using conventional coupling chemistry employed for commercial chromatography, enables us to utilize a new concept in screening complex samples containing particulate materials (cell culture, pharmaceutical and environmental samples) against different ligands in a fast and efficient manner. Designing small cryogel columns with incorporated imprints of small molecules (utilizing molecular imprint polymers, MIPs) could be explored as a new method for screening target molecules in complex media. The power of mass spectrometry could be combined with such a system for the rapid and efficient identification and quantification of target molecules.



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Name	Tag sequence	Ni-IDA	Ni-NTA	Zn-IDA	Co-IDA	Cd-IDA
Native GFP	MEFELGT					
His_6-GFP	MGH**HHHH**HGT	+	+	+	+	+
A	MEFH***VRLK***H	+				
B	MEFH***VCMH***H	+				
C	MEFH*QETE*H	+				
D	MEFH***PKLE***H	+				
E	MEFH*NWMD*H	+				
F	MEFH***FK***SH	+	+	+	+	+
G	MEFH*NAIL*H	+	+			
H	MEFH*N***RS***RH	+	+			
I	MEFH***ANHM***H	+	+	+	+	+
J	MEFH***WRS***RH	+	+			
K	MEFH*NGSE*H	+	+			
L	MEFH*T***R***SGH	+	+			
M	MEFH*NWMD*H	+	+			
N	MEFH*EIDV*H	+				
O	MEFH***WRAR***H	+				
P	MEFH*WGYL*H	+				
Q	MEFH*TSM***L***H	+	+	+	+	
R	MEFH*S***RL***SH	+				
S	MEFH*Q***KVL***H	+				
T	MEFH***ALR***GH	+				
U	MEFH*FQFD*H	+				
V	MEFH***RSLA***H	+				
W	MEFH***VWMR***H					

Positively charged amino acids (K, R) (fat letters). Negatively charged amino acids (D, E) (thin italic underline letters). Aromatic amino acids (F, W, Y)(fat italic underline letters). Histidine (fat underline letters). Uncharged polar amino acids (G, S, T, C, N, Q) (thin italic letters). Apolar amino acids (A, V, L, I, P, M) (fat and italic letters).

Figure 4. Schematic representation of the micro-scale bioseparation of a GFPuv-tagged peptide library using cryogel columns.
A) Construction of the tagged-GFPuv peptide library.
B) Following culture of individual clones on microtiter plates, selected clones were screened using metal-IDA cryogel mini-columns in 96-well plates (B 1), monolithic cryogel mini-column (B 2).
C) Screening the microtiter plates containing metal-IDA monolithic cryogel columns for fluorescence at 395 nm allows easy visualization of the target peptides throughout the capture process i.e. loading, washing and elution of the tagged peptides
D) The sequence of the peptide tags and binding efficiency of selected clones.

3.2. PREPARATIVE BIOSEPARATION: CAPTURE OF PLASMID DNA USING MACROPOROUS CRYOGEL

There is an increasing demand for pure preparations of plasmid DNA. Traditionally, plasmid DNA is produced from *E. coli* cells. The production of plasmid DNA involves three steps: upstream processing, fermentation of *E. coli* cells, then purification. Purification of plasmid DNA involves the following unit operations: cell harvest by centrifugation or filtration, cell lysis, primary recovery and adsorption followed by polishing and formulation. At the laboratory scale, cell harvesting can be achieved with centrifugation. However, at the industrial scale, the cells are normally harvested using well-developed continuous centrifugation or cross-flow filtration. The bacterial cell paste can be processed immediately or frozen for future use (Durland and Eastman 1998). Traditionally, for plasmid purification, alkaline lysis is the most common technique used for cell rupture. Alkaline conditions, in the presence of ethylenediamine tetraacetic acid (EDTA) and the detergent, sodium dodecyl sulfate (SDS), are traditionally employed for cell rupture and denaturation of host cell proteins with partial denaturation of the genomic DNA. This procedure usually results in a large volume of viscous, particulate-containing solution. Plasmid DNA accounts for less than 1% (w/w) of bacterial cell lysate while RNA, a major contaminant in plasmid DNA preparation, accounts for 6% (w/w) (Prazeres *et al.* 2001).

Cesium chloride/ethidium bromide (CsCl/EtBr) buoyant density gradient separation followed by ultracentrifugation (Prather *et al.* 2003) and alcohol precipitation using ethanol or isopropanol, are frequently used for laboratory-scale plasmid purification. However for large-scale plasmid purification, other techniques such as triple helix affinity precipitation (Costioli *et al.* 2003) and fractional precipitation from cell lysates using cetyltrimethylammonium bromide (CTAB) (Lander *et al.* 2002) have been developed. Selective precipitation using compaction agents such as spermine and spermidine (Murphy *et al.* 1999), precipitation by forming polyelectrolyte complexes (Wahlund *et al.* 2004b), and partitioning in thermoseparating aqueous two-phase polymer systems (Kepka *et al.* 2004) have also been used. Super-paramagnetic nano-particles coated with polyethyleneimine (PEI) were developed for plasmid DNA

purification (Chiang *et al.* 2005). However, a variety of chromatographic techniques are predominantly used for plasmid DNA purification such as ion exchange chromatography (Tseng *et al.* 2003), size exclusion chromatography (Horn *et al.* 1995), triple helix affinity chromatography (Wils *et al.* 1997), thiophilic interaction chromatography (Sandberg *et al.* 2004), hydrophobic interaction chromatography (Diogo *et al.* 1999), reverse phase chromatography (Green *et al.* 1997) and hydroxyapatite chromatography (Giovannini *et al.* 2002). EBAC has also been used for the capture of plasmid DNA (Varley *et al.* 1999) from filtered and centrifuged cell lysates (Ferreira *et al.* 2000; Theodossiou *et al.* 2001). More recently, monolithic columns were used for plasmid DNA purification from clarified cell lysate (Bencina *et al.* 2004; Branovic *et al.* 2004; Urthaler *et al.* 2005).

Ion exchange chromatography remains the method most commonly used in the DSP of biomolecules due to its robustness, rapid separation, organic solvent-free process, its ability to withstand sanitation with sodium hydroxide and a wide selection of industrial media. Phosphate groups located in the backbone of nucleic acids carry a negative charge at pH values above 4; hence, interactions between these groups and the positively charged anion exchanger groups may occur. The strength of this interaction depends on the density and the conformation of the negatively charged groups located on the plasmid DNA. Elution of the bound DNA is achieved with increasing salt concentrations. In addition to plasmid DNA, alkaline cell lysate contains host cell proteins, RNA, genomic DNA and endotoxin. Being negatively charged, some of these bio-molecules have physical and chemical similarities to plasmid DNA; hence, they compete for binding and are co-eluted with plasmid DNA during anion exchange chromatography.

Exploring the difference in binding affinities between RNA and plasmid DNA towards quaternary amines enables purification of plasmid DNA from its major contaminant, RNA. For example, the application of clarified cell lysate in the presence of 0.5 M NaCl greatly reduces non-specific binding of most of the cellular impurities (protein and RNA) to EBAC columns packed with Streamline QXL (Ferreira *et al.* 2000).

A significant problem associated with plasmid DNA chromatography in a packed-bed mode is pronounced mass-transfer limitations. Plasmid DNA is characterized by its large size (> 200 nm) with a helical length of about 370 nm, a diameter of double helix of 11.3 nm and a diffusion coefficient in the order of 10^{-8} cm^2/s, which is approximately an order of magnitude less than that of proteins (Diogo *et al.* 2005). Moreover, the high viscosity of the alkaline lysate generates high backpressures (between 15-60 MPa) in columns packed with beaded particles (Diogo *et al.* 2005). Monolithic chromatography has been used for plasmid DNA purification, and mass-transfer resistance was shown to be significantly reduced (Branovic *et al.* 2004; Urthaler *et al.* 2005). In columns packed with beaded particles, mass-transfer is governed by diffusion whereas in monolithic columns mass-transfer is predominantly due to convective transport. The macroporous monolithic cryogels used in this study (paper IV) were produced by radical polymerization of a monomer, acrylamide, and cross-linker, N,N'-methylenebisacrylamide, at subzero temperatures, as mentioned above. Large interconnected pores in monolithic cryogels allow for efficient convective mass-transport of plasmid DNA. Moreover, only a small backpressure is generated when a viscous solution is pumped through a macroporous monolithic cryogel column.

Viscous, particulate-containing solutions with a high content of contaminating substances such as RNA, genomic DNA and protein need to be processed for the capture of the target molecule (plasmid DNA), which is usually present in low amounts. Integration of a number of necessary unit operations, such as clarification, plasmid capture and primary purification, into a single step will both improve the final product recovery and the economy of the process. In addition, degradation of plasmid DNA is time-dependent, hence fast processing of non-clarified cell lysate is desirable (Urthaler *et al.* 2005). Due to the large pore size of the monolithic columns, it is possible to process particulate-containing solutions at high flow rates without increases in backpressure or a reduction in binding capacity (papers III and IV) (Figure 5).

Binding of large bio-molecules such as plasmid DNA to ion-exchange ligands depends strongly on the conformation of both the DNA and the ligand. Ligands located on flexible arms adopt preferable conformations for stronger binding. Grafting chromatographic matrices with layers of polymers enables the introduction of specific properties derived from the grafted layer while preserving the properties of the chromatographic matrix (Rohr *et al.* 2003). Grafting weak polyelectrolyte chains is interesting as it results in adsorbents with polymer brushes capable of significant changes in chain conformation (due to the co-operative nature of conformational transitions in the polymers) in response to small changes in environmental conditions, such as salt concentration and pH (Zhang *et al.* 2005). The polymer chains, which are flexible and protrude into solution, act as "tentacles" capable of multipoint interactions with negatively charged molecules such as DNA. Tentacle chromatography is superior to conventional ion exchange chromatography due to enhanced accessibility of the ligand to the oppositely charged groups located on target molecules (Muller 1990).

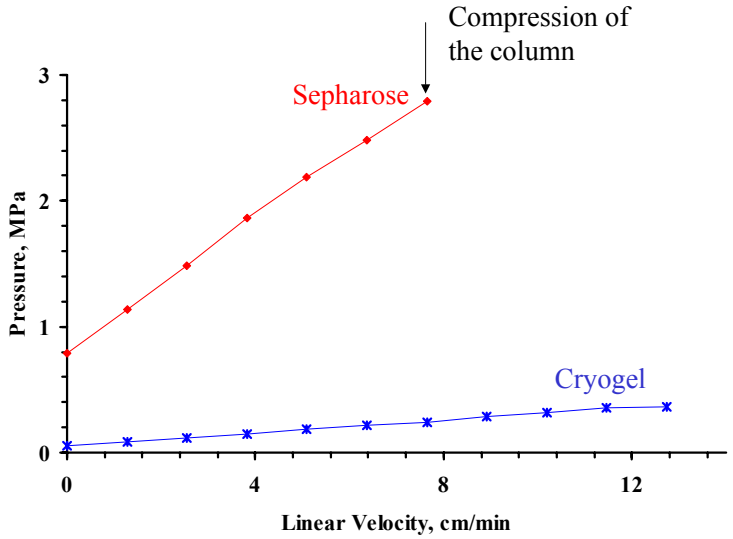

Figure 5. The difference in backpressure observed between a macroporous monolithic column (star) and a column of similar size (2.5 x 1 cm ID) packed with Sepharose CL-4B (closed rhombus). Chromatography was performed on a Bio-Rad FPLC system, and the flow rate was increased stepwise every minute. The backpressure was recorded and the experiment was stopped when the Sepharose CL-4B bed collapsed due to compression.

DNA binding to a grafted polycation results in the formation of so-called polyelectrolyte complexes (PECs). When formed in solution, PECs precipitate if the number of positive charges on the polycation is

equal to the number of negative charges on the DNA (Figure 6). The interaction depends on the polymer chain length, charge type (e.g., quaternary, tertiary or primary amino group) and density, pH, salt concentration and the nature of the counter ions (Izumrudov *et al.* 2003; Wahlund *et al.* 2003). To some extent, the salt concentration required for the solubilization of PECs, formed by the binding of DNA or RNA to polycation grafts, reflects the relative strength of the complex. The PEC formed between polycation and DNA is much stronger than that formed between polycation and RNA, as PECs formed by RNA are completely solubilized at 0.4 M NaCl, whereas salt concentrations higher than 0.7 M NaCl are required to solubilize PECs formed by DNA. Hence, there is an operational window between 0.5 M and 0.7 M NaCl allowing for the specific precipitation of DNA, while leaving the RNA predominantly in solution. This approach has been successfully used for the specific precipitation of plasmid DNA from clarified alkaline lysates (Wahlund *et al.* 2004a; Wahlund *et al.* 2004b). These results suggest the possibility of using grafted polycations on the macroporous cryogels for the selective capture of plasmid DNA.

The formation of PECs is an entropically driven process with free energy gain occurring via the release of immobilized counter-ions in the vicinity of the polymer chain into the bulk solution (Kabanov 2005). Concomitantly, PEC formation results in free energy loss due to the restricted mobility of the polycation and polyanion chains in the PEC compared with that in the free state. Following grafting, the mobility of the polycations is already restricted due to immobilization on the surface, and one may expect that PEC formation between the polyanion (nucleic acid) and immobilized polycation will be stronger as the entropy gain due to the release of counter-ions will remain essentially the same, whereas the loss due to the restriction mobility of polymer chains will be less as the chain mobility is already restricted prior to PEC formation. Thus higher salt concentrations are needed to dissociate the PECs formed with grafted polycations compared with PECs formed with soluble polycations.

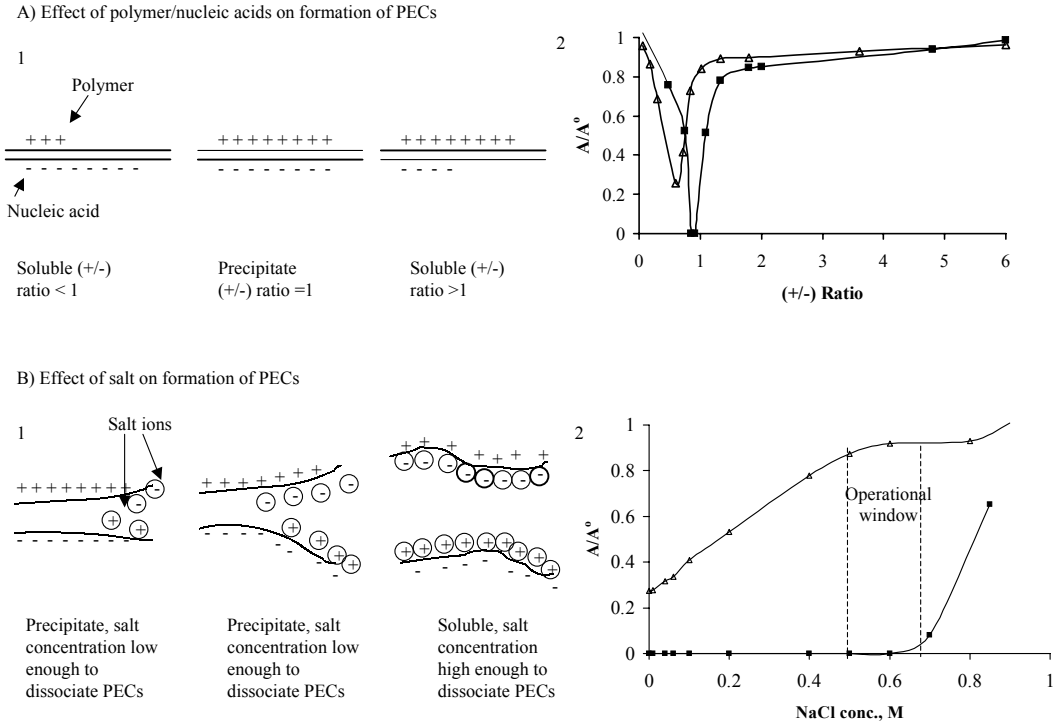

Figure 6. Polyelectrolyte complexes of nucleic acids with polycations.
A. Schematic representation of the effect of molar ratio (+/-) on the formation of PECs between polymeric chains carrying quaternary amines and phosphate groups respectively (1); relative residual absorbance of RNA (open triangle), and genomic DNA (closed square) remaining in solution after precipitation with polyDMAEMA as a function of the charge ratio (+/-) at pH 5 (2).
B. Schematic representation of the effect of salt concentrations on the solubilization of PECs (1); the effect of salt concentration on the solubilization of PECs between polyDMAEMA and genomic DNA (closed square) or RNA (open triangle) (2). The relative residual absorbance of nucleic acids remaining in the solution after precipitation with polyDMAEMA was expressed as (A/A^o).

In paper IV, polycations with tertiary and quaternary amine groups were introduced into the macroporous cryogel matrix by graft polymerization of N,N,-dimethylaminoethyl methacrylate (DMAEMA) and (2-(methacryloyloxy)ethyl)-trimethyl ammonium chloride (META) onto the pore surface of the chromatographic matrix. In addition, partial quaternization of polyDMAEMA was used (Figure 7).

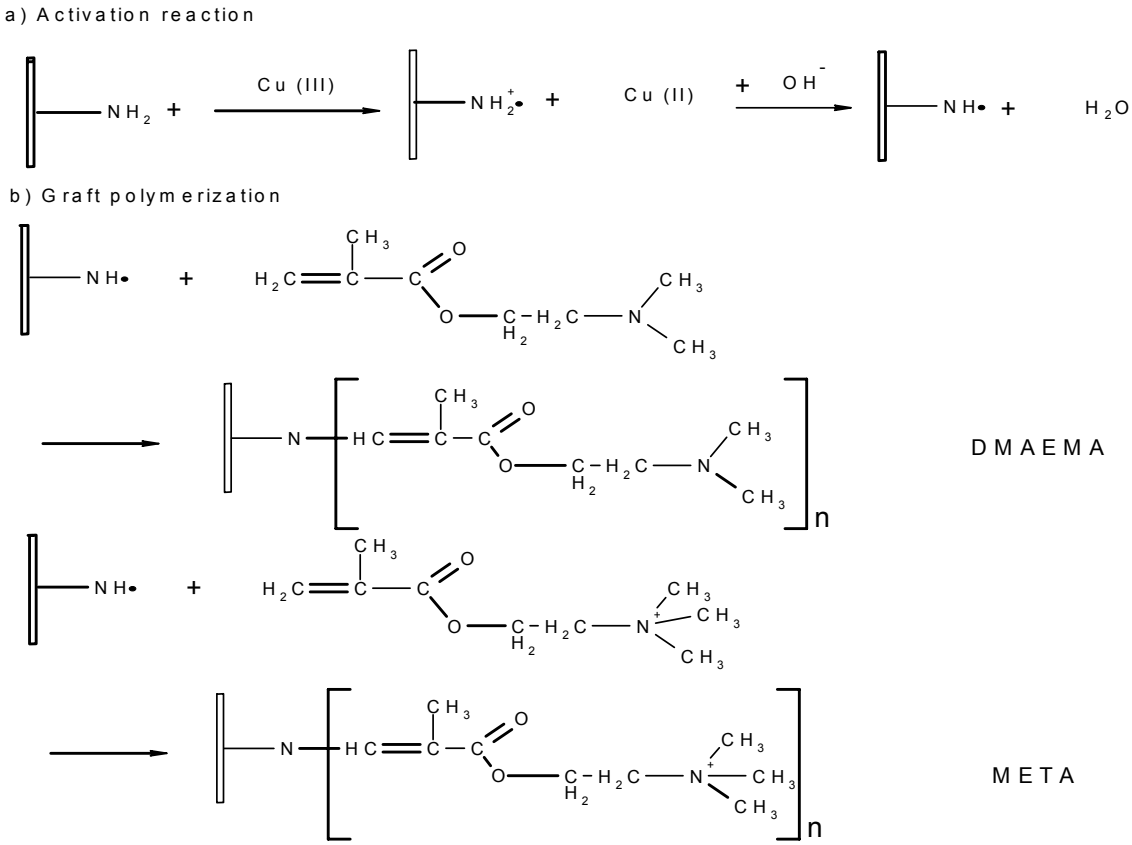

Figure 7. Polycation grafting onto a macroporous polyacrylamide cryogel.

Plasmid DNA was efficiently captured by polyMETA-grafted, polyDMAEMA-grafted and partially quaternized polyDMAEMA–monolithic macroporous cryogel columns directly from non-clarified alkaline lysate; bound plasmid DNA was eluted with a NaCl gradient. When eluted with a stepwise NaCl gradient from a polyMETA-grafted column, the plasmid DNA was free from RNA and contamination with host proteins was negligible (Figure 8). A small percentage of RNA and protein was co-eluted with plasmid DNA from both polyDMAEMA-grafted and partially quaternized polyDMAEMA–grafted columns (paper IV).

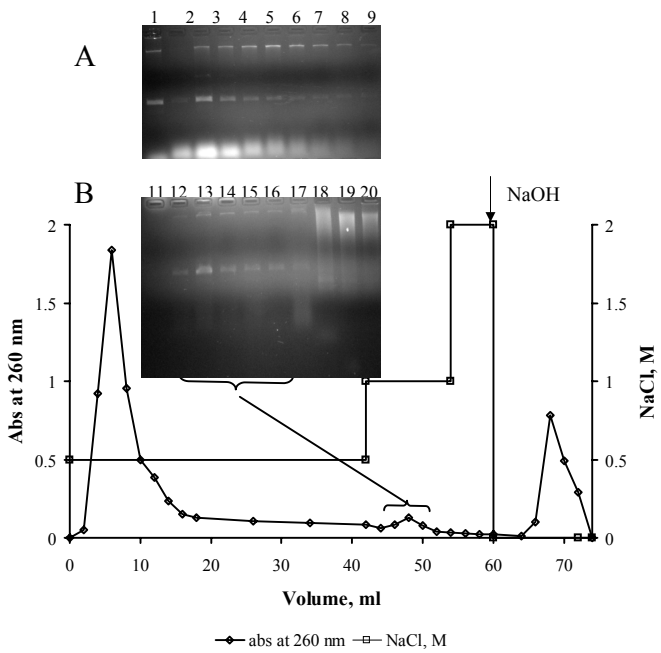

Figure 8. Chromatography of non-clarified alkaline *E. coli* lysate on poly-META-grafted monolithic macroporous cryogel columns. Experimental conditions: 1 ml of sample was loaded onto a poly-META-grafted macroporous monolith (grafting density 58%) in running buffer (20 mM sodium phosphate buffer pH 7.2 with 0.5 M NaCl), followed by washing at a flow rate of 4 ml/min. Stepwise elution was carried out using 1 and 2 M NaCl in running buffer at a flow rate of 1 ml/min. Finally isocratic elution was carried out with 0.275 M NaOH indicated by an arrow. Absorbance at 260 nm was measured (open rhombus). Arrows indicate the peaks with the highest plasmid content and their corresponding lanes in agarose gel electrophoresis.
Insert: Agarose gel electrophoresis. Gel A: applied (lane 1), breakthrough (lanes 2-10). Gel B: elution with 1 M NaCl (lanes 11-16), 2 M NaCl (lane 17) and elution with 0.275 M NaOH (lanes 18-20) fractions. Electrophoresis was carried out in 1% agarose with running buffer (40 mM Tris base, 20 mM sodium acetate, 2 mM EDTA, pH 8.3) containing 0.05 µg/ml ethidium bromide. Electrophoresis was carried out at 40 V for 3 hours.

4. REMOVAL OF CONTAMINANTS

The production of biologically active compounds via fermentation of *E. coli* cells is usually associated with the release of bacterial endotoxin (BE) into the fermentation broth, especially after cell lysis. Bacterial endotoxin, also called lipopolysaccharide (LPS), is an integral component of the outer cell wall of gram-negative bacteria and plays a key role in the pathogenesis of endotoxemia and septic shock (Fang *et al.* 2004) (Figure 9). At the end of the purification train for the recombinant protein, the removal of impurities such as BE is recommended. Indeed, fast and economical decontamination of protein solutions is required to preserve the activity of the labile protein. Authority guidelines for biopharmaceuticals

require that the level of endotoxin for intravenous application is not more than 5 endotoxin units (EU) per kg body weight per hour (Hirayama *et al.* 2002). One EU equals 100 pg of standard endotoxin.

Negative affinity chromatography has been employed for endotoxin removal. The biological activity of BE is not of concern, whereas the biological activity of the product (protein) should be preserved with 100% recovery (Anspach 2001). Selective binding between the immobilized ligand and endotoxin, without affecting the target protein, is required. The affinity ligand should be able to bind BE, which is present in very low concentrations, in the presence of protein of up to 6 orders of magnitude higher concentrations (Anspach 2001; Petsch *et al.* 1998). Bacterial endotoxin removal from protein-containing solutions also depends on the nature of the protein (Talmadge *et al.* 1989).

Polycationic ligands, such as polyethyleneimine (PEI) and poly(ε-lysine) (Sakata *et al.* 2002) have been reported to have a high affinity to BE and have been used for BE capture. Highly branched PEI with a ratio of primary, secondary and tertiary amines of 1:2:1, a molecular weight of 60 kDa, a pK > 9 for primary amino groups and a pK > 10.5 for secondary amines, has been also used (Petsch *et al.* 1997; Petsch *et al.* 1998).

Bacterial endotoxin has been removed from protein solutions using cellulose columns with immobilized poly(ε-lysine) at physiological pH (Bemberis *et al.* 2005). Efficient removal of BE from a bovine serum albumin (BSA) solution was reported using nylon flat-sheets with immobilized poly(L-lysine) (Petsch *et al.* 1997). Several reports on the capture of BE by using matrices with immobilized PEI showed efficient BE clearance (Anspach *et al.* 2000; Hirayama and Sakata 2002; Petsch *et al.* 1998).

The cyclic peptide antibiotic, polymyxin B, disorganizes the bacterial cell wall through its interactions with lipid A. Polymyxin B immobilized on a solid matrix has also been used as an affinity ligand for BE removal (Issekutz 1983; Liu *et al.* 1997). However, pronounced protein losses due to the binding of negatively charged protein to polymyxin B have been reported. Karplus *et al.* showed a loss of 24% of bovine catalase with a 10^3 BE reduction (Karplus *et al.* 1987).

Lysozyme from hen egg white, is an enzyme with a molecular weight of 14.4 kDa and a p*I* of 11.2 which catalyses the hydrolysis of peptidoglycan in the cell wall. Lysozyme possesses a positive charge under neutral conditions, while BE is negatively charged. Lysozyme binds BE through electrostatic forces and with a concomitant hydrophobic interaction with the acyl-chain region (Brandenburg *et al.* 1998). BE and lipid A bind to lysozyme with a 3:1 molar ratio (Brandenburg *et al.* 1998).

The appropriate selection of environmental conditions, such as pH and salt concentration, plays an important role in BE removal from a protein-containing solution. The pH values below that of the isoelectric point of a protein ensure best clearance and high recovery (Petsch *et al.* 1997). Basic proteins possess a net positive charge at pH values close to 7 and act as carriers for BE, hence decreasing BE clearance. The binding between the matrix with immobilized ligand and BE should be more efficient than the binding between BE and basic proteins.

In paper III, macroporous monolithic cryogel columns with immobilized polyethyleneimine, polymyxin B and lysozyme were employed for BE capture from BSA solution. Columns with immobilized polymyxin B showed nearly 100% recovery of BSA and almost 100% BE capture at pH 7.2. Bacterial endotoxin was quantitatively bound to the cryogel column with immobilized lysozyme at pH 4.7 with 100% BSA recovery, whereas at pH 7.2, 35% of the BE was in the breakthrough alongside the BSA. Lipopolysaccharide forms micelles due to the bridging of phosphate groups located on LPS monomers by divalent cations (Figure 9). Chelating agents such as EDTA bind divalent ions and promote dissociation of the micelles formed by LPS monomers. Addition of 0.01 M EDTA thus improved the BE recovery from the cryogel columns with immobilized lysozyme to 90% at pH 7.2. BE removal with a clearance factor of 10^4 from *E. coli* cell lysate at pH 3.6 was achieved using cryogel columns with immobilized PEI. BE was effectively removed at pH values of 3.6 and 7.2 and at low and high flow rates (Figure 10). Surprisingly, the dynamic binding capacity of cryogel columns with immobilized PEI toward BE, increased with increasing flow rate. The rationale behind this could be that, at low flow rates, the liquid only passes through the very large pores of the monolithic column; hence, the ligands immobilized at the

surface of smaller pores are essentially unavailable. At higher flow rates, the mobile phase may begin to pass through the smaller pores and thus be exposed to a greater area of immobilized ligand.

Negative chromatography using macroporous monolithic cryogel columns proved to be an efficient means of removing BE, both from protein solutions (e.g., target protein product), and from cell lysates (e.g., waste waters). Adjusting conditions such as the ionic strength and pH of the applied solution or adding a chelating agent eliminates retardation of the target protein on the column.

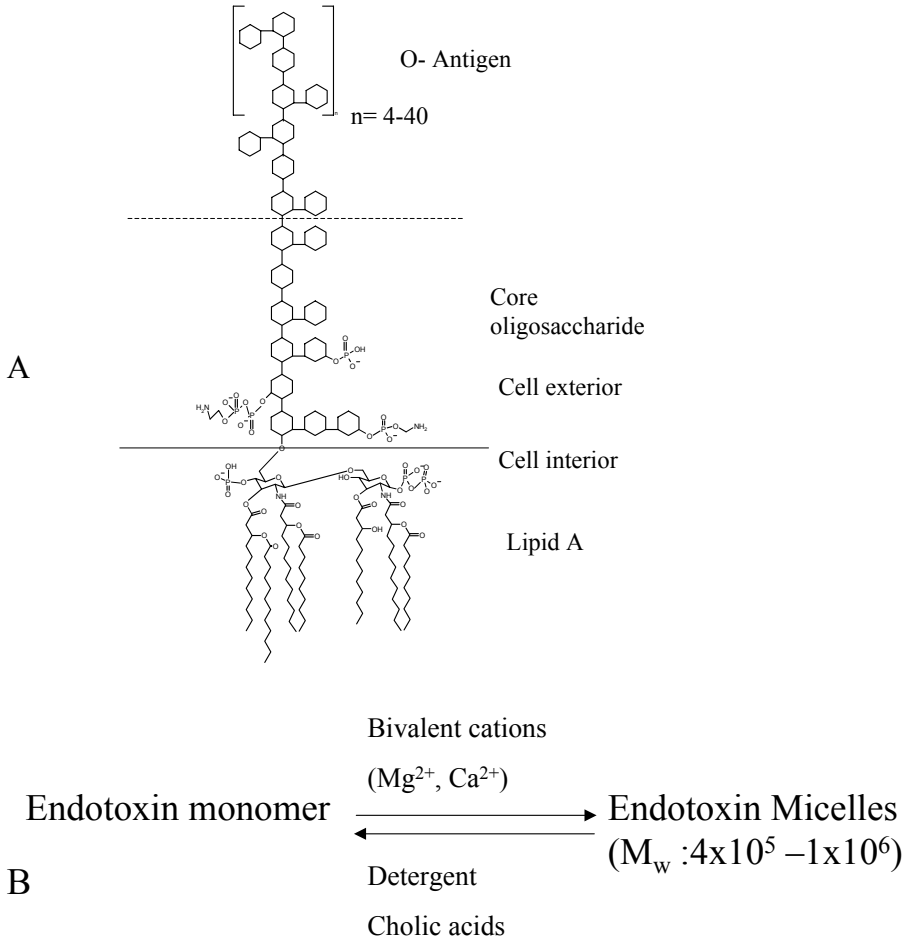

Figure 9. A: Chemical structure of bacterial endotoxin. B: Formation of endotoxin micelles.

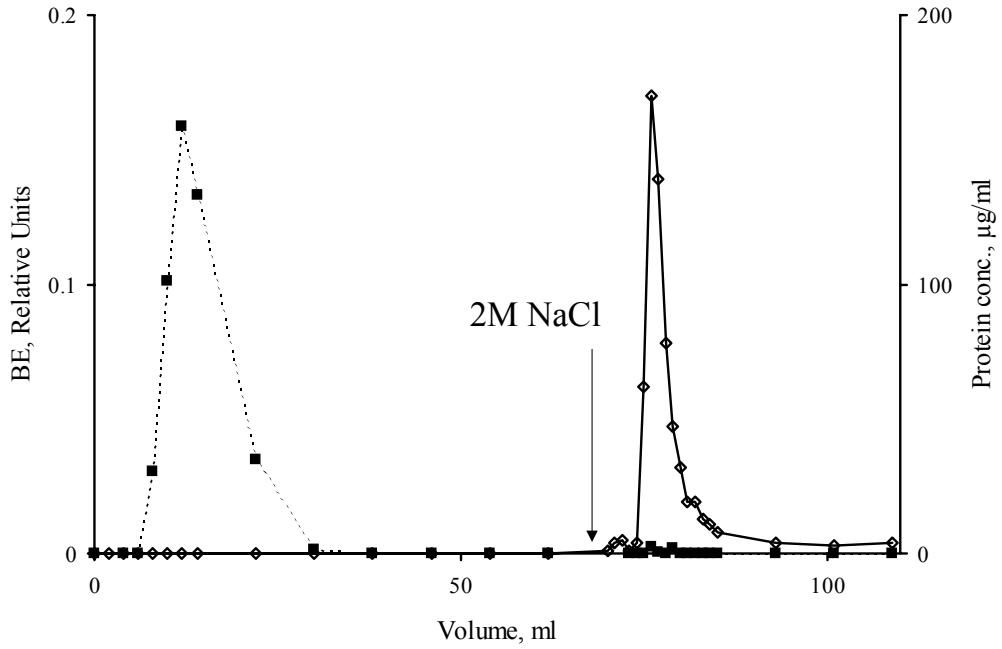

Figure 10. Capture of bacterial endotoxin (BE) from a BSA solution using macroporous monolith columns with immobilized PEI. Experimental conditions: BSA solution containing BE was applied in 1 ml of the running buffer followed by washing and elution with 2 M NaCl in the running buffer at a flow rate of 1 ml/min. BSA was assayed using BCA (closed square, dashed line) and BE was assayed by measuring absorbance at 280 nm with subtraction of the BSA contribution to absorbance (open rhombus, straight line).

CONCLUSIONS

The individual studies presented in this thesis clearly demonstrate that macroporous monolithic cryogel columns and composite cryogel matrices are materials with a large potential for the capture of biologically active compounds from a variety of biotechnological feeds, including those which contain particulate matter. The large interconnected pores typical of monolithic cryogels allow for minimal flow resistance and efficient convective mass-transport of large biomolecules and bio-aggregates, such as plasmid DNA and micelles formed by bacterial endotoxin. Further developments in this direction will concentrate on the design and production of highly selective, affinity ligands capable of the exclusive adsorption of the target biomolecule. Such ligands could be developed, for instance, using a triple-helix approach for plasmid DNA capture. On the other hand, an area where the full potential of cryogels could realize, may be the processing of even larger biological entities such as viruses, and microbial and even

mammalian cells. One may foresee the development of affinity chromatography methods for the integrated purification of viruses and the selective isolation of particular cell lines from microbial consortia or animal tissues. The challenge will be to isolate sub-populations of cells at different stages of development (e.g., lag-phase, exponential growth or a stationary phase for microorganisms) or differentiation (e.g., differentiated stem cells).

Handling bioparticles in a chromatographic mode presents a variety of potential complications when compared with handling soluble macromolecules, particularly large molecules such as plasmid DNA. The bioparticles have a negligible diffusing capacity in a chromatographic matrix and can be transported only via convective mass-transport. Moreover, the adsorption of particles to the affinity matrix is governed not only by specific affinity interactions of particular ligand-receptor pairs, but also by surface effects at the interface between the gel phase and the liquid phase. This imposes specific requirements for the development of affinity ligands for bioparticle separation. The brushes of grafted polymers carrying numerous ligands on flexible “tentacles” may be a solution. Such brushes may allow multiple ligand-receptor interactions ensuring strong binding of bioparticles, and, once bound to the brushes, bioparticles may be prevented from direct contact with the gel–liquid interface. Thus, non-specific surface effects may be avoided.

The ability of macroporous cryogels to retain liquid inside the pores via capillary forces (so-called *drainage-protected* property) offers another interesting perspective for their application in bioseparation. Handling samples using drainage-protected, monolithic cryogel mini-columns in a 96-well format is very convenient. Multiple samples of cell suspensions and cell homogenates could be easily processed in parallel. The captured biomolecules or bioparticles could be directly analyzed once bound to the cryogel mini-columns, for example through the quantitative measurement of fluorescence from markers such as green fluorescent protein. Alternatively, bound enzymes (provided binding is not interfering with the active site of the enzyme) could be quantified on the basis of their enzymatic activity after incubation with substrate and quantification of the substrate conversion. Bioparticles bound to cryogel mini-columns

could also be assayed directly using antibodies against specific antigens on the surface of the bioparticles in ELISA-like procedures. Alternatively, bound cells could be quantified on the basis of their metabolic activity after incubation with nutrients and quantification of the products formed.

In conclusion, the applications of macroporous monolithic cryogels in bioseparation described in this thesis represent merely the beginning of a systematic study of this interesting material. One could say that the limits are set only by the imagination and creativity of the researchers.

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