BI-LAYERED CHROMATOGRAPHY MATRICES FOR THE PURIFICATION OF BIOLOGICAL NANOPLEXES

by

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Dedication

I would like to dedicate this thesis to myself (1981-present)

For tears, sweats and blood that had been shed along the way

September, 2012

Abstract

The preparations of SEC/IEC supports from commercially available, underivatised base matrices by AGE activation-partial bromination technique via two different approaches; (i) viscosity enhanced-reaction diffusion (VE-RD) and (ii) microwave-assisted reaction diffusion, were studied and optimised. Selected supports produced by both approaches were further evaluated by applying to packed bed chromatography system in order to purify target pDNA from neutralized *E.coli* cleared lysates.For VE-RD approach, viscosity enhancement by sucrose ($\leq$ 0.032 Pa.s) was found to greatly aid the creation of thin inert outer layer. The optimum condition for SEC/IEC Sepharose CL-6B production observed was 10% single bromination at room temperature in 64% (w/v) aqueous sucrose without sodium acetate addition. The effects of different base matrices, conductivities, linear flow rate, target pDNA sizes and support preparations on chromatography performance were investigated. Microwave irradiation heated up the reaction in a rapid controlled manner compared to conventional heating. SEC/IEC Sepharose CL-6B produced via microwave-assisted reaction diffusion approach at 80^{o}C with 10% partial bromination showed almost complete surface charge elimination with the highest SI value of 57.4. This support showed the high core binding capacity. However, a delayed pDNA breakthrough was also observed. It was noted that the plasmid forms remain unchanged after SEC/IEC column purification.

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Last but not least, I would like to thank my head of chemical engineering department in Thailand for enlightening me that my whole PhD project, Bioseparation, is NOT engineering.

I also would like to remark the similarity of the propose of this thesis which is to capture impurities from cell lysate loaded onto SEC/IEC column without binding to plasmid DNA to the words of a Cistercian church leader, Arnaud Amalric, in 1208; *"Kill them all, God will recognise his own"*.

Contents

Chapter 1: Introduction

List of figures

Chapter 1: Introduction

Chapter 2: Viscosity enhanced, reaction-diffusion balancing for the preparation of SEC/IEC supports

Chapter 3: Evaluations of SEC/IEC supports prepared by viscosity enhanced, reaction-diffusion balancing for the separations of RNA from *E. coli* neutralised lysates containing pDNA

Appendix

List of tables

Abbreviations

SEC	Size exclusion chromatography
IEC	Ion exchange chromatography
EBA	Expanded bed adsorption
VE-RD	Viscosity enhancement-reaction/diffusion balancing
pDNA	Plasmid DNA
gDNA	Genomic DNA
mRNA	Messenger RNA
HM-RNA	High molecular weight RNA
kb	Kilobase
AV	Adenovirus
AAV	Adeno-associated virus
HSV	Herpes simplex virus
LV	Lentivirus
SI	Selectivity index
k/D	Reaction/diffusion rate ratio
CV	Column volume
ESEM	Environmental scanning electron microscopy
EDS	Energy dispersive spectroscopy
AGE	Allyl glycidyl ether
Q	Quaternary amine
DMSO	Dimethyl sulfoxide
BSA	Bovine serum albumin
DPA	Diphenylamine
BCA	Bicinchoninic acid

Chapter 1

Introduction

1.1 Nanoplex

Nanoplexes are biotechnological products which are nanoparticulate in nature and generally composed of sophisticated biological structures with the large particle size of 10 - >300 nm (Zhang et al., 2001b, Jahanshahi, 2004). Examples of nanoplexes includes non-viral vectors i.e. plasmid DNA, polyplex and lipoplex; viral vectors, mega molecular vaccines and virus-like particles (VLPs) (Arpanaei et al., 2010, Zhang et al., 2001b). Main applications of nanoplexes are drug and gene delivery vehicles, especially for gene therapy (Azari et al., 2011, Bonoiu et al., 2009, Thomas et al., 2010) which dictate the requirements of high product purity in order to be administered safely in human clinical treatments. Purification of nanoplexes have several challenges due to the similarity of target molecules and impurities for example, in plasmid DNA purification where impurities such as RNA, genomic DNA, proteins, and endotoxins possess the same charge properties as plasmid, worsen by similarity in size between pDNA, RNA and genomic DNA resulting in co-purification of impurities alongside the plasmid of interest (Prazeres and Ferreira, 2004, Wicks et al., 1995). Other challenges in nanoplex purification are caused by the large size of the molecules, fragility and complex chemical properties on the surface (Arpanaei et al., 2010). The large size of nanoplexes results in a compromised mass transfer especially for chromatography purification where commercially available matrices are designed to accommodate much smaller molecules such as proteins. For example, a study on direct visualization of plasmid

DNA adsorption on individual Q Sepharose XL particles revealed that plasmid adsorption is restricted on the surface of the support (Ljunglöf et al., 1999). The fragility of nanoplex molecules is another factor to consider in purification process. Shear-induced structural damage can occur in some nanoplexes i.e. budding viruses such as lentiviral vectors (Sheridan, 2011) and plasmid DNA (Levy et al., 1999). This shear sensitivity property led to difficulties in process design since some unit operations i.e. homogenization or freeze-thaw are not applicable (Carlson et al., 1995, Kong et al., 2008), making chromatography more preferable due to its considerably mild process.

In this chapter, the main applications of nanoplexes, i.e. gene therapy and DNA vaccination, are further discussed, as well as gene delivery vectors. Since plasmid DNA was used as the test system representing nanoplexes in the entire study, the detailed information and discussions will be mainly focused on plasmid DNA purification and especially on chromatography techniques. Lastly, the idea of bilayered SEC/IEC supports is also explained and discussed.

1.2 Gene therapy and DNA vaccination

1.2.1 Overview

Since the success of the first approved clinical trial on a four year-old girl, Ashanti DeSilva, suffering from severe combined immunodeficiency (SCID) in 1990 (Blaese et al., 1995, Sheridan, 2011, Bess, 2008), gene therapy and DNA vaccination have raised huge interests as the new choices for preventing or treating a disease via gene transfer, with great promises to fulfill current requirements worldwide for therapeutic usages. The prospect of genetic medicine that could treat diseases by compensating the errors in an individual's DNA

sequence emerged from the combination of the successful development of virus-based method transformation in mammalian cells in the1960s and the advent of recombinant DNA technology in the 1970s (Friedmann, 1992, Sheridan, 2011). In 1990, the first approved gene therapy was performed by Blaese and coworkers in order to treat SCID using *ex-vivo* retroviral mediated gene transfer into T-cells. One of the two children undergone the treatment, DeSilva, exhibited a temporary response. Although this procedure did not correct the defective genes or enable the withdrawal of enzyme-replacement therapy, this very first attempt showed that the procedure was feasible and safe, led to the persistence of transduced T-cells as much as 15-20 years later (Fischer et al., 2010). On October 16, 2003, another milestone in gene therapy was established when a recombinant human adenovirus-p53 encoding the p53 tumor suppressor gene (Gendicine™) developed by Shenzhen SiBiono GeneTech (Shenzhen, China) was approved by the State Food and Drug Administration of China (SFDA) for the treatment of head and neck squamous cell carcinoma (Peng, 2005). Gendicine™ was formally launched in April 2004 and became the world's first gene therapy product approved by a governmental agency (Peng, 2005, Thanou et al., 2007). Up to date, Gene therapy trials have been carried out worldwide in hopes for the approved clinical applications. Examples of clinical trial status of gene therapy products up to 2011 are presented in Table 1.1.

Table 1.1 Examples of clinical trial status of gene therapy products up to 2011 (Sheridan, 2011)

Company	**Therapy**	**Indication**	**Phase of development**
Retrovirus			
San Raffaele	ADA-SCID GT: CD34+ cells transduced with Moloney murine leukemia virus carrying ADA gene	Primary immunodeficiencies	Phase 1/2
Neurologix	NLX-P101: GAD in virus injected into subthalmic nucleus of the brain	Parkinson's disease	Phase 2
Ribozyme CO, USA	CD34+ cells transduced with retrovirus vector with multiple ribozymes	Non-Hodgkin's lymphoma HIV/AIDS	Phase 2
Tocagen San Diego	Toca-511: replication competent retrovirus with prodrug activator cytosine deamidase gene injected into tumor	Glioma	Phase 1/2
Lentivirus			
Bluebird Bio	Bluebird Bio LentiGlobin: introduces globin gene into patient hematopoietic stem cells	β-thalassemia and sickle cell anemia	Phase 1/2
Lentigen	LG-740: T cells treated *ex vivo* with lentivirus with chimeric T-cell receptor gene	B-cell leukemia and lymphoma	Phase 1
Oxford BioMedica	ProSavin: lentivirus with three genes required for dopamine biosynthesis injected into striatum of brain	Parkinson's disease	Phase 1/2
Adenovirus			
Advantagene MA, USA	ADV-tk: replication-deficient adenovirus with HSV thymidine kinase gene injected into tumor during biopsy	Glioma Pancreatic cancer	Phase 1 Phase 1
Applied Genetic Technologies FL, USA	rAAV1-CB-hAAT: AAV with alpha-1-antitrypsin gene	Alpha1-antitrypsin deficiency	Phase 2
	rAAV2-CB-human retinal pigment epithelium specific 65 dalton protein (RPE65)	Congenital amaurosis (blindness with mutation in RPE gene)	Phase 1/2
Amsterdam Molecular	AMT-101: adeno-associated virus with human lipoprotein lipase gene	LPL deficiency	Filed
Aventis Paris	Ad5CMV-p53	Head and neck cancer	Phase 2
Biogen	Adenoviral mediated interferon-β	Pleural mesothelioma Colon cancer, glioma	Phase 1 Phase 1/2
Ceregene San Diego	CERE-120: adeno-associated virus with neurotrophic factor, neurturin	Parkinson's disease	Phase 1/2
	CERE-110: adeno-associated virus with gene for nerve growth factor	Alzheimer's disease	Phase 1/2

Table 1.1 (continued) Examples of clinical trial status of gene therapy products up to 2011 (Sheridan, 2011)

Company	Therapy	Indication	Phase of development
Adenovirus (continued)			
Celladon CA, USA	SERCA-2a: sarcoplasmic reticulum Ca2+ATPase gene with AAV vector	Congestive heart failure	Phase 1/2
Genzyme	AAV2-sFLT01: adeno-associated virus with anti-VEGF	Wet macular degeneration	Phase 1
GenVec	TNFerade: replication deficient adenovirus with TNF-a controlled by radiation-induced promoter	Esophageal cancer	Phase 2
Shenzhen SiBiono GeneTech	rAd-p53: replication deficient adenovirus encoding hu recombinant p53	Advanced thyroid tumors, oral, maxillofacial tumors	Phase 4
Targeted Genetics Seattle	tgAAG76: AAV with human RPE65	Congenital amaurosis (blindness with mutation in RPE gene)	Phase 1/2
	tgAAC94: AAV2 with TNF-a –IgG1 fusion gene	Arthritis	Phase 2 completed
Plasmid			
AnGes, Tokyo	Hepatocyte growth factor-plasmid	Arterial disease	Phase 2
Genexine Seoul, Korea	GX-12: plasmid plus IL-12 mutant, given with HAART	HIV-AIDS	Phase 1
ScanCell Nottingham, UK	SCIB1: plasmid with tyrosine-related protein	Melanoma	Phase 1/2
Vical San Diego	Allovectin-7: plasmid with gene for HLA-B7 and b2microglobulin genes, injected into tumors	Melanoma	Phase 3
ViroMed MN, USA	VM202: plasmid with two isoforms of hepatocyte growth factor, HGF728 and HGF 723	Limb ischemia Myocardial ischemia	Phase 2 Phase 1/2
Other			
Diamyd Medical Stockholm, Sweden	Nerve Targeting Drug Delivery System: HSV vector with enkaphalin administered intradermally	Pain	Phase 1
Epeius Biotechnologies CA, USA	Rexin-G: nanoparticle delivering cyclin-G1 gene	Advanced pancreatic, metastatic breast, osteosarcoma, and soft tissue sarcoma	Phase 1/2
MultiGene Vascular Systems Nesher, Israel	Patient cells modified with four angiogenic genes	Peripheral artery disease	Phase 1/2

Gene therapy targets to treat genetic defects by making functional genes available to assist or replace defective or mutant genes (Tente, 2011). Example of genetic defects that have been studies for a cure by gene therapy varies from innate genetic defects such as severe combined immunodeficiency (SCID) (FISCHER et al., 2011, Blaese et al., 1995, Fischer et al., 2010), to acquired diseases i.e. cancer (Frederiksen et al., 1999, Horn et al., 1995, Jia et al., 2012, Wysocki et al., 2002), AIDS (Lisziewicz, 1997, Sorg and Methali, 1997, Zaia, 2003, Delgado and Regueiro, 2005, Kitchen et al., 2011), parkinson's disease (Dass and Kordower, 2007, Fiandaca et al., 2008, Lewis and Standaert, 2008, Lim et al., 2010, Rodnitzky, 2012) even heart failure (Kawase et al., 2011, Rapti et al., 2011, Hajjar, 2012, Delgado and Regueiro, 2005, Kitchen et al., 2011) etc. Gene therapy treats diseases via three different mechanisms; (i) by introducing therapeutic genes into a host to replace defective genes (replacement therapy); (ii) by suppressing expression of certain undesirable genes (antisense therapy); or (iii) provide additional biological activities (supplement therapy) (Yang et al., 1996).

For inserting genetic material into human chromosomes, two different methods have been employed. The first is '*ex vivo*' technique in which genetic alterations of target cells occur outside of the body (Fig 1.1a). Cells from the affected tissue area are surgically removed, injected with therapeutic DNA to correct the disease and grown in cultures. The corrected tissues are then transferred back into patient's affected organ. This method is time consuming and causes pain in patients therefore, raises more interest on another technique. The second method, namely '*in vivo*' technique (Fig 1.1b), does not require surgery. Therapeutic DNA is directly introduced into the body by cell-specific carrier, usually via gene transfer vectors, i.e. viruses or plasmids.

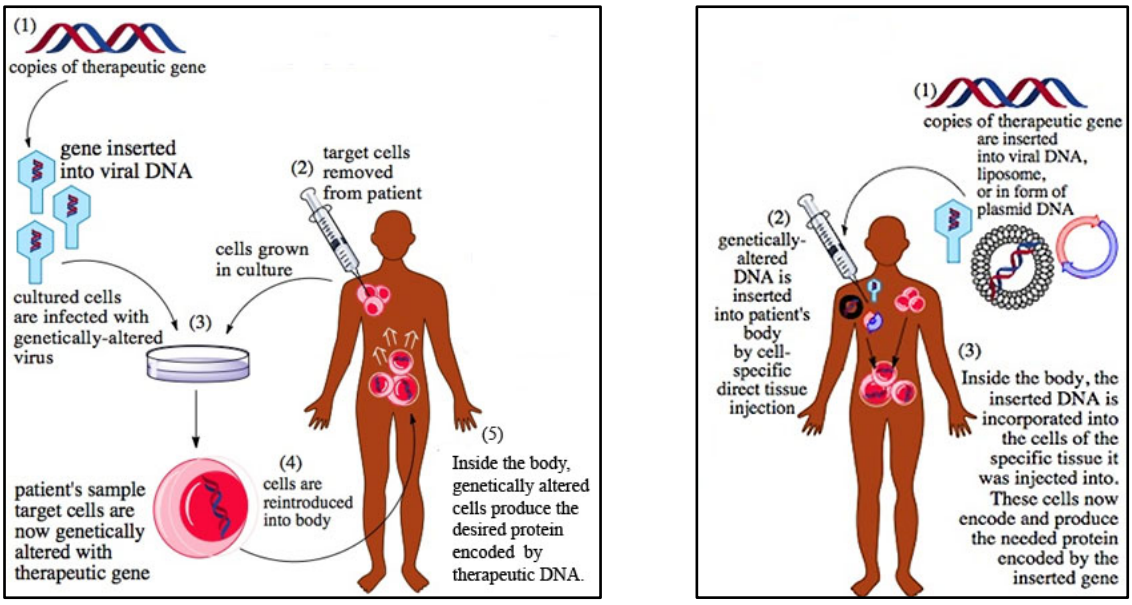

Fig. 1.1 In gene therapy, genetic materials can be inserted into human chromosomes by; a) *ex vivo* or, b) *in vivo* technique. The figure was retrieved and modified from http://gene-therapy.yolasite.com/process.php, last accessed on Sept, 3rd, 2012.

DNA vaccines bases on plasmid DNA containing specific genes encoding target proteins and can activate both cell-mediated immunity and humoral responses (Prather et al., 2003). DNA vaccinations have yielded highly attractive results against parasitic infections (Da'dara and Harn 2005); (Ivory and Chadee, 2004, Carvalho et al., 2010) such as malaria (Doolan and Hoffman, 2001, Doolan et al., 2003) and viral infection such as AIDS (Mascola and Nabel, 2001, Smith et al., 2004). Recent developments of DNA vaccine for cancer treatment have been also reported (Anderson and Schneider, 2007, Cohen, 2001, Ma and Yang, 2010). The main advantages of DNA vaccination compared to other types of vaccination (i.e. weakened bacteria or virus, modified exotoxins or modified attenuated viral vaccines) are the more cost effective process for large scale production, moderate storage conditions (stable at ambient temperature) as well as being generally regarded as safer for administration (Ferreira et al., 2000). The main disadvantage with DNA vaccine is the less efficiency compared to its viral counterparts therefore, high dose is required. It is estimated

that milligram amounts may be needed for full treatment (Ferreira et al., 2000). Fig. 1.2 demonstrates the principle and overall process of DNA vaccination. Antigen coding genes from pathogen are isolated and cloned into a vaccine plasmid. The resulting pDNA is grown in bacteria, purified and dissolved before being injected into the host, usually by a direct intramuscular (i.m.) injection. After injection the pDNA will, in theory, be taken up by a cell (e.g. myocytes) and the pDNA encoded gene(s) will be transcribed and translated into protein(s), resulting in immune responses (Gillund et al., 2008).

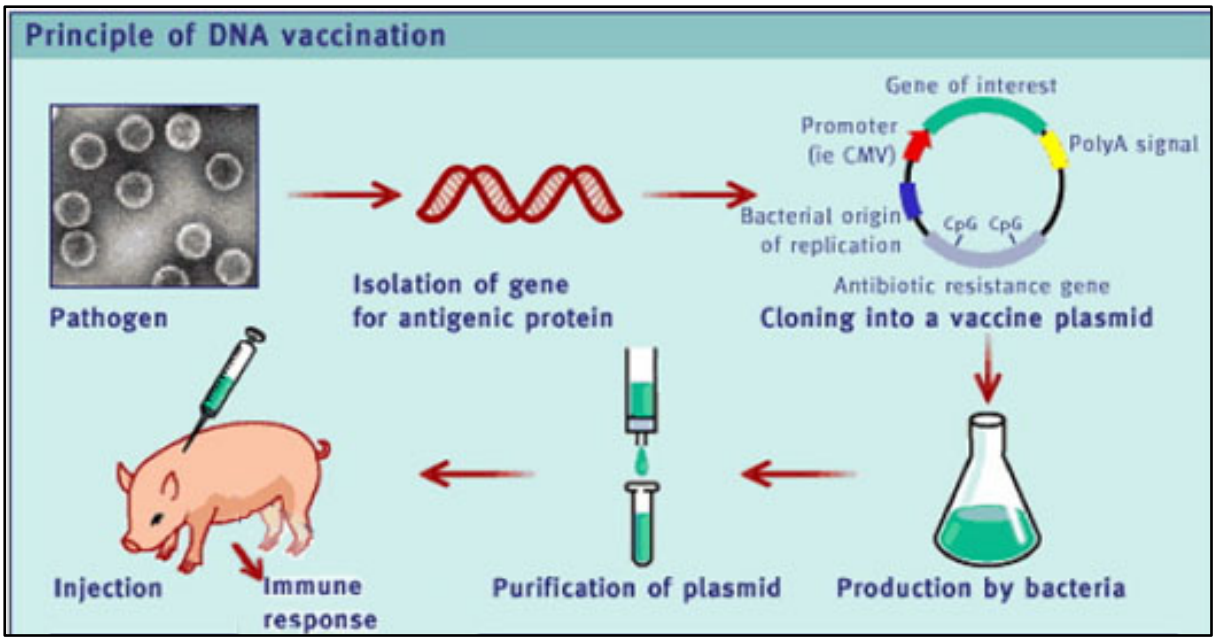

Fig. 1.2 The principle of DNA vaccination, from isolation of the gene encoding the antigenic protein to final immune responses. The figure is modified from publication of Gillund et al. (2008).

1.2.2 Vectors used in gene therapy

Vectors used for gene therapy can be categorized as viral and non-viral vectors. According to The Journal of Gene Medicine clinical trial site, vectors utilized in gene therapy clinical trials by June 2012 is presented in Fig. 1.3.

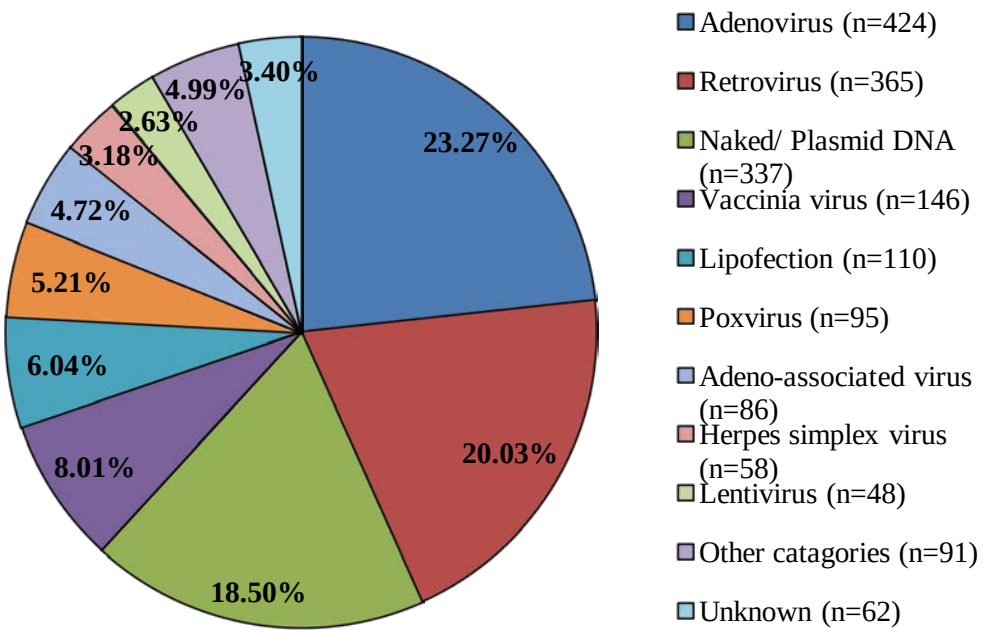

Fig.1.3 Vectors used in gene therapy clinical trials up to 2012. (Data source: The journal of gene medicine clinical trial site; http://www.abedia.com/wiley/vectors.php; last accessed on June, 17th, 2012)

1.2.2.1 Viral vectors

Viral vectors have been designed based on the advantage of the efficiency of viruses to enter and insert genetic material into target cells, with the assuring ability to transport its genomic DNA to the nucleus of the host cell without being degraded by lysosomes (Wivel and Wilson, 1998). Viral genome insertion can potentially remove the pathological functions by delivering a normal copy of defective genes and in some cases, involving the inactivation of deleterious functions (Cearley and Wolfe, 2009). Delivering the gene of interest into the target cells by viral vector is one of the basic methods of gene transfer. Many successful in gene delivery and therapy has been achieved with viral vectors due to their high transduction efficiency in cells *in vivo* (Selvam et al., 2006). However, viral vectors also possess several disadvantages, such as immunogenicity towards host, restricted

target cell types, production problems, and limited DNA carrying capacity (Wolf et al., 2009).

The major criteria in the design of viral vectors are safety, target cell specificity and availability of efficient permissive cell lines (Wu and Ataai, 2000). For viral vectors development, the usual approach is to remove the unneeded or pathogenic features of the virus while retaining the efficiency of gene delivery, expression, and persistence wherever appropriate. Designing viral vectors addresses many common considerations for both safety and biological activity including removal of virulent genes, elimination of the replication component of the parental virus, and responses to components of the vector by host (Patel and Misra, 2011). Viral-encoded functions can be separated into *cis* and *trans* elements. The *cis* elements, such as the origin of replication or the packaging signal, must be carried by the virus itself. Whereas the *trans* elements can be complemented from DNA sequences inserted into the host genome or carried on transfected plasmid. In viruses with small viral genomes, usually all *trans* genes are removed to create space for insertion of the therapeutic gene and to render the vector replication defective. The deleted genes are either stably transfected into packaging cell lines or transiently expressed by transfection of helper plasmids or helper virus. Packaging cell lines must be carefully selected and modified to avoid the presence of overlapping regions between the vector and the cell genome, thus eliminating the possibility of rescuing the replication competent virus from integrated complementary genes or provirus (Wu and Ataai, 2000). Some of the different types of viruses used as gene therapy vectors are briefly discussed as followed:

1.2.2.1.1 Retrovirus

Retroviruses are small RNA viruses that can perform reverse transcription to produce proviral double stranded DNA from its RNA genome by the activity

of the enzyme reverse transcriptase (Geiger et al., 2010, Robbins and Ghivizzani, 1998). The infection of retrovirus to target cells occurs through a specific interaction between the viral envelope protein and a receptor on the surface of the target cell (Robbins and Ghivizzani, 1998). The virus is then internalized by fusion or via endosome formation, depending on the envelope protein (Baum et al., 2006). After entering the cell, retroviruses can deliver three forms of genetic information (i) if reverse transcription does not occur, the mRNA may be subject to immediate translation; (ii) if integration of viral DNA to the host chromosome is blocked, episomal circles can be generated and may persist in non-dividing cells; (iii) if all steps of the retroviral transduction process are completed, a double-stranded DNA integrates in cellular chromosomes by an virally coded integrase enzyme and expresses therapeutic product as part of the host cell's DNA (Baum et al., 2006, Robbins and Ghivizzani, 1998). The ability of retroviruses to integrate their genome into the host chromosome enables a stable genetic modification to remain in host cell for life, unlike some other viral vectors, i.e. adenovirus, herpes simplex virus (HSV) or papilloma virus (Robbins and Ghivizzani, 1998). Delivery of nucleic acids by retroviral particles is demonstrated in Fig. 1.4.

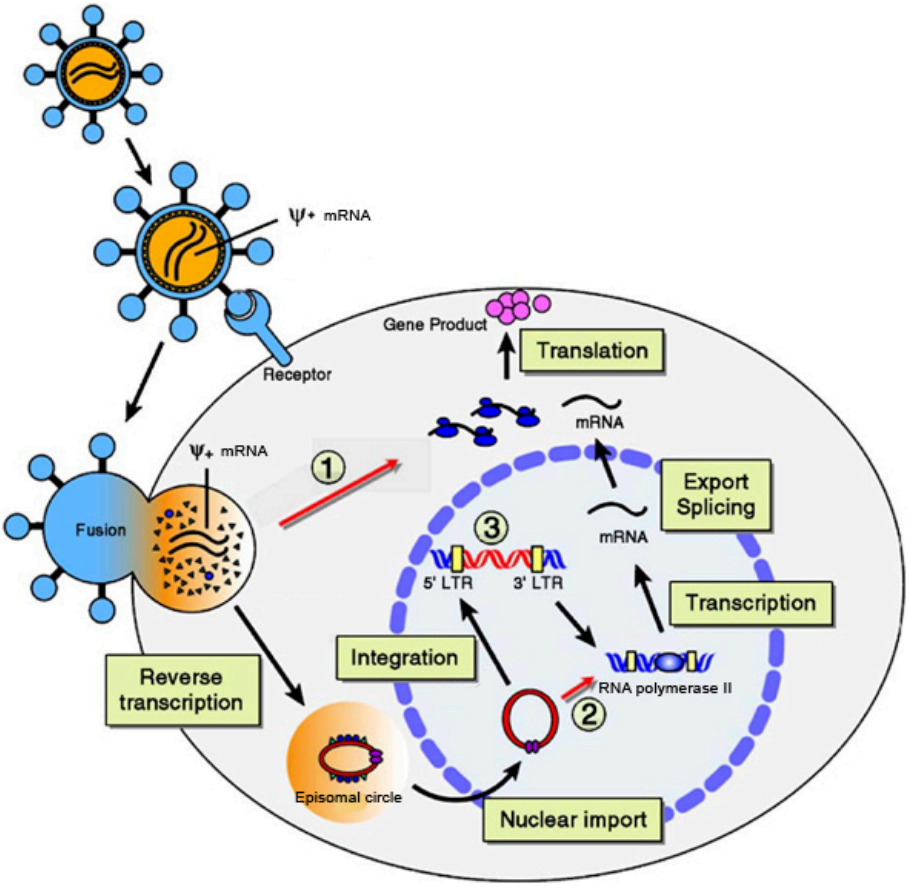

Fig. 1.4 Delivery of nucleic acids by Retrovirus. Retroviruses can deliver three forms of genetic information after receptor-mediated uptake,: (1) if reverse transcription does not occur, the mRNA may be subject to immediate translation; (2) if integration is blocked, episomal circles can be generated that may persist in non-dividing cells; (3) if all steps of the retroviral transduction process are completed, a double-stranded DNA integrates in cellular chromosomes. Diagram was modified from (Baum et al., 2006). Abbreviation; Ψ : Retroviral packaging sequence, LTR : Long terminal repeat.

1.2.2.1.2 Adenovirus (AV)

Adenovirus is a nonenveloped virus with a moderate size of 60-90 nm (Rapti et al., 2011), consists of a linear double strand DNA at the size of ~30-35 kbp with capacity for 4.5–30 kb of foreign DNA, which accommodates most of the known cDNA sequences (Lim et al., 2010). Adenovirus has been intensively used as a non-integrating vector for gene therapy due to its episomal nature which eliminates the risk of insertional

mutagenesis or oncogenesis that might occur in integrating vectors (Robbins and Ghivizzani, 1998, Geiger et al., 2010, Rapti et al., 2011). Adenovirus has a broad tissue tropism, which means it can infect a wide range of cell types, including non-dividing cells such as neurons (Wu and Ataai, 2000, Robbins and Ghivizzani, 1998, Lim et al., 2010). The cell entry pathway of adenoviral vectors is shown in Fig. 1.5.

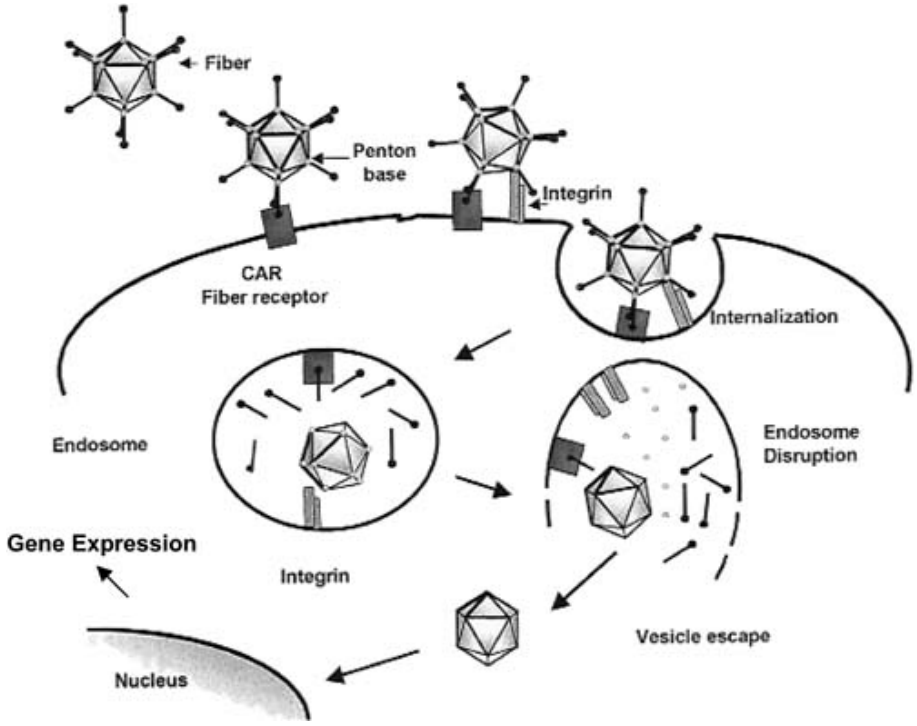

Fig. 1.5 Cell-entry pathway of the adenoviral vector. The adenovirus vector initially binds to the cell via the specific cellular receptor, coxsackievirus and adenovirus receptor (CAR). After binding, the virion achieves internalization via receptor-mediated endocytosis pathway. After internalization, the virus is localized within the endosomes. Acidification of the endosomes allows the virions to be released within the cytosol and consequently the virion will be translocated into the nucleus to begin gene expression (Contreras et al., 2004). Abbreviation; CAR: Coxsackievirus and adenovirus receptor.

Although adenoviral vectors demonstrate prolonged gene expression, its major problem is the ability to rapidly trigger strong host immune responses which potentially cause a harmful side effects (Wu and Ataai, 2000). Therefore, the use of recombinant adenoviral vectors has gained more interest.

The genome of wild type adenovirus contains five early genes responsible for the activation of viral DNA replication and the expression of viral structural proteins. These genes consist of E1A, E1B, E2, E3, and E4. In recombinant Ad vectors, portions of the early genes are replaced with the transgenic DNA of interest. The first generation adenoviral vectors was lacking only one or two early genes; E1 and/or E3 resulting in the impaired replication ability. Despite the absence of E1 gene, the first generation adenoviral vector still replicated and expressed other adenoviral gene at decreased level and was found to induce strong cellular immune response (Byrnes et al., 1996a, Byrnes et al., 1996b). The second and third generations of adenoviral vectors were created by the additional deletions of genes E2 and/or E4 and were reported to exhibit decreased immunogenicity and increased transgene capacity compared to the first generation vectors (Lusky et al., 1998, Moorhead et al., 1999).

Another development of adenoviral vectors is ‘gutless adenovirus’ which only contains a packaging signal and can carry up to 37 kb of foreign DNA (Hardy et al., 1997, Lim et al., 2010, Mandel et al., 2008). In these vectors, the expression of viral proteins was eliminated, led to an improved stability of transgene expression and reduced potential immune response. However, these vectors were reported to still induce capsid-mediated inflammatory response (Lim et al., 2010).

1.2.2.1.3 Adeno-associated viruses (AAV)

Adeno-associated virus is a small virus at a size of ~20 nm carrying a 4.7 kb single stranded DNA genome (Wu and Ataai, 2000, Geiger et al., 2010, Mandel et al., 2008). AAV vector is non-pathogenic and non-immunogenic which means that the inflammatory response toward these vectors is minimal compared to adenoviral vectors. These features, combined with the capability to infect both dividing and non-dividing cells including muscle cells and neurons with prolonged gene expression make AAV an attractive vector for gene therapy (Rapti et al., 2011, Lim et al., 2010).

The wild-type AAV genome comprises of two open reading frames encoding the four replication and packaging function genes (*rep*) to control viral replication, structural gene expression and integration into the host genome and three capsid (*cap*) genes encoding capsid structural proteins, *rep* and *cap* genes are flanked by two inverted terminal repeats (ITRs).The *rep* and *cap* genes alone are insufficient to cause a productive infection. Once infects a cell, wild type AAV normally integrates into the host cell genome and goes into latency at a specific site on human chromosome 19q13.4. In order to enter a lytic cycle in which progeny are produced, wild type AAV requires superinfection of a helper virus, i.e. adenovirus or herpes virus (Lim et al., 2010, Mandel et al., 2008, Geiger et al., 2010, Robbins and Ghivizzani, 1998, Daya and Berns, 2008) or cotransfection of helper plasmid containing DNA regions mediating AAV vector replication from adenovirus (Matsushita et al., 1998).

Recombinant AAV (rAAV) vectors are created by replacing the 4.7 kb viral genome with the expression cassette of choice, flanked by the viral ITRs (Rapti et al., 2011). Since *rep* and *cap* genes were removed, rAAV genome losses the ability to integrate to host genome and remain episomal. This non-integrating property eliminates the risk of insertional mutagenesis. Another advantage of rAAV is the long term expression even in

episomal form. It has been observed in several clinical trials that rAAV showed sustainable transgene expression for 6 months to 3.7 years in patients (Rapti et al., 2011). Moreover, AAVs are very resistant to extreme conditions of pH, detergent and temperature, making them easy to manipulate (Galibert and Merten, 2011). Despite of these advantages, the small size of AAV, however, limits the size of the therapeutic DNA that it can carry. Other major problems of AAV include low production yield and contamination associated with the helper virus (Wu and Ataai, 2000, Kawase et al., 2011). The transduction of AAV into host cell is demonstrated in Fig 1.6.

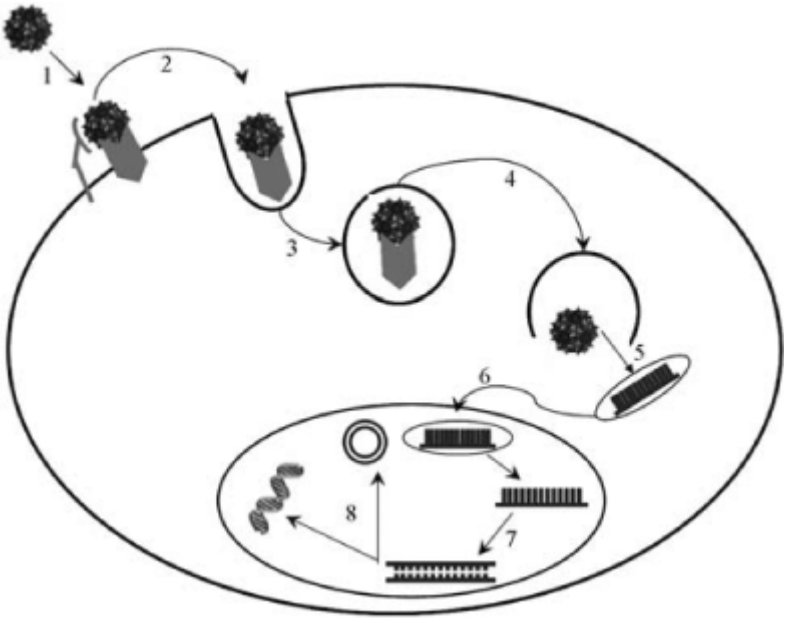

Fig. 1.6 AAV transduction into host cell: (1) binding to a membrane receptor/coreceptor; (2) endocytosis by host cell; (3) endosome formation; (4) escape from the endosome; (5) uncoating to release the viral genome; (6) entering host nucleus; (7) conversion of viral genome from single to double stranded; and (8) integration to host genome or remaining in episomal form (Coura and Nardi, 2008).

1.2.2.1.4 Herpes-simplex virus (HSV)

Herpes simplex virus is an enveloped double-strand linear DNA virus. HSV is rather complicated virus with a ~150 kb viral genome encoding at least 84 almost entirely contiguous (unspliced) genes. Approximately half of these genes are nonessential for viral replication in cell culture. These nonessential genes can be removed to provide multiple sites of foreign gene insertion capable to carry at least 30 kb of foreign genes either a large single gene or multiple transgenes for coordinately or simultaneously expression (Wu and Ataai, 2000, Kay et al., 2001, Robbins and Ghivizzani, 1998, Mata et al., 2002, Aravindaram and Yang, 2009). Wild type HSV is pathogenic, associating with cold sores, corneal blindness, and encephalitis (Mandel et al., 2008). HSV can infect a wide range of cells, both dividing and non-dividing, since its cellular receptors; heparan sulfate (HS), herpesvirus entry mediator (HVEM), and nectin-1 and -2, are widely expressed on the cell surface of numerous cell types (Patel and Misra, 2011). After endocytosis, the wild type HSV is retrogradely transported to the cell body and eventually to the nucleus where it replicates. Upon infection, wild type HSV can either undergo lytic cycle or establish latency, especially in neurons (Millhouse and Wigdahl, 2000). Due to its attractive features such as neural tropism, retrograde transport, and large transgene capacity properties, HSV has gained a large interest for gene therapy application towards the nervous system (Lim et al., 2010, Mandel et al., 2008).

HSV-1 is currently the most extensively engineered herpesvirus for gene transfer purposes. HSV-1 is neurotrophic and can establish lifelong presence in sensory neurons (Lim et al., 2010). Two types of HSV-1 vector systems; recombinant virus and amplicons, have been developed. The recombinant HSV (rHSV) vectors are created by replacing nonessential viral genes with a transgene(s) of interest at different sites in the viral

genome. These vectors can usually accommodate up to 40 kb of transgenic DNA (Lim et al., 2010). rHSV can be divided to two catagories; replication-competent attenuated vector and replication-incompetent attenuated vector. Replication-competent attenuated vectors still contain essential genes for *in vivo* replication whereas the genes that are required for replication in replication-incompetent vectors are deleted (Lim et al., 2010). The properties of rHSV vectors are highly dependent on the presence of immediate early (IE) genes. The absence of multiple IE genes led to a diminished toxicity but the transgene expression was also found to be severely decreased (Krisky et al., 1998) while the presence of IE gene products results in cytopathic effects (Johnson et al., 1992).

The HSV-1 amplicon is similar to the gutless adenoviral vector system. The viral genome only contains a viral origin of replication and a packaging signal. The genes necessary for viral production and replication are provided in *trans*, by a helper virus/transfection system (Mandel et al., 2008, Lim et al., 2010, Robbins and Ghivizzani, 1998). Advantages of using amplicon are simple vector construction, reduced cellular toxicity and immunogenicity compared to the rHSV-1 vectors and the capacity to carry large fragments of foreign DNA. The latter feature not only allows for the insertion of entire genomic loci but also the addition of various elements, i.e. promoters, inducible systems for regulated gene expression or several separate expression cassettes (Lim et al., 2010, Wu and Ataai, 2000).

1.2.2.1.5 Lentivirus (LV)

Lentivirus (LV) is subclass of retrovirus. In contrast to more commonly studied oncoretroviruses, LVs are capable of integrating their genome into both mitotic cells and post-mitotic cells (Naldini and Verma, 2000). LV genome is a single-stranded RNA (~9.7 kb) encoding cis-acting sequences for packaging, reverse transcription,

nuclear localization and integration. LV genome also includes *gag*, *pol* and *env* encoding the core proteins, the virion-associated enzymes, and the envelope glycoprotein, flanked by the long terminal repeats (LTR). The LTRs are important for integration, transcription and polyadenylation. LV genome also contains regulatory genes (*rev* and *tat*) and accessory genes (*vpu*, *vpr*, *vif*, and *nef*) (Lim et al., 2010, Mandel et al., 2008, Naldini and Verma, 2000).

Human Immunodeficiency Virus type I (HIV-I) is classified as a lentivirus and has been engineered for gene therapy applications since it has been widely and thoroughly studied (Naldini et al., 1996). The first generation of LV vectors based on the human immunodeficiency virus 1 (HIV-1) was generated using a three-plasmid system consisting of a packaging plasmid, vector plasmid and envelope plasmid. The packaging plasmid contains the viral genes under the Cytomegaloviral (CMV) promoter except for the *env* and *vpu* open reading frames (ORFs), which have been deleted and provided by the envelope plasmid that encodes vesicular stomatitis virus G protein (VSV-G) instead of the HIV-1 *env* proteins. The vector plasmid contains HIV-1 *cis*-acting elements that enable the transgene and its promoter to be packaged (Lim et al., 2010). In second and third generation, the additional viral sequences were removed from the vector plasmid in order to decrease the possibility of replication-competent recombinant virus. One of the third generation LV called self-inactivating lentivirus was produced by inactivating the 5′ LTR. The absence of the promoter and enhancer sequences in the LTR prevents possible replication of the virus during vector production, resulting in a vast reduction of recombination probability after the vector genome has integrated into the host (Miyoshi et al., 1998). Lentiviral vectors can accommodate a transgenic DNA at the size up to 18 kb proviral length (Lim et al., 2010).

The oncogenic mutation caused by the random integration of a lentiviral vector genome into the host cell genome is the primary safety concern for its application in

gene therapy. The non-integrating lentiviral (NIL) vectors were developed by removal of integrase activity which is required to catalyze the integration of viral genome into the host genome. As a result, NIL genome remains in the nuclease of target cell as a linear or circular double stranded DNA (Philpott and Thrasher, 2007, Sarkis et al., 2008). However, integration properties of lentiviral genome into the host genomes holds great promise for *ex vivo* gene delivery allowing a stable transduction in cell lines, primary cells or stem cells (Lim et al., 2010). Lentiviral vector transduction is illustrated in Fig. 1.7.

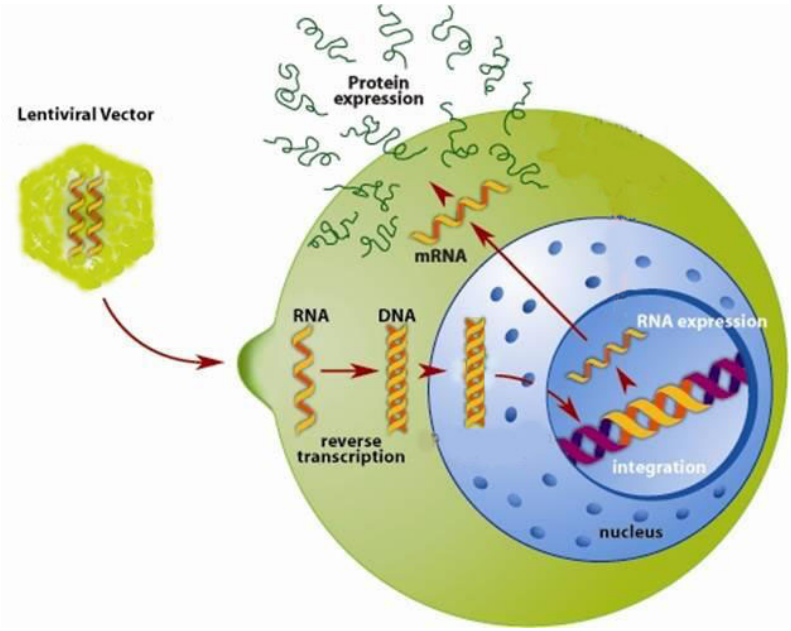

Fig. 1.7 The mechanism of Lentiviral (LV) vector transduction. First, LVs bind to target cells using an envelope protein which allows for release of the LV RNA containing the transgenes into the cell. The LV RNA is then converted into DNA using reverse transcriptase by a process called reverse transcription. The DNA then enters the nucleus and integrates into the target cell's chromosomal DNA. (Illustration was modified from: Lentigen; http://www.lentigen.com/technology/vectors; last accessed on September, 21st, 2012)

1.2.2.2 Non-viral vectors

Despite the high gene transfer efficiency, the potential dangers of using of viral vectors in gene therapy have been highlighted in clinical trials history (Glover et al., 2005, Park et al., 2006, Fang et al., 1996, Madsen and Mooney, 2000, Sheridan, 2011, Wolf

et al., 2009). In 1999, the death of an 18 year-old volunteer, Jesse Gelsinger, caused by vector-associated toxicity in a gene therapy trial using adenoviral vector has triggered the interest on the safer, non-viral vectors (Glover et al., 2005, Sheridan, 2011). Advantages of non-viral vectors over viral vectors are their low immunogenecity, absence of endogenous virus recombination, low production cost and reproducibility (Wolf et al., 2009, Lee and Kim, 2005). Moreover, non-viral vectors have no limitation in DNA size for packaging and the possibility of modification with ligands for tissue- or cell-specific targeting (Lee and Kim, 2005). Non-viral gene delivery vectors are briefly discussed as followed;

1.2.2.2.1 Plasmid DNA

Plasmid-based gene expression system for use in gene therapy and DNA vaccination minimally consists of three major components, (i) a prokaryotic plasmid vector, essentially containing origin of replication (*ori*) which enables plasmid to replicate in production host (normally *E. coli*); (ii) eukaryotic regulatory elements to control the expression of therapeutic gene inserted to target cells; and (iii) a gene or genes encoding therapeutic or antigenic protein (s) (Durland and Eastman, 1998).

Plasmid DNA can be injected directly into a tissue without additional help from either a chemical agent or a physical force. Gene transfer with naked DNA is attractive to many researchers because of its simplicity and lack of toxicity. However, local injection of plasmid DNA leads to low-level gene expression since naked DNA is susceptible to degradation by serum enzymes such as endonucleases, thereby reducing the amount of intact functional pDNA that is available in hosts to express antigen. The transportation of pDNA across cell membranes while entering into a host cell is limited by its net negative surface charge and large hydrodynamic diameter (Han et al., 2009, Ledley, 1996). In

addition, DNA may be degraded during being uptaken by endocytosis. These mechanisms reduce the chance that DNA entering nuclei will be intact and functional. The strategy to improve gene transfer of naked plasmid DNA is to include substances, capable of enhancing the efficiency of DNA internalization by target cells, in DNA solution (Gao et al., 2007). For example, transferrin was observed to enhance transfection *in vitro* (Sato et al., 2000), the addition of water-immiscible solvents (Schughart and Rasmussen, 2001, Schughart et al., 1999, Desigaux et al., 2005) non-ionic polymers (Desigaux et al., 2005), surfactants (Freeman and Niven, 1996), hypotonic solution (Lemoine et al., 2005) has also been reported to increase gene transfer across cell membranes. Furthermore, several nuclease inhibitors have been shown to enhance naked DNA–mediated gene transfer in cultured cells, muscle, and lungs (Gao et al., 2007). Plasmid DNA can also be introduced into target cells by physical methods such as gene gun (Aravindaram and Yang, 2009, Dileo et al., 2003), electroporation (Li et al., 2012, Peng et al., 2012), ultrasound-facilitated gene transfer (Saliba et al., 2012, Song et al., 2012) and hydrodynamic gene delivery (Crespo et al., 2005, Herweijer and Wolff, 2006).

1.2.2.2.2 DNA lipoplex

Low gene transfer efficiency in naked pDNA is mainly caused by its susceptibility to degradation by serum and cellular enzymes such as endonucleases and difficulty to travel across cell membrane due to its size and charge. Lipoplexes solve these disadvantages by facilitating the transportation of DNA into target cells and at the same time, protecting DNA from being attacked by nuclease (Gregoriadis et al., 2000). However, some types of liposomes are subject to nonspecific binding by a large range of different cell types (Wivel and Wilson, 1998).

Due to similarity between liposomes and lipid bilayer structure of cell membrane, lipoplexes can fuse with the cell membrane and directly release contained DNA or be internalized by endocytosis –mediated mechanism. The result of endocytosis –mediated mechanism is the formation of a double-layer inverted micellar vesicle called endosome. During the maturation of the endosome into a lysosome, the rupture of endosome's wall might occur resulting in a release of the contained DNA into cytoplasm and potentially towards the nucleus. DNA imported into the nucleus might result in gene expression. Alternatively, DNA might be degraded within the lysosome (Parker et al., 2003, Ferrari et al., 2001, Tros de Ilarduya et al., 2010). Transfection of lipoplexes can be promoted by adjusting vesicle surface charge, size and lipid composition, or by the co-entrapment of plasmids expressing appropriate cytokines (e.g., interleukin 2), or immunostimulatory sequences (Gregoriadis et al., 2000). Transfection mechanism of lipoplex is shown in Fig. 1.8.

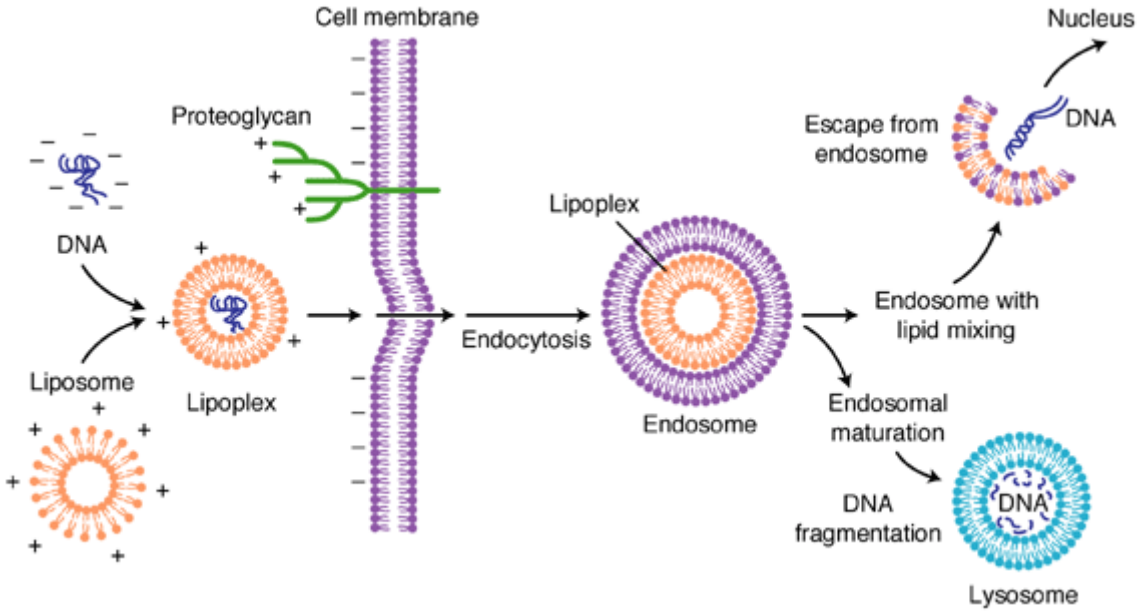

Fig. 1.8 Lipoplex-mediated transfection and endocytosis (Parker et al., 2003).

1.2.2.2.3 DNA polyplex

Polyplexes (polyelectrolyte complexes) are formed by self-assembling of cationic polymers, i.e. polyethylenimine (PEI), with DNA by involving ionic interactions (Howard et al., 2004, Duarte et al., 2007). Polyplex enables gene delivery by condensing and shielding DNA into small particles and facilitating cellular uptake via endocytosis through charge-charge interaction with anionic sites on cell surfaces (Gao et al., 2007, Tros de Ilarduya et al., 2010). Main difference on delivery mechanism between polyplexes and lipoplexes is that the release of DNA from polyplexes into cytoplasm cannot occur unassisted (Gao et al., 2007). Therefore, co-transfection with endosome-lytic agents such as inactivated adenovirus, is required. However, some cationic polymers, i.e. PEI, exhibit endosomolytic properties and consequently are powerful transfection agents (Boussif et al., 1995). Example of cationic polymers widely used in polyplex formation includes PEI, polyallylamine, polyamidoamine, and polypropylamine dendrimers, cationic dextran, chitosan, cationic proteins (polylysine, protamine, and histones) and cationic peptides (Gao et al., 2007). The principle of gene delivery using polyplexes is demonstrated in Fig. 1.9.

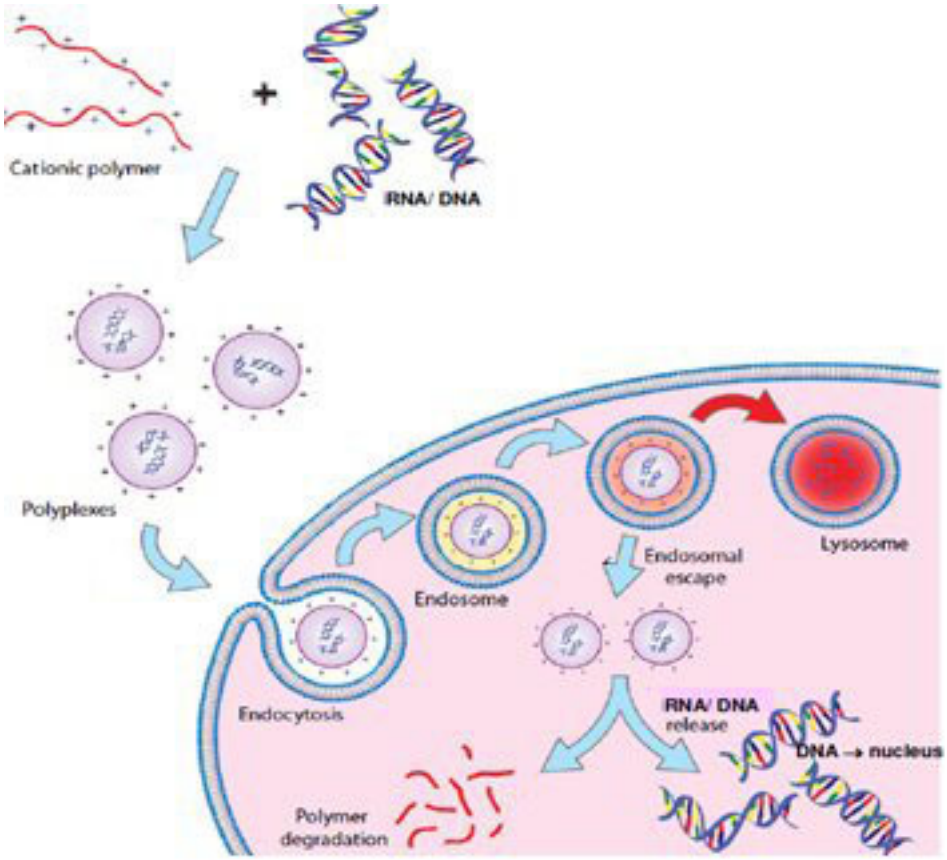

Fig. 1.9 Principle of gene therapy using polyplexes as carriers. Therapeutic nucleic acid (DNA or RNA) is mixed with a cationic polymer to form polyplex particles which can enter target cells by endocytosis. The polymer promotes the release of polynucleotide from the endosomes and the entrance of DNA to the nucleus while protecting the target nucleic acid from lysosomes degradation. (Reprinted with permission from Biomedical Chemistry group, University of Twente; http://www.utwente.nl/tnw/bmc/; last accessed on September, 6th, 2012)

1.3 Chromatography of plasmid DNA

Although nanoplexes include wide range of nanoparticulate bioproducts such as protein nanoparticles, plasmid DNA, viruses, virus-like particles and macromolecular assemblies (Zhang et al., 2001a, Jahanshahi and Ebrahimpour, 2009, Liu and Zhang, 2011), plasmid DNA had been used as the test system in this thesis. Therefore, in this section, the detailed discussion will be solely focused on plasmid DNA.

1.3.1 Characteristics of plasmid DNA

Plasmid DNA (pDNA) is double stranded extrachromosomal circular DNA molecule containing at least one origin of replication (*ori*) which results in an ability to undergo DNA replication independently from chromosomal DNA (Lara and Ramirez, 2012). Each strand of DNA is a polymer of deoxyribonucleotide monomers linked to each other by 3'5'phosphodiester bonds to create a so-called 'backbone'. Deoxyribonucleotide molecule consists of a deoxyribose and a nitrogenous base. Two single-strand DNA connect to each other by hydrogen bond between two complementary nitrogenous bases (Peter et al., 1998, Urthaler et al., 2005a), exposing sugar-phosphate backbone to the outside. These two strands wind around each other and around a common axis in antiparallel fashion to form right handed double helix structure (Hames, 2000, Alberts, 2002) stabilised by hydrogen bonds between A-T and G-C base pairs and by stacking forces (Sinden, 1994). The interior of the double helix is highly hydrophobic due to the close packing of the aromatic bases while the exterior is rich in phosphate groups which are negatively charged at $pH > 4$ (Diogo et al., 2005). The structure of DNA is demonstrated in Fig. 1.10.

In order to modulate the potential energy of the molecule, pDNA helix can be coiled in space to form a supercoiled (SC) pDNA (see Fig 1.11). DNA supercoiling is important for DNA packaging within all cells since the length of DNA can be thousands of times longer than a cell. Moreover, supercoiling also plays an important biological function of facilitating local- and global-strand separation of DNA molecules during transcription and replication, respectively (Witz and Stasiak, 2010, Marians et al., 1977, Nollmann et al., 2007). On the othe hand, another portion of pDNA population can be in a non-supercoiled or open circular (OC) form where breakages of one of double strand (single strand nick) occur. In case of the cell lysate, linear or denatured pDNA can also be found. Linear pDNA results from cleavage

of the phosphodiester bonds in opposite site of double strand DNA (double strand nick) while denatured forms are caused by disruption of hydrogen bonds of complementary base pair on each strand (Diogo et al., 2005, Urthaler et al., 2005a). Diagram showing three different forms of pDNA is shown in Fig 1.12 .

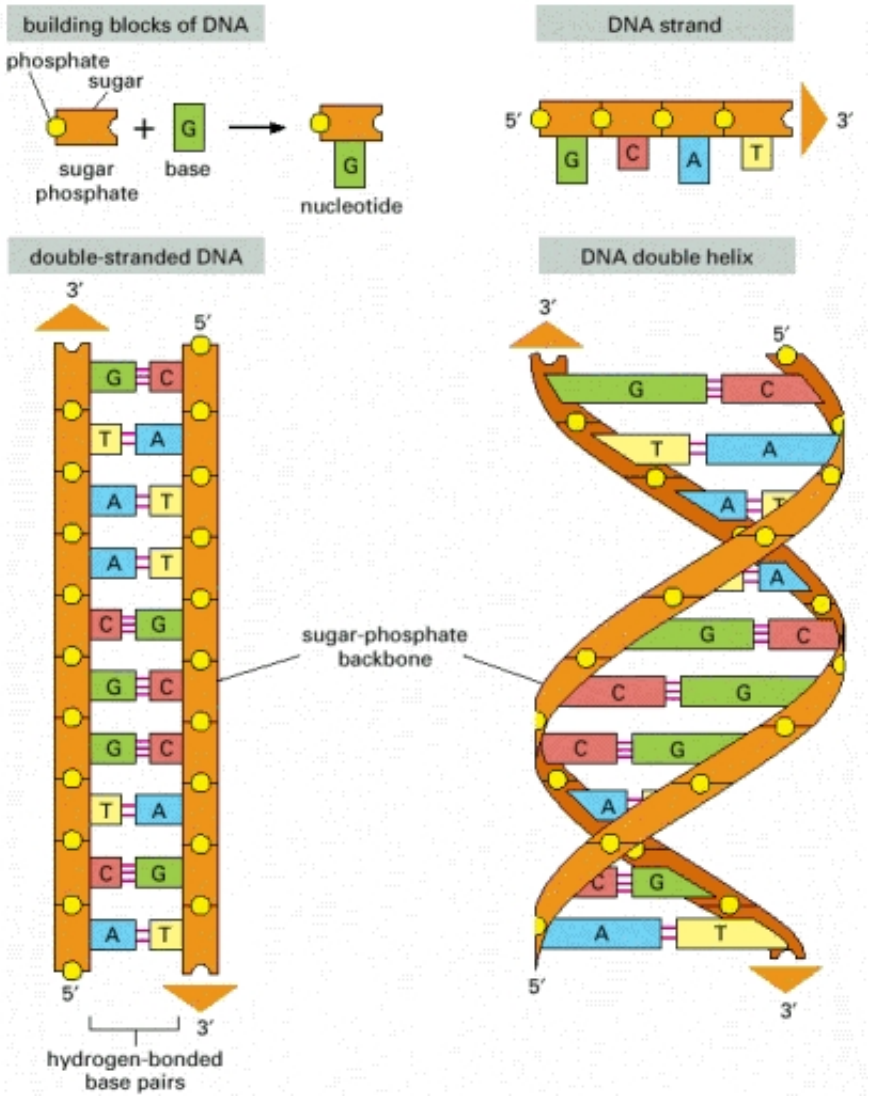

Fig. 1.10 DNA structure. (Alberts, 2002)

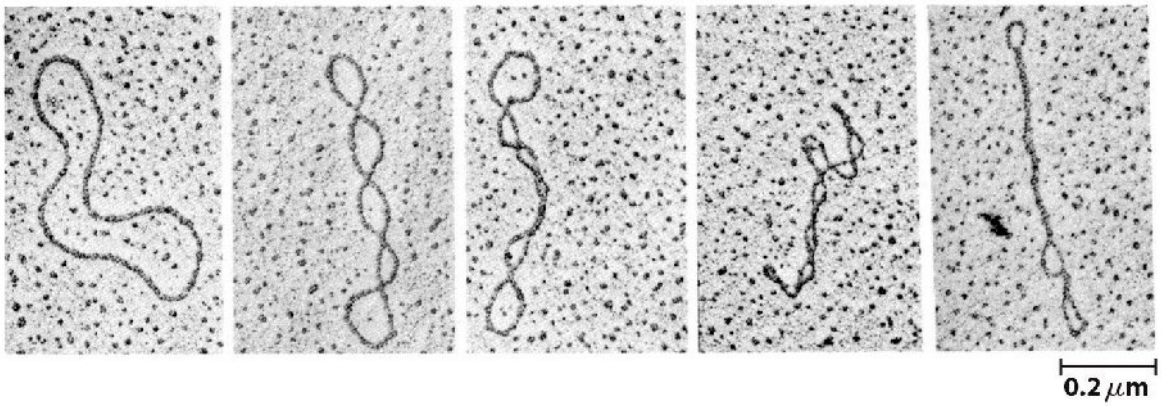

Fig. 1.11 Electron micrographs of relaxed and supercoiled plasmid DNAs. The molecule at the left is relaxed, and the degree of supercoiling increases from left to right. (Kornberg and Baker, 2005)

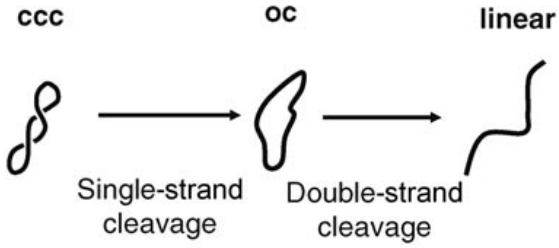

Fig 1.12 Three different forms of plasmid DNA; covalently closed circular (CCC) or supercoiled (SC), open circular (OC) and linear. (Uji-i et al., 2006)

1.3.2 Large scale purification of plasmid DNA

The demands for manufacture of large amounts of high-purity therapeutic or pharmaceutical grade supercoiled-form pDNA have been raised due to the increasing use of pDNA vectors in pre-clinical and clinical trials of gene therapy and DNA vaccination (Przybylowski et al., 2007). Since transfection efficiency of pDNA is considerably low (only one in every 1000 plasmid DNA molecules presented to the cells reaches the nucleus and is expressed) (Varley et al., 1999), full treatments therefore require milligram quantities of plasmid DNA (Ferreira et al., 2000, Deshmukh and Lali, 2005). Thus, the development of

cost effective large-scale processes for the production of plasmid DNA has become one of the challenges associated with therapeutic pDNA technology.

For the industrial scale production of pharmaceutical-grade plasmid DNA, three main requirements have to be met. First, the therapeutic DNA produced must be of highest purity, free from contamination of host cell components. Second, the process has to produce a cost-effective yield. Last, for safety, the whole process must be compliant to regulatory guidelines (Urthaler et al., 2005a, Epstein, 1996). The overall process should be designed specifically to deliver a pDNA product that meets quality specifications set or recommended by regulatory organizations, such as the World Health Organization (WHO), Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA) (Prazeres et al., 2001, Stadler et al., 2004). Summary of these specifications for final pDNA product is shown in Table 1.2.

Table 1.2 Specifications of accepted levels of impurities for the final pDNA product, adapted from FDA and WHO guidance. (Diogo et al., 2005, Stadler et al., 2004, Urthaler et al., 2005a, Sousa et al., 2008)

Issue in focus	**Range of acceptance**	**Determined by**
Endotoxin	< 0.1 E.U./mg pDNA	LAL test
Host genomic DNA	< 0.05 µg/ mg pDNA	TaqMan-PCR
RNA	undetectable	Analytical HPLC
Host protein	undetectable	BCA test
SC over total pDNA	> 97%	CGE

FDA guidance for cellular and gene therapy can be accessed via the U.S. Food and Drug Administration officaial website;
http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/CellularandGeneTherapy/default.htm

WHO regulatory policy for gene therapy can be accessed via WHO official website; http://www.who.int/en/

Abbreviations; LAL : Limulus amebocyte lysate, PCR : Polymerase chain reaction, HPLC : High performance liquid chromatography, BCA : Bicinchoninic acid, CGE : capillary gel electrophoresis.

The supercoiled (SC) form is considered as most stable and most appropriate for therapeutic applications due to the highest transfection efficiency compared to the other plasmid isoforms and is therefore the most desirable form (Urthaler et al., 2005a, Urthaler et al., 2005b, Han et al., 2009, Latulippe and Zydney, 2009). According to regulatory organizations guidelines, percentage of supercoiled form compare to the total pDNA is one of the main parameter to consider the quality of a pDNA preparation for therapeutic use (Table. 1.2).

Generally, a process for production of therapeutic pDNA mainly consists of 3 steps (see Fig. 1.13); (i) fermentation, (ii) cell lysis and (iii) purification (Urthaler et al., 2005a). This chapter will only focus on the downstream processes; cell lysis and chromatographic purification.

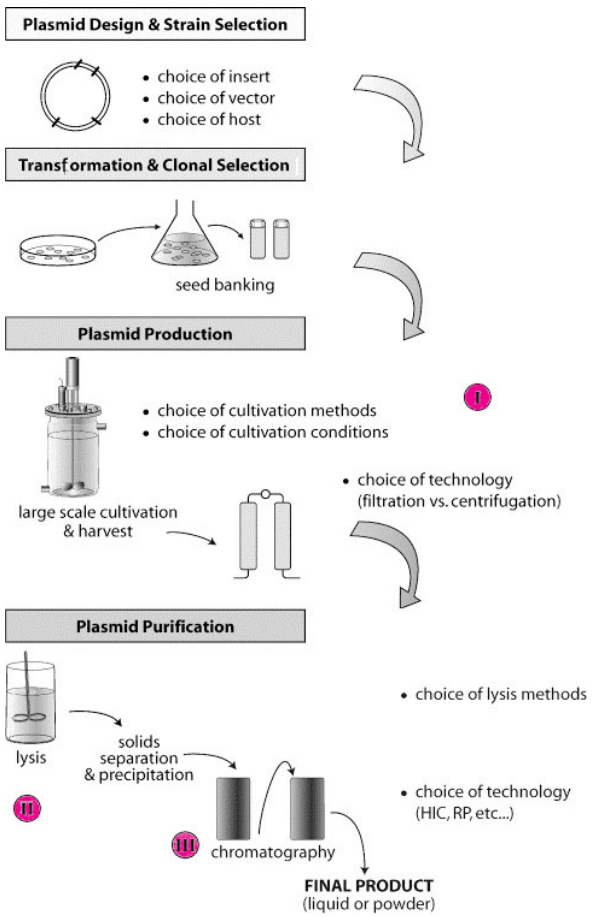

Fig. 1.13 Overall process for therapeutic plasmid DNA production. Modified from Prather et al. (Prather et al., 2003).

1.3.2.1 Cell lysis

Large scale productions of pDNA are normally conducted via fermentation, using *E. coli* as the production host. The efficiency of fermentation process is strongly influenced by the host strain, fermentation mode, the medium compositions and harvesting point (Urthaler et al., 2005a). After fermentation, harvested cells undergo the lysis step where the cells are disintegrated in order to release the intracellular components (Fig. 1.14). Currently, most of bacterial cell lysis techniques (homogenization, freeze/thaw, lysozyme digestion etc.) have been optimized for the purification of recombinant proteins and mostly, are not suitable to plasmid DNA purification. Homogenization operates at high shear rate therefore, inapplicable on shear-sensitive polynucleotide such as pDNA (Carlson et al., 1995). Freeze/thaw process was reported for causing the loss of supercoiled form pDNA (Kong et al., 2008). Lysozyme digestion is not applicable for therapeutic proposes since it relies on the use of lysozymes which are normally acquired from animal origins, i.e. egg white. For therapeutic DNA production, animal originated substances are prohibited by regulatory agencies due to safety concerns. Considering these disadvantages of the other methods, chemical lysis seems to be the method of choice for industrial-scale production of pDNA. Usually, bacterial cell disruption is performed using alkaline lysis method, first introduced by Birnboim and Doly (1979). This procedure is based on the cell disruption by the combination of sodium hydroxide and the detergent, sodium dodecyl sulfate (SDS) which leads to cell lysis and release of the intracellular components. Proteins and genomic DNA are subsequently precipitated by the following neutralization step involving potassium acetate addition (Birnboim and Doly, 1979). During neutralization, supercoiled plasmid DNA anneals from its pH-denatured state while large molecular weight (~200 kb) genomic DNA cannot diffuse and anneal properly, resulting in single-stranded genomic DNA precipitation (Prather et al., 2003) which can be removed by filtration or centrifugation. The clarified cell

lysates normally appear as a diluted, viscous plasmid mixture in the presence of high salt concentration containing plasmid DNA at concentrations of ≤ 3% of the clarified cell lysate even when high copy number plasmid was applied (Kepka et al., 2004b, Stadler et al., 2004, Sousa et al., 2008). The downfall of this method is the conversion of supercoiled pDNA to the alternative forms (e.g. denatured supercoiled, multimeric, open circle, and linear) since the DNA had actually denatured during this process. Also, if vigorous mixing was applied, shear force can cause fragmentation of genomic DNA which leads to the difficulty in the subsequence purification (Prather et al., 2003). Hence, it is critical to avoid shearing the chromosomal DNA into small fragments or breaking the precipitate network to prevent contamination of gDNA into the cleared lysate. This can be accomplished by avoiding vigorous mixing after addition of the alkaline detergent and potassium acetate solutions. At large scales, thorough mixing without substantial shearing presents a challenge. Once the precipitate has been removed, there is less concern about shearing unless the shear stress is very high or the plasmid is large (>15 kb) (Durland and Eastman, 1998, Levy et al., 1999).

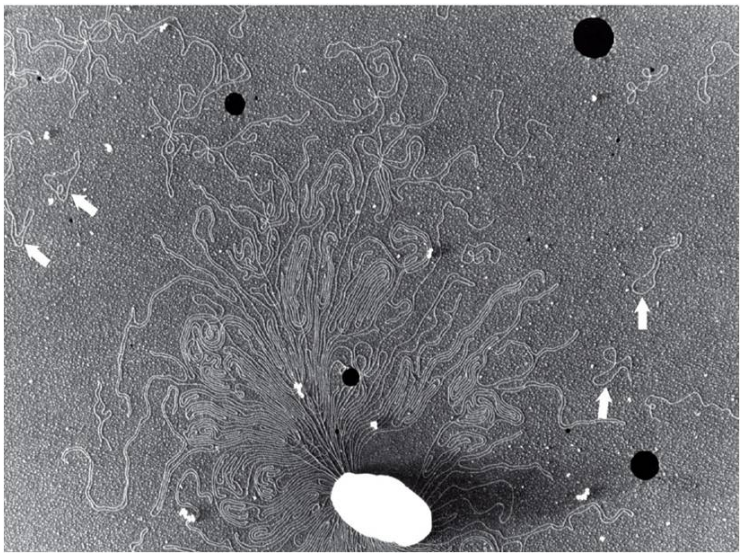

Fig. 1.14 Electron micrograph of DNA from a lysed *E. coli* cell. White arrows indicate circular plasmid DNA. (Nelson and Cox, 2008)

1.3.2.2 Chromatography

After alkaline lysis, the major impurities remaining in the process stream are RNA, gDNA fragments, endotoxins, and proteins. Generally, RNA can be reduced by addition of RNA-digesting enzymes, such as RNase. However, this approach cannot be used for the purification of therapeutic pDNA since the application of animal-derived materials, such as commercially available RNases, is prohibited by regulatory organizations (Sousa et al., 2008). The removal of endotoxins is particularly critical since these lipopolysaccharide components of the *E. coli* cell wall can produce symptoms of toxic shock syndrome if present in sufficient quantities *in vivo* (Wei et al., 2007). Moreover, in terms of physiochemical properties, the similarities of these impurities' molecules to plasmid DNA and their wide molecular weight range makes purification difficult (Prazeres et al., 1999). Apart from impurities, the separation of the undesirable pDNA isoforms to achieve high purity supercoiled plasmid is also essential (Urthaler et al., 2005a, Sousa et al., 2008). For pDNA purification, most of the available processes for pDNA purification i.e. organic solvent precipitation (Murphy et al., 1999), organic solvent extraction or ultrafiltration are time-consuming and lack of scalability (Han et al., 2009). Moreover, product specification guidelines stated by the regulatory authorities dictate that the application of materials that are not certified for administration in human or application of enzymes of animal origins or toxic reagents such as phenol, CsCl, CsBr, etc., are unacceptable (Miller et al., 1997; Ferreira et al., 2000; Shamlou, 2003; Prather et al., 2003; Eon-Duval and Burke, 2004). Hence, chromatography, considered as the method with highest resolution , becomes essential for production of pDNA suitable for therapeutic applications (Urthaler et al., 2005a). Moreover, chromatography processes are well established, scalable and normally provide proven track record which make it highly preferable for pharmaceutical products (Przybylowski et al., 2007).

The most commonly used chromatography techniques for therapeutic pDNA purification are anion exchange (AEC), size-exclusion (SEC), hydrophobic interaction (HIC) and affinity chromatography (AC) (Eon-Duval and Burke, 2004, Diogo et al., 2000a, Horn et al., 1995). These chromatography techniques are fundamentally discussed as presented below, along with their advantages and disadvantages towards pDNA purification.

1.3.2.2.1 Anion exchange chromatography (AEC)

Ion-exchange chromatography plays an important role in downstream processing of bio-molecules by offering the advantages of robustness, rapid separation, no solvent requirement, and a wide selection of process-grade stationary phases (Hanora et al., 2006, Diogo et al., 2005). Since DNA displays the negatively-charged property, anion exchange chromatography (AEC) mode is applied.

AEC is based on the interaction between positively charged-anion exchange ligands c.g. quaternary amine (Chandra et al., 1992), Diethylaminoethyl (DEAE) (Muller, 1986), on the stationary phase and negatively charged phosphate groups in the DNA backbone (Prazeres et al., 1998, Prazeres and Ferreira, 2004). Elution patterns of nucleic acids bound to supports are functional to increasing charge density and was also found to depend on nucleotide sequence and nitrogenous base composition (Yamakawa et al., 1996, Muller, 1986). In most cases, the more compact supercoiled pDNA binds more tightly to the supports therefore, elutes later than other forms due to its higher overall charge density (Prazeres et al., 1998). It has been reported that some other polyanionic molecules i.e. gDNA fragments (Prazeres and Ferreira, 2004) and endotoxins (Wicks et al., 1995) may co-purify with the plasmid due to their similar binding affinities and limited diffusion of the macromolecules inside the adsorbent pores (Lyddiatt and O'Sullivan, 1998, Prazeres and Ferreira, 2004). These high molecular weight impurities can also compete with target pDNA

for binding sites and tenaciously adhere to the anion-exchange matrices resulting in decreased binding capacity of target pDNA (Prazeres and Ferreira, 2004). Moreover, ion exchange chromatography can also suffer from low pDNA binding capacity since most of the IEC supports available commercially were designed for protein purification while pDNA is too large to enter the pores being restricted to bind only at the support surface (Eon-Duval and Burke, 2004, Prazeres et al., 1999, Gustavsson et al., 2004, Tiainen et al., 2007b, Mao et al., 1993, Ljunglöf et al., 1999, Grunwald and Shields, 2001). It was reported that dynamic capacities higher than 3 mg/mL gel, as can be expected in the field of protein purification while pDNA binding capacity is much less due to the large size (Prather et al., 2003). Ljunglöf et al. (1999) reported a study on direct visualisation of plasmid DNA adsorption on individual Q Sepharose XL particles by employing confocal scanning laser microscopy. Plasmid DNA visualization was aided by the fluorescent dye, YOYO-1, which forms a highly fluorescent complex with double stranded DNA. The results revealed that adsorption of plasmid DNA mainly takes place in an outer layer of the particles (Fig. 1.15) (Ljunglöf et al., 1999). Another disadvantage for ion exchange chromatography is similarity between the charge properties of target products and the charge properties of some impurities, resulting in binding and elution of impurity species alongside the targets (Diogo et al., 2005).

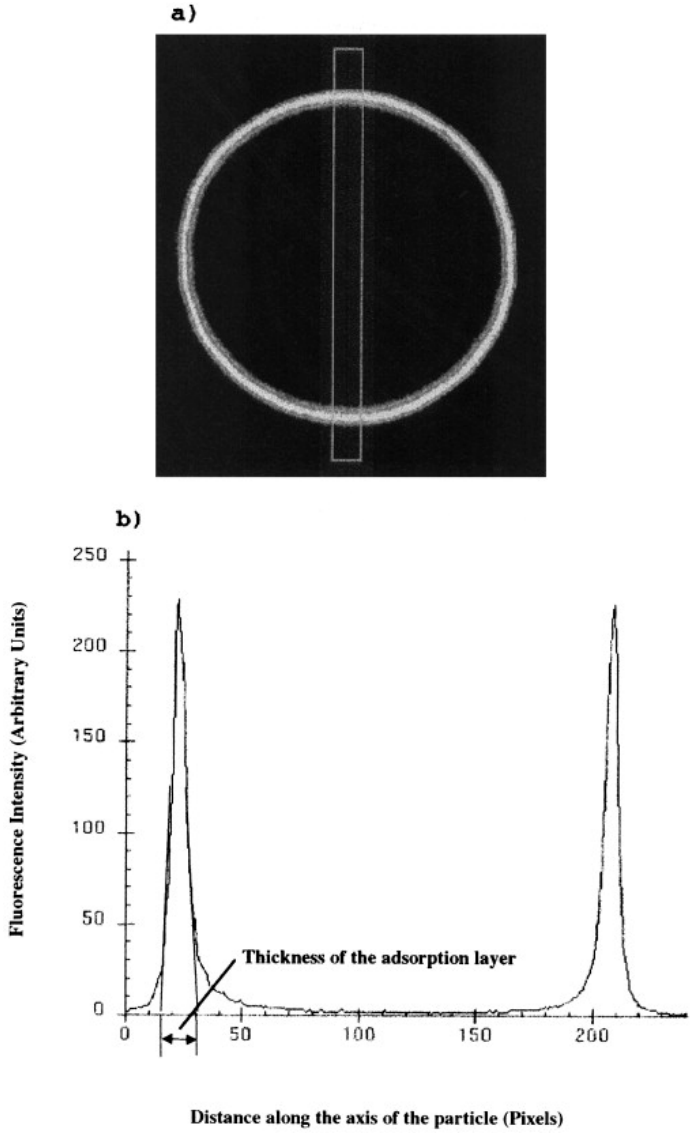

Fig. 1.15 A study on direct visualisation of plasmid DNA adsorption on individual Q Sepharose XL particles published by Ljunglöf et al. showing plasmid adsorption being restricted on the surface of the support. (a) Confocal image of Q Sepharose XL saturated with plasmid DNA. The rectangular box represents the area that was evaluated. (b) Fluorescence intensity profile after image analysis: *x*-axis shows distance along the axis of the particle (in pixels of 0.42 μm) and *y*-axis fluorescence intensity (in arbitrary units) (Ljunglöf et al., 1999).

1.3.2.2.2 Size exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) (also known as 'gel filtration') fractionates and purifies plasmids on the basis of size and conformation (Prazeres and

Ferreira, 2004, Sousa et al., 2008). Typically, the different DNA isoforms are eluted as a broad, non-Gaussian peak i.e.; gDNA elutes first as the leading edge, followed by open circular and then supercoiled plasmid (Yoshinaga and Suzuki, 1983, Moreau et al., 1987). Smaller molecules such as RNA, DNA fragments, and salts can be easily separated from the leading DNA peak due to their differences in size affect their retention times. The choice of the fractions to be collected can be identified easily, enables the recovery of almost pure supercoiled plasmid (Prazeres and Ferreira, 2004); free from proteins, RNA, and gDNA (Moreau et al., 1987, Stadler et al., 2004, Urthaler et al., 2005b). Moreover, substantial reduction in endotoxin loads as well as isoforms separation between supercoiled and open circular form also achieve (Prazeres and Ferreira, 2004, Horn et al., 1995).

Due to the compact structure which leads to a reduction in size, supercoiled pDNA tends to elute later than the other isoforms. Yoshinaga and Suzuki (1983) published the effect on retention time of plasmid isoforms by purifying different 4.36 kbp plasmid isoforms using a Toyopearl HW75S column and the greater retention volume of supercoiled isoform compared to the open-circular isoform was observed (Yoshinaga and Suzuki, 1983). Moreau et al. (1987) reported that separations of different plasmid isoforms showed a reduction in the resolution between the supercoiled and open-circular isoforms with increasing plasmid size when applied alkaline lysis-SEC purification procedure using Fractogel TSK75S column on different plasmids with the size ranging from 2.9 to 17.5 MDa (Moreau et al., 1987). Retention behaviors of different pDNA isoforms during SEC purification was found to depend on the effective molecular size (Potschka, 1991).

Disadvantages of SEC are its limited capacity (< 2% column volume) (Diogo et al., 2005), reduced resolution at higher loading and the dilution of the plasmid product which consequently requires an extra step for concentration. Furthermore, few SEC

resins available commercially have limited selectivity to be used in plasmid purification (Prazeres and Ferreira, 2004). Gomez-Marquez et al (1987) reported an observation of a limited selectivity between plasmid isoforms obtained from purification of pDNA (2.7 kbp) from clarified bacterial lysates on a Sephacryl S-1000 column. All three isoforms eluted as a single peak (Gómez-Márquez et al., 1987). The same observations was noticed by Latulippe and Zydney (2009) when different isoforms of plasmids (sizes ranging from 3.0 -17.0 kbp) was purified by Sephacryl S-1000 SF. The elution peaks of the three isoforms overlapped on one another, indicated the limitation of isoform separation (Latulippe and Zydney, 2009).

For pDNA purification, SEC can be applied alone (Horn et al., 1995, Bywater et al., 1983, Ferreira et al., 1997, McClung and Gonzales, 1989, Raymond et al., 1988, Latulippe and Zydney, 2009) or in combination with other chromatographic techniques such as anion exchange (Varley et al., 1999, Prazeres and Ferreira, 2004). The use of a simulated moving bed chromatography system employing SEC for plasmid purification was also published (Paredes et al., 2005).

1.3.2.2.3 Affinity chromatography (AC)

Affinity chromatography is based on the specific reversible interaction between a particular structure in the target molecule and a ligand immobilised on the stationary phase (Prazeres and Ferreira, 2004, Diogo et al., 2005). This method specifically purifies biomolecules on the basis of their specific biological function or individual chemical structure (Lowe et al., 2001, Kanoun et al., 1986, Sousa et al., 2008). The specific interactions occurring between the ligand and target molecule can occur by electrostatic interactions, hydrophobic interactions, van der Waals forces and/or hydrogen bonding (Sousa

et al., 2008). As a consequence, the specific nature of the employed interactions resulting in a high selectivity and resolution, the major advantage of AC (Platonova and Tennikova, 2005).

The choice of stationary phase and operating conditions depend on the molecular properties of target biomolecules and the physicochemical and thermodynamic nature of the molecular interactions between the two (Mallik and Hage, 2006, Sousa et al., 2008). Elution can be performed either specifically by using a competitive ligand or non-specifically by changing the pH, ionic strength or polarity depending on the support used and the chemical characteristics of the biomolecules in which perform the interaction (Sousa et al., 2008). Different types of AC have been reported and are briefly discussed below;

i). Triple-helix affinity chromatography (THAC)

Triple-helix affinity chromatography is based on the formation of a triplex between an oligonucleotide covalently linked to a chromatographic matrix, and a specific duplex sequence in the target pDNA (Wils et al., 1997, Diogo et al., 2005). This triple-helix interaction is only possible if a suitable target homopurine sequence has been previously inserted into the pDNA (Wils et al., 1997, Schluep and Cooney, 1998). Stationary phase is covalently attached by pyrimidine oligonucleotide in order to bind to duplex DNA via the major groove and through the formation of Hoogsteen hydrogen bonds (Sousa et al., 2008). The preferable operating pH is mild acidic since cytosine protonation is required to maintain stability of the triple-helices (Schluep and Cooney, 1998). In alkaline condition, the dissociation of triple-helices occurs (Sousa et al., 2008).

THAC is claimed to purify plasmid DNA in one step while significantly reduce the levels of impurities (Prazeres et al., 1999). Despite highly selective property, THAC has a low versatility since each ligand targets only specific base sequence which can lead to a prohibitively high cost for scaling-up. Moreover, low binding kinetics

was also noticed. (Prazeres and Ferreira, 2004, Han et al., 2009). Another limitation is that when the ligands of biological origin were used, a safety issue arises since these ligands tend to be fragile and may leak out and contaminate the product (Kanoun et al., 1986, Diogo et al., 2005, Sousa et al., 2008).

ii). Protein–DNA affinity chromatography

A bi-functional protein was prepared by fusing a zinc finger (ZF) DNA binding domain with glutathione *S*-transferase (GST) as the N-terminal domain (Woodgate et al., 2002). The ZF protein is a consensus sequence ZF protein that binds to the sequence 5-GGG-GCG-GCT-3, while the GST domain is able to bind to matrix immobilised glutathione. In this way, a complex can be formed between a target pDNA, containing the recognition sequence and glutathione-Sepharose. The protein–pDNA complexes can then be recovered from glutathione-Sepharose by competitive elution with reduced glutathione buffer (Diogo et al., 2005).

iii). Immobilised metal affinity chromatography (IMAC)

Immobilised metal affinity chromatography has been recently applied to pDNA purification, using an imino diacetic acid (IDA) resin charged with Cu(II) (Murphy et al., 2003). The resin was found to bind exposed purine bases in solution. In this way, denatured DNA and RNA bind to the IMAC column whereas the pDNA, not having exposed purine, is not absorbed (Diogo et al., 2005).

iv). Boronate affinity chromatography (BAC)

One of the few differences between RNA and DNA which can be used to facilitate the separation is the presence of a vicinal 2,3*cis*-diol at the 3’ end of RNA molecules but absent in DNA molecules (Singh and Willson, 1999). Boronate ligands

(e.g. *m*-aminophenylboronic acid) attached to chromatographic matrices are able to recognise and bind RNA molecules via this feature. BAC has been used essentially in applications in which RNA is the desired product (e.g. ribozyme science and rRNA probe methods). Although the use of BAC has not been described in the context of pDNA purification, its application as a way of reducing the RNA content in pDNA-containing clarified or pre-purified lysates should not be overlooked (Liu, 2006, Singh and Willson, 1999, Diogo et al., 2005).

v). Polymyxin B affinity chromatography

Polymyxin B is a cationic polypeptide antibiotic that has bactericidal activity against gram-negative bacteria. This activity is based on the ability to disorganize the bacterial cell wall due to the specific interaction of polymyxin B with the lipidic structure of endotoxins (Lipid A) (Petsch and Anspach, 2000). This ability has been explored by affinity chromatography with polymyxin B functionalized onto Sepharose to significantly reduce endotoxin contamination (200–100,000-fold) in pDNA solutions (Montbriand and Malone, 1996, Wicks et al., 1995). However, low yields were obtained as a result of non-specific ionic interactions between pDNA and polymyxin B (Wicks et al., 1995). Another disadvantage of the technique is related with the neuro- and nephrotoxicity of polymyxin B and stimulation of monocytes to release interleukin-1(Petsch and Anspach, 2000).

1.3.2.2.4 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography (HIC) has been used for pDNA purification (Diogo et al., 2000a, Diogo et al., 2001, Diogo et al., 2000b) and is considered to be the methods of choice for selective separation of pDNA isoforms and

endotoxin reduction (Stadler et al., 2004). Stationary phases derivatised with mildly hydrophobic ligands allow the separation of supercoiled and open circular plasmid DNA from protein, RNA, gDNA, endotoxins, denatured plasmid forms and oligonucleotides (Latulippe and Zydney, 2009, Prazeres and Ferreira, 2004, Iuliano et al., 2002b). HIC separates pDNA from these impurities by relying on their difference in hydrophobicity. Double helix structure of plasmid are formed by exposing hydrophilic sugar-phosphate backbone to the outside while hydrophobic aromatic bases are packed and shielded inside (Li et al., 2005). As long as double strand of the plasmids remain intact (i.e. in supercoiled or open circular form), their interactions with the HIC support are minimal (Prazeres and Ferreira, 2004). Contrarily, denatured gDNA and RNA are single-stranded having hydrophobic bases exposed, resulting in higher hydrophobicity and stronger interactions with the hydrophobic ligands compared to circular or supercoiled pDNA (Li et al., 2005). Endotoxins are polyanionic amphiphilic molecules that can form multimolecular aggregates with a complex supramolecular structure. High hydrophobicity content enables endotoxins to interact with the HIC media even more strongly via the lipidic moiety (Diogo et al., 2000a, Petsch and Anspach, 2000, Li et al., 2005). This endotoxin binding property make HIC become attractive for endotoxin removal. Since the hydrophobicity of pDNA are rather small, binding to stationary phases requires high salt concentration which is commonly considered as a disadvantage, especially for industrial application since the use of salt elevates the cost and associates with environmental impact. Elution is generally performed by decreasing the salt concentration of the mobile phase in order to weaken the hydrophobic interactions (Jungbauer et al., 2005, Iuliano et al., 2002a, Xiao et al., 2007, Diogo et al., 2005). In the cases of nucleic acids, retention on HIC is mainly affected by their size, base composition and structure (Ferreira et al., 2000).

During purification process, HIC media mainly adsorb impurities (protein, RNA, gDNA, endotoxins, denatured plasmid forms and oligonucleotides) while intact pDNA does not bind due to its low hydrophobicity (Li et al., 2005). Diogo et al. described the application of HIC for purification of supercoiled plasmid DNA by using sepharose gel derivatized with 1,4-butanedioldiglycidylether under non-denaturing condition. All contaminants were absorbed on HIC matrices and separated from non-binding plasmid DNA (Diogo et al., 2001, Diogo et al., 2000b). Other cellulose and Sepharose-based HIC matrices were also found to promote the binding of poly A, denatured DNA and viral RNA via hydrophobic interactions (Cashion et al., 1980). This binding behavior may become a solution for the commonly encountered problem of low DNA binding on common media caused by inadequate pore sizes as discuss earlier by facilitating the 'negative mode' chromatography where impurities bind to supports while target DNA appears in the flowthrough.

1.4 Bilayered SEC/IEC supports

Many efforts on chromatography support development have been made by researchers in order to solve the surface-restricted binding of such large biomolecules as nanoplexes on particulate chromatographic supports. Matrices properties have been improved by various approaches such as; extending the support's surface area by grafting extender polymers to the beads (Theodossiou et al., 2001, Müller, 1990, Theodossiou et al., 2000a), using small non porous support particles to get rid of pore flow problem (Levy et al., 2000, Sumita et al., 1994, Tiainen et al., 2007b). Another attempt has been made on exploring new choices of matrix formats which contain networks of large flow pores to allow plasmids to be transported into the interior of the supports by convective flow i.e.; 'superporous' matrices

(Deshmukh and Lali, 2005, Tiainen et al., 2007a), membranes (Teeters et al., 2003, Chang et al., 2008, Grunwald and Shields, 2001), non porous silica fibre (Tiainen et al., 2007b) and monolith (Urthaler et al., 2005c, Yamamoto et al., 2009, Sousa et al., 2011, Savina et al., 2005, Branovic et al., 2004, Plieva et al., 2004). However, these solutions still have some drawbacks. Small beads lead to pressure drop limitation (Teeters et al., 2003). Membranes possess problems such as uniform flow distribution, a relatively large dead volume and scalability (Urthaler et al., 2005c). Large pore monoliths only provide limited surface areas on their pore walls (Plieva et al., 2004) and the high operation pressure leads to high shear rate which may harm some fragile target molecules.

Amongst the various chromatography modes available for the purification of nanoplexes, ion exchange (IEC) and size exclusion (SEC) chromatography have been the most widely used (Ferreira et al., 2000, Gustavsson et al., 2004). However, due to the disadvantages of these techniques as mentioned earlier, an increase interest in 'negative modes' of adsorption chromatography has emerged. In negative mode chromatography, small impurities are bound and target species pass through the column (Jahanshahi et al., 2005). The negative mode also benefits pDNA purification due to the minimal interaction between nanoplexes and matrices resulting in the minimal changes on target nanoplex structure. HIC also provide a satisfying separation power but the main disadvantage is the use of high salt concentration. Hence, the idea of bilayered SEC/IEC supports which combine the strengths of SEC and IEC, possessing a functionalized core to capture small impurities and inert, non-stick surface to exclude large charged micromolecules (Fig. 1.16) becomes more attractive.

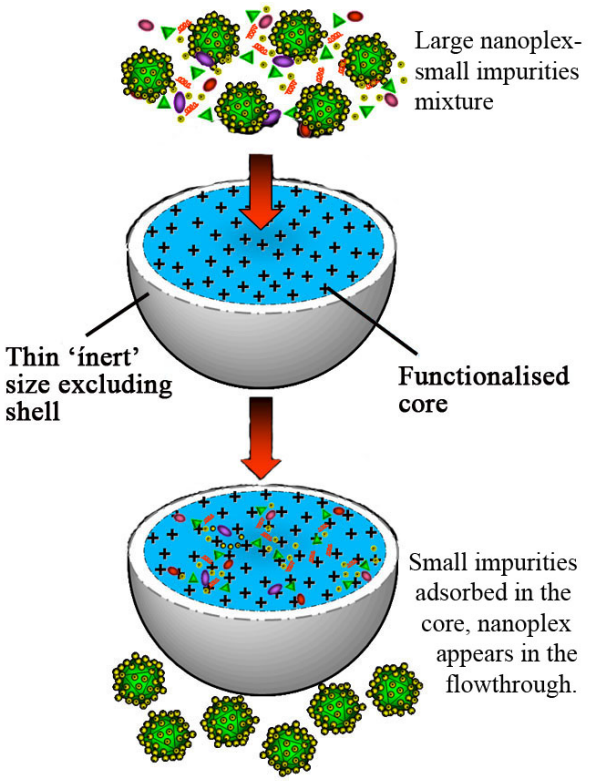

Fig. 1.16 Schematic showing separation of nanoplex from smaller chemically similar contaminants using bi-layered SEC-AEC hybrid chromatography supports.

Existing approaches to produce bilayered SEC/IEC supports in order to exclude large biomolecules mostly have been done on expanded bed adsorption (EBA) supports i.e. laminating IEX base matrices with a non- charged polymer layer i.e. agarose and cross-linked agarose. Dainiak et al. (2002) reported coating Amberlite resins with cell-repelling polymer, polyacrylic acid (PAA) for a direct capture of shikimic acid from fermentation broth. The negatively charged polymer, PAA, was physically absorbed onto the anion exchanger surface followed by a covalent cross-linking. The target molecule, shikimic acid, is small enough to travel across the PAA layer while large negatively charged structures such as cell and cell debris are repelled from binding to the support (Dainiak et al., 2002). In 2004, Viloria-Cols et al. reported the effect of shielding an adsorbent with a layer of agarose on reducing the cell binding while still allowing the low-molecular-mass bioproducts to be adsorbed. An AEX

resin, Amberlite IRA-400, was coated with agarose followed by cross-linking the agarose layer. Resulting SEC/IEC support was seen to effectively prevent the binding of *E. coli*, *Saccharomyces cerevisiae*, and *Lactobacillus casei* cells but at the same time, allowed the binding of lactic acid to the adsorbent (Viloria-Cols et al., 2004). Jahanshahi et al. (2008) introduced a novel prototype polymer-coated adsorbent (PCA) manufactured by using the three-phase emulsification process to laminate anion exchange base matrix with agarose gel. The non-stick exterior coating acts as a sieve reducing the non-specific binding of cell and cell debris without diminution of selective capture of target protein from complex particulate feedstocks such as whole microbial broths and cell debris (Jahanshahi et al., 2008). However, the lamination of adsorbents with gel forming polymer possesses a fundamental problem of difficulty in casting sufficiently thin, uniform, mechanically robust layers around core particles, resulting in the compromised bed expansion properties, hydrodynamics and intraparticle mass transfer (Arpanaei et al., 2010).

Another approach to created bilayered SEC/IEC supports is applying low temperature plasma discharge treatment. In 2010, Arpanaei et al. introduced the application of low temperature glow discharge plasma for preparation of bilayered SEC/IEC from commercial strong anion exchanger, Q HyperZ. Plasma treatments were employed to either: (i) shave off the surface charges from the support particles (plasma etching): or (ii) coat the support particles with nano-thin polymer (plasma polymerization). The modified Q Hyper Z exhibited surface charge elimination without any damage on support structure. The DNA induced inter-particle cross-linking was not observed during dynamic binding study in EBA mode while the core protein binding capacity remained unchanged (Arpanaei et al., 2010).

However, these existing approaches are based on EBA systems while only few researches were published on packed bed column chromatography systems. In 2004, using the AGE

activation-partial bromination approach based on Bergstorm's methods (Bergstrom, 2002), Gustavsson et al. produced a bilayered SEC/IEC for packed bed chromatography. This so-called 'lid bead' was produced by chemically functionalisation of commercially available SEC base matrix, Sephacryl S500 HR. Conclusively, this process consists of (i) introduction of allyl groups (CH_2=CH-CH_2-) throughout the structure of supports by reaction of allyl glycidyl ether (AGE) with hydroxyl groups on the support; (ii) partial bromination of allyl groups on the surface of each particle to create an outer layer via addition reaction; (iii) hydrolysis of the resulting outer layer of bromo-alkyl groups to create an inert outer layer; (iv) full bromination of the remaining allyl groups on support's core; and finally (v) coupling of a quaternary amine ligand, trimethylamine (Q), to the core. This lid bead was further applied to an integrated process for purification of a 6.1 kbp plasmid from a clarified *E. coli* lysate. This integrated process consisted of ultra/diafiltration step combined with polymer/polymer aqueous two-phase system and finally, lid bead was used for polishing step. The overall process yield for plasmid DNA of 69% was achieved (Kepka et al., 2004a, Gustavsson et al., 2004). However, some problems were noticed in these bi-functional supports. For example, in tests with clarified alkaline lysate feedstocks at high ionic strength, it was necessary to sacrifice over 30% of the support's RNA binding capacity in order to prevent pDNA binding. Such problem appears to result from an imperfect ability to control the thickness and inertness of the outer size excluding layer, combined with a lower than ideal definition between the inert outer layer and charged core. Due to the poor control of the thickness of inert outer layer, the ineffective mass transfer is also expected (Arpanaei et al., 2010).

It has been realized that for AGE activation- partial bromination approach, in order to obtain the maximum elimination of surface pDNA binding while maintain the maximum core binding for impurities, the inert outer layer needs to be very thin where charge elimination

occurs densely and restricted on the outermost surface of support particle. Therefore, core binding is not compromised. This can be achieved by optimization of the bilayer-defining step, partial bromination.

1.5 Aims

The aim of this study is to explore the preparation of SEC/IEC support for 'one column-one bead' nanoplex purification using AGE activation/partial bromination route via two different approaches; viscosity enhancement-reaction/diffusion balancing (VE-RD) and microwave assisted reaction diffusion balancing. Plasmid DNA, representing the nanoplexes, was used as a test system. Parameters affecting the properties of SEC/IEC matrices produced were assessed for the impacts on supports' characteristics. Characterizations of SEC/IEC supports were performed by means of physical, chemical and biological approaches to determine the most effective production conditions. Supports candidates were further evaluated by application on column chromatography for plasmid DNA purification from neutralized *E. coli* cleared lysate. Effects of different chromatographic operating parameters on the performance of SEC/IEC columns were also tested.

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Chapter 2

Viscosity enhanced, reaction-diffusion balancing for the preparation of SEC/IEC supports

Abstract

SEC/IEC support preparation via an AGE activation-partial bromination route, using viscosity enhancement to achieve a balanced reaction-diffusion rate (VE-RD), was studied on Sepharose CL-6B. Parameters examined included: types of solvent and viscosity enhancers, viscosities, and temperatures used for partial bromination reaction as well as degrees of partial bromination and numbers of bromination-hydrolysis cycles. Single 10% partial bromination at room temperature in 64% (w/v) aqueous sucrose without sodium acetate addition was found to be the the most suitable condition for SEC/IEC Sepharose CL-6B production due to the high selectivity indices seen in the bilayered SEC/IEC products and the simplicity of process scale up compared to double 10% bromination.

2.1 Introduction

Since the success of the first approved trial on a four year-old girl suffering from severe combined immunodeficiency (SCID) in 1990 (Blaese et al., 1995, Sheridan, 2011), gene therapy and DNA vaccination have raised huge interest as techniques for prevention of treatment of diseases via gene transfer, and show great promise for fulfilling current worldwide requirements for therapeutic usage. DNA vaccines bases on plasmid DNA

containing specific genes encoding target proteins and can activate both cell-mediated immunity and humoral responses (Prather et al., 2003). DNA vaccinations have yielded highly attractive results against parasitic infections (Ivory and Chadee, 2004, Carvalho et al., 2010) such as malaria (Doolan and Hoffman, 2001, Doolan et al., 2003) and viral infections such as AIDS (Mascola and Nabel, 2001, Smith et al., 2004). Recent developments of DNA vaccine for cancer treatment have been also reported (Anderson and Schneider, 2007, Cohen, 2001, Ma and Yang, 2010). Gene therapy aims to treat genetic defects such as severe combined immunodeficiency (SCID) (FISCHER et al., 2011, Blaese et al., 1995, Fischer et al., 2010) i.e. cystic fibrosis (Desigaux et al., 2005, Diogo et al., 2000a, Prazeres et al., 1999), or acquired diseases i.e. cancer (Frederiksen et al., 1999, Horn et al., 1995, Jia et al., 2012, Wysocki et al., 2002), AIDS (Lisziewicz, 1997, Sorg and Methali, 1997, Zaia, 2003), Parkinson's disease (Dass and Kordower, 2007, Fiandaca et al., 2008, Lewis and Standaert, 2008, Lim et al., 2010, Rodnitzky, 2012) etc.

Although the therapeutic genes can be transferred by several types of viral vectors, plasmid DNA (pDNA) vectors have been considered to be safer, simpler to use and easier to produce on a large scale at reasonable cost (Diogo et al., 2005, Wolf et al., 2009). The safety aspect of pDNA vector has been emphasized by several cases of viral vector-associated toxicity during clinical trials. In 1999, an 18 year-old volunteer, Jesse Gelsinger was the first person who died on a gene therapy trial using adenoviral vector (Glover et al., 2005, Sheridan, 2011). In 2008, Hacein-Bey-Abina et al. reported that 4 out of 10 patients developed T cell leukemia 31-68 months after a gene therapy using retroviral vector (Hacein-Bey-Abina et al., 2008). The increasing use of pDNA vectors in pre-clinical and clinical trials of gene therapy and DNA vaccination has raised the demand for manufacture of large amounts of high-purity

therapeutic or pharmaceutical grade supercoiled-form pDNA. Therefore, a need for more effective processes for large scale pDNA purification has arisen.

Liquid chromatography plays an important role in process-scale manufacturing of therapeutic macromolecules for downstream processing due to its high resolution, capacity and the ability to provide the product purity required (Tiainen et al., 2007b). Chromatography has come a long way since the 1950's, when Sober and Peterson first introduced a cellulose based ion exchanger for protein separation (Sober and Peterson, 1958). However, despite five subsequent decades of chromatographic process development, commercially available chromatography supports still perform only one function for one given purpose. From this perspective, the development of chromatographic support design could be considered to be static. With the development of improved methods for including high-level expression of recombinant *E. coli*, combined with successful production process optimization, it can be predicted that feedstock yield in biopharmaceutical processes may soon be too high for current chromatographic techniques to deal with (Przybycien et al., 2004, Lyddiatt and O'Sullivan, 1998). For example, it has been considered possible that the yearly production of pDNA would reach the scale of kilograms or even tonnes. Therefore, downstream processing has become the bottleneck for pharmaceutical production and accounts for the majority of the manufacturing costs (Aldridge, 2006, Prazeres et al., 1999).

Of the various chromatography modes available for the purification of plasmid DNA, ion exchange (IEC) and size exclusion (SEC) chromatography have been the most widely used (Ferreira et al., 2000, Gustavsson et al., 2004). However, each technique possesses some disadvantages. Ion exchange chromatography can suffer from low pDNA binding capacity since most of the IEC supports available commercially were designed for protein purification while pDNA is too large to enter the pores being restricted to bind only at the support surface

(Eon-Duval and Burke, 2004, Prazeres et al., 1999, Gustavsson et al., 2004, Tiainen et al., 2007b, Mao et al., 1993, Ljunglöf et al., 1999, Zöchling et al., 2004). As the result, binding capacities for pDNA are usually at approximately hundreds of micrograms of plasmid per mL of chromatographic support while the binding capacity of ~200 mg/mL was reported for proteins (Shamlou, 2003). Another disadvantage for ion exchange chromatography is similarity between the charge properties of target molecules and the charge properties of some impurities, resulting in binding of impurity species alongside the targets (Diogo et al., 2005).

In order to solve the problems caused by surface-restricted binding of pDNA, many efforts on chromatography support development have been made. Various approaches have been studied in order to improve the matrices properties. For example, grafting the extending polymers on to the support particle (Theodossiou et al., 2001, Müller, 1990). Small non-porous particles were also applied to solve the pore flow problem (Levy et al., 2000, Sumita et al., 1994, Tiainen et al., 2007b) New choices of matrix formats which contain networks of large flow pores to allow plasmids to be transported into the interior of the beads by convective flow also have been explored i.e.; 'superporous' matrices (Deshmukh and Lali, 2005, Tiainen et al., 2007a), membranes (Teeters et al., 2003, Chang et al., 2008, Grunwald and Shields, 2001), non porous silica fibre (Tiainen et al., 2007b) and monolith (Urthaler et al., 2005c, Yamamoto et al., 2009, Sousa et al., 2011, Savina et al., 2005, Branovic et al., 2004, Plieva et al., 2004). However, these matrixes still have some drawbacks. Small particles lead to pressure drop (Teeters et al., 2003), disadvantages of membranes are the lack of uniform flow distribution, a relatively large dead volume and scalability (Urthaler et al., 2005c). Large pore monoliths only provide limited surface areas on their pore walls (Plieva et al., 2004) and the high operation pressure leads to high shear rate which may harm target molecules.

Size exclusion chromatography separates large biomolecules from smaller impurities based on their size differences, regardless of charges. However, problems still exist due to the comparatively low selectivity toward different isoforms of pDNA, limited capacity, reduced resolution at higher loading and dilution of the plasmid product which consequently requires an extra step for concentration (Lemmens et al., 2003). Co-purification of the other pDNA species along with the most desirable form, supercoiled pDNA has been reported by many researchers (Gómez-Márquez et al., 1987, Latulippe and Zydney, 2009, Prazeres and Ferreira, 2004).

The drawbacks of both chromatographic techniques, combined with difficulties in the production of adsorbent material with sufficiently high capacities have increased interest in 'negative modes' of adsorption chromatography in which small impurities are bound and target species pass through the column (Jahanshahi et al., 2005). The negative mode also benefits pDNA purification due to the minimal interaction between pDNA and matrices resulting in the minimal changes on target pDNA structure. For subtractive adsorption chromatography to be successful in the separation of large biomolecules from smaller contaminants sharing similar or identical surface chemistry, new matrices to be produced should possess 'non-stick' exteriors or barriers to exclude large entities (such as pDNA) while leaving the charged cores freely accessible to smaller impurities. The idea of a bilayered SEC/IEC support involves combining the strengths of IEC and SEC, having charged core which absorbs negatively charged small impurities while inert outer layer excludes pDNA, resulting in an effective 'one column-one bead' separation where impurities are ionically captured inside the beads while target plasmids are excluded by size and instantly come out in the flowthrough. Attempts to achieve a complete separation between small and large biomolecules possessing the same charge properties have resulted in different bilayered

SEC/IEC support prototypes reported, mostly on EBA system. For example, Viloria-Cols et al. (2004) and Jahanshahi et al (2008) reported laminating IEX base matrices with a non-charged polymer layer i.e. agarose and cross-linked agarose in order to exclude large biomolecules. Only small target molecules are allowed to enter the pores and bind to the support's ligands (Viloria-Cols et al., 2004, Jahanshahi et al., 2008). The application of low temperature plasma discharge treatment was reported by Arpanaei et al. in 2010. This treatment eliminates the support's surface charges by either (i) shaving off the surface charges from support particles (plasma etching) or (ii) coating the support particles with nano-thin polymer (plasma polymerization) (Arpanaei et al., 2010). Although these methods have shown some promising results, only EBA supports have been applied and tested on these studies while only a few publications dedicated to column chromatography systems. In 2004, a bilayered SEC/IEC for column purification of plasmid DNA was introduced by Gustavsson et al. This so-called lid-bead was produced by functionalising a commercially available SEC matrix, Sephacryl S500HR using via AGE activation-partial bromination approach. Conclusively, this process consists of (i) introduction of allyl groups (CH_2=CH-CH_2-) throughout the structure of supports by reaction of allyl glycidyl ether (AGE) with hydroxyl groups on the support; (ii) partial bromination of allyl groups on the surface of each particle to create an outer layer via addition reaction; (iii) hydrolysis of the resulting outer layer of bromo-alkyl groups to create an inert outer layer; (iv) full bromination of the remaining allyl groups on support's core; and finally (v) coupling of a quaternary amine ligand, trimethylamine (Q), to the core. This lid bead was further applied to polishing step in an integrated process for pDNA purification from a clarified *E. coli* lysate. (Kepka et al., 2004a, Gustavsson et al., 2004). However, in order to prevent pDNA binding, over 30% loss in core binding capacity was necessary. Such problem seemed to caused by the inability to control the

thickness and inertness of the outer size excluding layer and the lack of definition between the outer layer and the core. Clearly, the production procedure needs to be improved.

For AGE activation-partial bromination approach, it is important to achieve a thin brominated outer layer in partial bromination step in order to minimize compromisation of core charge by deeper penetration of bromine into the supports. This can be accomplished by, in this chapter, slowing the diffusion of bromine into the pores so that the bromination reaction only occurs on the outermost site of the supports. Fine-tuning of viscosity and temperature of the solution used in partial bromination reactions can aid in optimization of conditions for creating an SEC/IEC support structure with a thin inert outer layer to exclude pDNA without compromising the core binding capacity. Viscosity enhancement was mentioned in Gustavsson's report (2004) by applying high concentration of sucrose during partial bromination. However, the effects of different viscosity values on performances of SEC/IEC supports have not been studied. In this chapter, the effects of different viscosity values applied during partial bromination on surface and core binding capacities were systematically examined and discussed.

Against the above, the aim of this study is to optimise the conditions used for SEC/IEC supports preparation using VE-RD balancing approaches on a commercially available underivatised chromatographic support, Sepharose CL-6B. Various solvents, viscosity enhancers and temperature conditions were tested for partial brominations, as well as different degrees of partial bromination and numbers of bromination-hydrolysis cycles. The SEC/IEC supports produced were characterised physically, chemically and biologically by means of electron scanning microscopy (SEM), chemical assays and binding studies with pDNA and bovine serum albumin (BSA).

2.2 Materials and methods

2.2.1 Materials

The SEC base matrix, Sepharose CL-6B, was purchased from GE Healthcare Bio-Sciences (Lot number 10027527, Uppsala, Sweden). Viscosity enhancers; sucrose and glycerol, were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Allyl glycidyl ether (AGE), 50% (w/v) sodium hydroxide solution, sodium hydroxide pellets, sodium borohydride ($NaBH_4$, 99%), bromine, sodium acetate anhydrous, sodium chloride, trimethylamine hydrochloride (Q) and Dimethyl sulfoxide (DMSO) used in SEC/IEC supports preparations were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Ethanol was purchased from Fisher Scientific (Loughborough, UK).

E. coli DH5α containing the 27379 bp plasmid pITT3 was kindly provided by Dr. Eirini Theodosiou, Department of Chemical Engineering, Loughborough University, UK. This plasmid is a pPR633-based high copy number plasmid (4579 bp) and containing a 22800 bp insert from *Saccharomyces cerevisiae* chromosome III at BamHI site. Luria Bertani (LB) broth, LB agar, D-glucose, ampicillin, polypropylene glycol (PPG) antifoam used for culture and fermentation were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). QIAfilter Plasmid Giga Kits for plasmid purification were purchased from Qiagen GmbH (Hilden, Germany).

For bromine assay, standard potassium bromate solution (0.1 M) was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). H_2SO_4 was purchased from Fisher Scientific (Loughborough, UK). For ionic capacity assay, mercury II thiocyanate was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA) and ammonium iron III sulphate was purchased from Fisher Scientific (Loughborough, UK). For binding studies,

bovine serum albumin (BSA), Tris HCl and Trizma base were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). For diphenylamine assay, diphenylamine, perchloric acid, acetaldehyde, glacial acetic acid and calf thymus DNA were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Pierce® BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL, USA). For agarose gel electrophoresis, 100x TE (10 mM Tris-Cl, pH 7.5. 1 mM EDTA) buffer, agarose, Lambda- HinDIII marker, 6x gel loading dye, 10x Tris borate EDTA (TBE) buffer and ethidium bromide were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Distilled water was used in all experiment unless stated otherwise.

2.2.2 Viscosity measurements

Rheological behavior of solutions used for viscosity enhancing was measured using an Advanced Rheometer AR1000 from TA Instruments (New Castle, Delaware, USA) equipped with a 40 mm, 2^o angle stainless steel cone geometry. Viscosity changes were measured during the increasing temperature from 2 ^oC to 90 ^oC (applying a temperature ramp rate of 4^oC/min) at a shear rate of 10 s^{-1}.

2.2.3 Preparation of SEC/IEC supports

SEC/IEC supports can be produced by employing an AGE activation/ partial bromination route, briefly described in Fig. 2.1. The detailed procedures are also presented hereby.

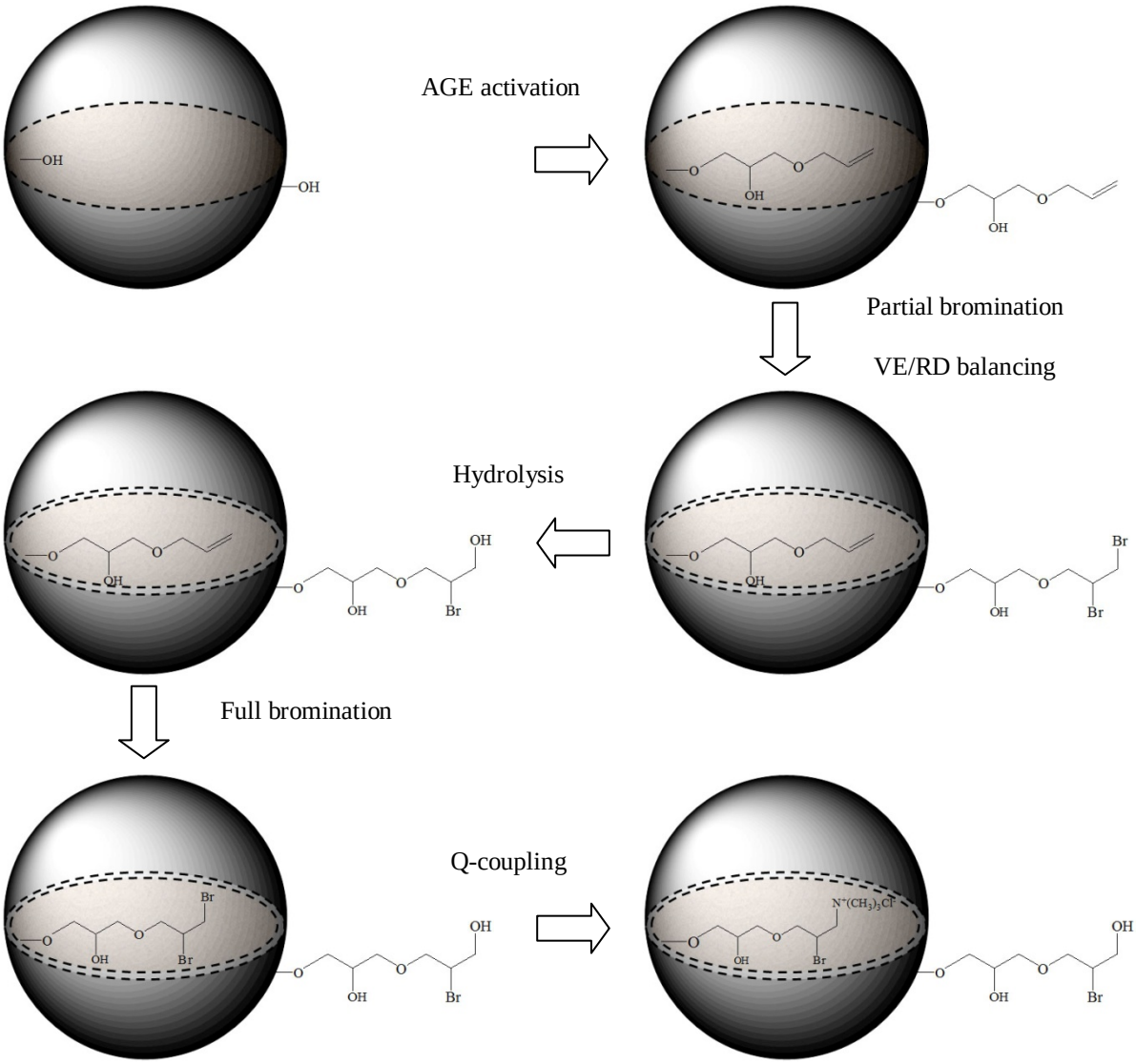

Fig.2.1 Schematic diagram of SEC/IEC supports preparation via AGE activation-partial bromination route, using VE/RD approach. Trimethylamine chloride (Q) was used for ligand coupling in this experiment.

2.2.3.1 AGE activation

AGE activation (Adapted from Gustavsson et al., 2004) was carried out by washing 60 mL settled volume of Sepharose CL-6B with MilliQ water under vacuum. The suction drained support was then transferred to a 250 mL conical flask containing 0.25 g of

sodium borohydride and 6.7 g of sodium sulphate in 24 mL of 50% (w/v) NaOH. The flask was immediately immersed in a 50^{o}C reciprocal water bath shaker (Grant OLS 200, Grant Instruments Ltd., Cambridge, UK), and was left to mix at 170 rpm for 1 h. Supports slurry was then suction dried to remove exceed NaOH solution and transferred back to the flask. Fifty one millilitres of AGE was then added to the supports and the reaction was left to proceeded overnight (16 h) in the water bath at 40^{o}C and 170 rpm. The AGE activated support was subsequently washed with: water; 70% (v/v) ethanol; and finally water once again, in order to eliminate the residual chemicals and solvents used in the reaction. The suction drained support was assayed for allyl groups content by the bromine assay. The AGE activated support was stored in 20% ethanol at 4^{o}C. Allyl contents of AGE activated supports were 0.341 $\pm$ 0.026 µg/mL supports (n=16, $\pm$ standard deviation).

2.2.3.2 Partial bromination

A 15 mL portion of allylated supports was equilibrated 3 times in a viscosity enhanced solution. The equilibrated supports were then suction dried, added to a screw cap tube containing 12 mL of viscosity enhanced solution and mixed thoroughly. For partial brominations performed in the presence of sodium acetate, 0.33 g of sodium acetate was added at this point with thorough mixing. A calculated amount of 1% (v/v) bromine solution in water, aiming for 10% elimination of total allyl content, was added. The tube was immediately sealed and shaken vigorously until the yellow colour disappeared. Supports were then washed thoroughly with water. The remaining allyl content of the supports was measured by bromine assay.

The variations of viscosity in experiments using sucrose as a sole viscosity enhancer were conducted by varying sucrose concentrations from 0-80% (w/v). Viscosities at these concentrations were further increased by reduction of temperature from room temperature to 2^{o}C by placing reaction vessels in an ice bath.

2.2.3.3 Hydrolysis of the partially brominated Sepharose CL-6B

Ten millilitres of partially brominated supports were suction dried and added to a screw cap tube containing 10 mL of water. An aliquot of 1.12 mL of 50% (w/v) sodium hydroxide solution was added, followed by addition of 0.04 g sodium borohydride. The tube was sealed immediately and left to be shaken overnight at 40^{o}C in an orbital shaker incubator (New Brunswick Scientific, New Jersey, USA). After time elapse, hydrolysed supports were washed thoroughly with water.

2.2.3.4 Full bromination of hydrolysed supports and coupling of trimethylamine chloride (Q)

Eight millilitres of hydrolysed supports were transferred to a screw cap tube containing 3.48 mL of water. After mixing, 0.39 g of sodium acetate was added and dissolved. Bromine was added to the mixture until a permanent yellow colour was obtained. The supports were then washed thoroughly with water and transferred to a clean screw cap tube containing 3.48 mL of water. A 2.61 mL portion of 50% (w/v) sodium hydroxide was added to the mixture followed by 0.035 g of sodium borohydride. After mixing, 5.07 mL of 65% (w/v) trimethylamine chloride was added. The tube was sealed immediately and was left

at room temperature overnight on a blood tube rotator SB1 (Stuart Scientific, Staffordshire, UK).The supports were then washed with water, 1 M NaCl and water once again. Modified supports were stored at 4^{o}C in 20% (v/v) ethanol.

Fully Q-coupled supports were produced by directly applying the full bromination and Q-coupling step step (Method 2.2.3.4) to allylated supports directly after AGE activation.

2.2.4 Production of plasmid used for binding studies

Starting cultures were prepared by inoculating 10 mL aliquots of Luria Bertani (LB) broth containing 50 µg/mL ampicillin with a fresh single colony of *Escherichia coli* DH5α cells containing the plasmid pITT3 and shaking overnight at 37°C and 220 rpm. A 1 mL portion of inoculum culture was then added to a 0.25 L shake flask containing 80 mL of LB broth supplemented with 50 µg/mL ampicillin and the contents were shaken on an orbital shaker at 220 rpm at 37°C until an OD_{600nm} of 0.59. LB broth (final volume of 4 L) containing 30 g/L glucose, 100 µg/mL ampicillin and Prolypropylene glycol (PPG) antifoam was used for the growth of *E. coli* in a 5 L fermenter (FerMac 360, Electrolab, Tewkesbury, UK) equipped with two six-blade Ruston turbines. The fermenter was operated in batch mode and the starting conditions employed were: temperature 37°C; agitator speed 400 rpm; air–flow rate 1.0 VVM; pH 7. The pH was maintained at 7.0 throughout the fermentation by the automatic addition of either 2 N H_2SO_4 or 4 N NaOH. The dissolved oxygen tension (DOT) was maintained above 50% by increasing the aeration and agitation. After the cells reached late exponential phase, the fermentation was terminated.

Cells were harvested by centrifugation in a J2-21 centrifuge (Beckman, High Wycombe, UK) operated at 10,000 rpm and 4^{o}C for 0.25 h. The cell paste (total wet weight of

19 g; dry cell weight of 0.872 g/L; plasmid yield of 476.76 µg/g wet cell) was stored at -20^{o}C. The plasmid produced at the end of the fermentation was purified by QIAfilter Plasmid Giga Kit. Purified plasmid was visualised by agarose gel electrophoresis and measured for pDNA content using diphenylamine (DPA) assay.

2.2.5 Analysis

2.2.5.1 Environmental scanning electron microscopy (ESEM)

ESEM visualization of SEC/IEC supports was kindly assisted by Mrs Theresa Morris, School of Metallurgy and Materials, University of Birmingham. Samples were prepared by dehydrating in ethanol followed by critical point drying. Imaging of samples was carried out on Philips XL-30 FEG Environmental SEM (FEI Company, OR, USA).

2.2.5.2 Bromine assay

One millilitre of an acidified bromine solution was added to 0.05 mL samples of suction dried supports. The mixture was shaken vigorously by a vortex mixer for 10s, the supports were separated by centrifugation at 10,000 g for 10s and the supernatants were measured for absorbance at 410 nm using a Cecil CE2040, 2000 series spectrophotometer (Cecil Instruments, Cambridge, UK). Sulfuric acid solution at a concentration of 0.5 M was used for dilutions. Sepharose CL-6B supports were used as a control and the original acidified bromine solution was used as blank. A bromine standard curve was prepared by measuring A_{410} of bromine in water at a concentration range of 0-10 mM with the number of moles of bromine that disappeared corresponding to the number of mols of allyl groups present on 1

mL settled bed of allylated support. The acidified bromine solution was prepared by mixing 4 mL of standard potassium bromate solution (0.1 M) with 8 mL of 0.5 M H_2S0_4, leave to develop the yellow colour for 600 s. The solution was mixed again by 3-4 times inversion.

2.2.5.3 Ionic capacity assay (Adapted from Theodossiou and Thomas, 2002)

Half millilitre of settled bed volume of support was incubated with 25 mL of 2 M NaCl for 1.5 h. Supports were then washed five times under vacuum with 25 mL water on a sintered glass filter funnel (16-40 µm nominal max. pore size) to remove excess acid. The drained supports were subsequently transferred to 50 mL centrifuge tubes containing 25 mL of 0.1 M NaOH and left overnight on a blood tubes rotator SB1 at room temperature. During incubation with NaOH, the excess hydroxide ions (OH^-) displace the Cl^- ions on the beads. The supports were left to settle and 1 mL of liquid phase was taken for Cl^- ion determination.

Cl- ion determination was performed as follows: 100 µL of 0.25 M ammonium iron (III) sulphate in 9 M HNO_3 and 100 µL of mercury (II) thiocyanate saturated in 96% ethanol were added to 1 mL samples, then mixed vigorously using a VM20 vortex mixer (Chil Tern Scientific Instrumentation, London, UK). The reaction was allowed to proceed for 600 s at room temperature and then the absorbance of each sample was measured at 460 nm using UVIKON 922 spectrophotometer (KONTRON Instruments, Bletchley, UK).

2.2.5.4 Static binding studies

Supports (0.05 mL settled bed volume) were equilibrated 3 times with 1 mL of 0.05 mM Tris-HCl pH 8.0. Drained supports were then incubated for 0.5 h at room temperature on a blood tube rotator SB1 with either 15 µg/mL pITT3 plasmid solution or 5 mg/mL BSA solution in 0.05 mM Tris-HCl pH 8.0. The supernatants were then taken to be analysed for DNA or protein contents using DPA assay or BCA assay, respectively.

2.2.5.5 Diphenylamine (DPA) assay

A 175 µL portion of pDNA binding supernatant was added to a 1.5 mL microcentrifuge tube followed by 25 µL of 2 mg/mL BSA solution. After vigorous mixing by a vortex mixer, 200 µL of 0.4 M perchloric acid (PCA) was added. The samples were vigorously mixed again before being incubated at 4^{o}C for 0.5 h. Samples were then centrifuged at 10,000 g for 1200 s and supernatant was discarded. A 250 µL portion of 1 M PCA was then added to the pellet, followed by vigorous mixing on a vortex mixer and incubation in a water bath at 70^{o}C for 0.5 h. Samples were then cooled to room temperature and 500 µL of freshly prepared chromogenic reagent was added followed by vigorous mixing. Samples were then incubated for 18 h in a water bath at 37^{o}C. Following incubation, the samples were measured for absorbance at 600 nm. Calf thymus DNA solutions in TE buffer, pH 8.0 at concentration range of 0-16 µg/mL were used to construct a standard curve for DNA. Water was used as blank. Chromogenic reagent was prepared by mixing 20 mL of diphenylamine solution (contains 1.5 g of diphenylamine, 100 mL of glacial acetic acid and 1.5 mL of conc. H_2SO_4) with 0.1 mL of 2% (v/v) acetaldehyde.

2.2.5.6 Bicinchoninic acid (BCA) assay

BCA assay for protein was carried out using a Pierce® BCA Protein Assay Kit (Pierce, USA) in accordance with the assay kit manual. A working reagent was prepared by mixing 50 parts of reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with 1 part of reagent B (containing 4% cupric sulfate). To perform the assay, 1 mL of working reagent was added to a 1.5 mL microcentrifuge tube containing 50 µL of sample. The tubes were then vigorously mixed by a vortex mixer and incubated in a water bath at 37^{o}C for 0.5 h. After cooling down to room temperature, samples were measured for absorbance at 562 nm using a UVIKON 922 spectrophotometer (KONTRON Instruments, Bletchley, UK). A standard curve was constructed as described in the assay kit manual using the standard 2 mg/mL BSA solution provided.

2.2.5.7 Agarose gel electrophoresis

Agarose gel electrophoresis was performed on 0.8% (w/v) agarose gel prepared with 1x TBE buffer. The gel was run in 0.5x TBE buffer in an iMyRun gel tank (Helixx Technologies, Ontario, Canada). Three µL of samples or Lambda- HinDIII marker was mixed with 7 µL of 0.5x TBE buffer and 2 µL of 6x loading dye. Twelve µL of the mixture was loaded onto 18 µL well. Horizontal electrophoresis was operated at 75 V for 2.5 h. The gel was then stained in 0.5 µg/ml ethidium bromide for 0.5 h. The ethidium bromide stained gel was visualised using a UVP UV-transilluminator at a wavelength of 304 nm. Images were taken by a Kodak DC290 digital camera (Kodak, NY, USA) via Kodak 1D software (Eastman Kodak Company; SIS, NY, USA).

2.3 Results and discussion

The preparation of SEC/IEC supports via an AGE activation/ partial bromination route involves: (i) allylation of the supports, introducing allyl groups (CH_2=CH-CH_2-) throughout the structure of Sepharose CL-6B by reaction of allyl glycidyl ether (AGE) with hydroxyl groups on the support; (ii) partial bromination of allyl groups on the surface of each particle to create an outer layer via addition reaction; (iii) hydrolysis of the resulting outer layer of bromo-alkyl groups to create an inert outer layer; (iv) full bromination of the remaining allyl groups on the support; and finally (v) coupling of trimethylamine (Q), a quaternary amine ligand, to the support's inner core.

In the partial bromination step, it is important to achieve a thin brominated outer layer in order to minimize compromisation of core charge by deeper penetration of bromine into the supports. This can be accomplished by, in this experiment, slowing the diffusion of bromine into the pores so that the bromination reaction only occurs on the outermost site of the supports. The idea of viscosity enhancement to facilitate reaction-diffusion rate (VE-RD) balancing can be explained by the Arrhenius equation, Einstein-Stoke equation and Arrhenius-Guzman equation as shown below;

Arrhenius equation

$$k = A\, exp^{(-Ea/RT)} \qquad \text{(Eq. 2.1)}$$

Einstein-Stoke equation

$$D = \frac{k_B T}{6\pi\eta r} \qquad \text{(Eq. 2.2)}$$

Arrhenius-Guzman equation

$$\eta = 0.5\, x\, 10^{-3} \exp^{(\frac{B}{RT})} \qquad \text{(Eq. 2.3)}$$

Where;

	k	is the rate coefficient (mol/L.s)
	A	is the frequency factor (s^{-1})
	E_a	is the activation energy, ~ -120 kJ/mol for bromination of double bond (Lister, 1941, Conn et al., 1938)
	R	is the gas constant (8.314 x 10^{-3} kJ/mol/K)
	T	is a temperature (K)
	D	is the diffusion constant (m^2/s)
	k_B	is Boltzmann's constant (1.381 x 10^{-23} J/K)
	η	is viscosity (kg/m/s)
	r	is the radius of the spherical diffusing species, 228 pm for Br_2 (Mountain, 2000)
	B	is an emphirical constant related to nature of the liquid (K)

In this chapter, Sepharose CL-6B was modified by the AGE activation-partial bromination route with VE-RD balancing to create SEC/IEC supports. Several parameters affecting thickness and inertness of supports' outer layer were investigated. The supports were analysed at various stages of preparation for allyl contents, ionic binding capacity as well as pDNA and protein binding capacities. The relative values, % reduction, were used to express the changes in each parameter after modifications as described below;

The reductions in allyl content was calculated by comparing the allyl content of partially brominated supports to that of the original allylated supports as shown in Eq. 2.4.

$$\%\ Reduction\ in\ allyl\ content = \frac{(\text{allyl contents of original allylated supports}) - (\text{allyl contents of partially brominated supports})}{\text{allyl contents of original allylated supports}} \text{ x } 100\% \quad \text{(Eq. 2.4)}$$

% Reductions in ionic capacity were calculated by comparing ionic capacities of modified supports to fully Q-coupled supports as shown in Eq. 2.5.

$$\%\ Reduction\ in\ ionic\ capacity\ =$$

$$\frac{(\text{ionic capacity of fully Q–coupled supports}) - (\text{ionic capacity of sample})}{\text{ionic capacity of fully Q–coupled supports}} \text{ x } 100\% \qquad \text{(Eq. 2.5)}$$

Effectiveness of surface or core charge elimination is expressed by % reductions in pDNA or protein binding capacities, respectively. % Reductions in binding capacity were calculated by comparing the binding capacities of modified supports to fully Q-coupled supports as shown in Eq. 2.6.

$$\%\ Reduction\ in\ binding\ capacity\ =$$

$$\frac{(\text{binding capacity of fully Q–coupled supports}) - (\text{binding capacity of sample})}{\text{binding capacity of fully Q–coupled supports}} \text{ x } 100\% \qquad \text{(Eq. 2.6)}$$

For Eq. 2.5 and 2.6, fully Q-coupled supports were produced by directly applying the full bromination and Q-coupling steps to the supports directly after AGE activation.

The effectiveness of the bilayer creations can be indicated by selectivity index (SI) value. SI value was expressed by comparing the remaining protein binding capacity to the remaining DNA binding capacity which therefore, indicates the ratio of residual core charge to the degree of surface charge elimination, respectively. From this aspect, SI can therefore be used to virtually demonstrate the 'thinness' of the inert outer layer as well as the depth of bromine penetration into support's pores. Selectivity indices (SI) were calculated using equation;

$$SI\ = \frac{100\% - (\%\ \text{reduction in protein binding capacity})}{100\% - (\%\ \text{reduction in pDNA binding capacity})} \qquad \text{(Eq. 2.7)}$$

ESEM visualisations of SEC/IEC Sepharose CL-6B confirm that support's structure remain unchanged compared to unmodified support even at a magnification as high as 8000x (Fig.

2.2). Unbound pDNA supernatants collected from static binding studies were visualised by agarose gel electrophoresis to check the stability of plasmid forms after binding (Fig. 2.3), with results indicating that plasmid following binding to SEC/IEC Sepharose CL-6B compared were the same as those seen following binding to unmodified supports. Plasmid band intensities on agarose gel after binding to SEC/IEC and unmodified supports were fairly similar, suggesting that surface exclusion of pDNA by the SEC/IEC supports was successful. In contrast, a large proportion of the pDNA applied to fully Q-coupled Sepharose CL-6B was bound.

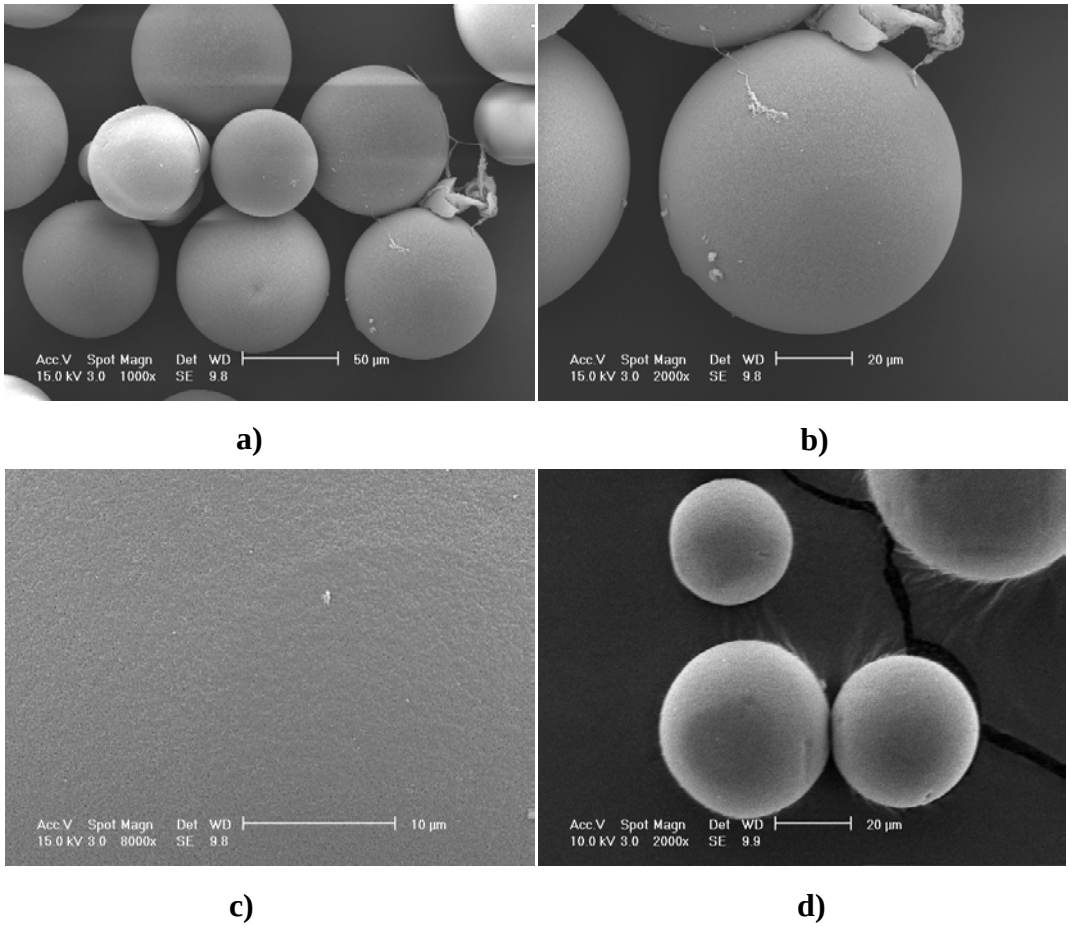

Fig.2.2 ESEM images of SEC/IEC Sepharose CL-6B, modified by VE-RD approach at; a) 1000x; b) 2000x; c) 8000x magnification and d) unmodified Sepharose CL-6B at 2000x magnification.

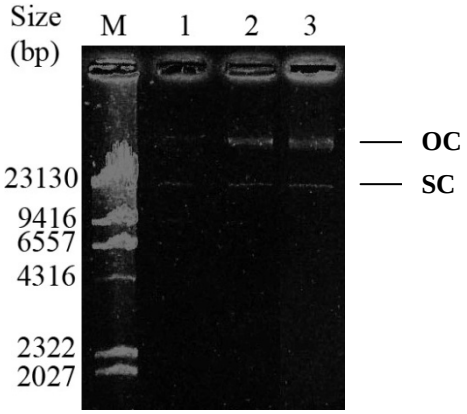

Fig.2.3 Agarose gel electrophoresis of unbound pITT3 plasmid after binding to SEC/IEC Sepharose CL-6B. Lane; M) Lambda HinD III marker; 1) fully Q-coupled; 2) 10% partial bromination in 64% (w/v) sucrose; 3) unmodified. Electrophoresis was operated in 0.8% (w/v) agarose at 75 V for 2.5 h. The gel was stained with 0.5 µg/mL ethidium bromide.

2.3.1 Effects of different viscosity enhancers, solvents and sodium acetate addition

In previous work by Liddy (2009), DMSO was reported to be the best solvent used for partial bromination reaction (Liddy, 2009). Therefore, two solvent systems were tested: (i) water or (ii) 85% (v/v) DMSO. Two viscosity enhancers tested in this experiment were sucrose and glycerol. Sucrose solution at concentrations as high as 2 g/mL was used during partial bromination reaction to produce bilayered Sephacryl S-500 HR by Gustavsson et al. in order to increase viscosity of reaction mixture (Gustavsson et al., 2004). Glycerol was chosen due to its inertness, water solubility and low toxicity. In this experiment, viscosity enhancers were added to the solvent to obtain a viscosity of 0.1 Pa.s. The effect of sodium acetate addition was also tested at a concentration of 2.75% (w/v) as described by Liddy (2009). In water, viscosity enhancement by either sucrose or glycerol addition resulted in dramatic improvement of surface charge elimination, as indicated by an improved reduction of pDNA binding capacities of supports (Fig. 2.4). Only a small difference was observed when using

40% (w/v) sucrose as a viscosity enhancer with a 85% (v/v) DMSO solvent. These results indicate that sucrose is a preferable choice compared to glycerol for viscosity enhancement. The best compositions for reaction mixture are 80% w/v sucrose in water without sodium acetate, yielding 60.8% reduction in pDNA binding capacity, 10.5% reduction in protein binding and SI of 2.28.

Sodium acetate addition was investigated due to its inclusion in previous partial bromination studies. The very first reports on chromatographic support modification using partial bromination reaction i.e. Bergstorm et al. (2002) and Gustavsson et al. (2004) added sodium acetate at a final concentration of 1% (w/v); a final concentration of 0.11% (w/v) was used by Berg et al. (2007) and 2.75% (w/v) final concentration was reported by Liddy (2009). In this study, it was observed that sodium acetate has a negative effect on the partial bromination reaction, resulting in less effective elimination as reflected by a smaller % reduction in pDNA binding capacities and allyl contents (Fig. 2.4).

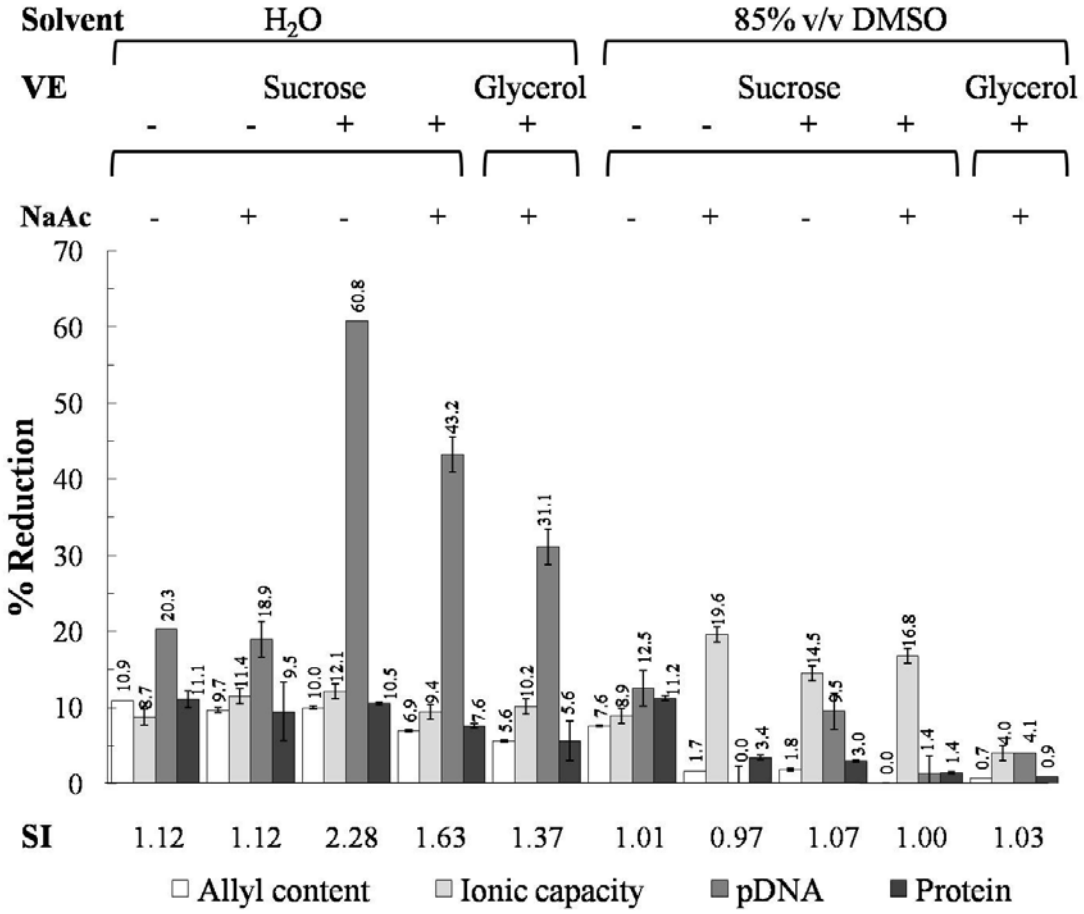

Fig.2.4 Chemical and biochemical characterisation of SEC/IEC supports produced at 10% bromination in different solvents, viscosity enhancers (VE) and NaAc addition. The concentrations of sucrose in water and DMSO are 80% and 40% (w/v), respectively. Glycerol was added to obtain the concentrations of 98.5% (v/v) in both solvents.

Bromine colour was observed to disappear more rapidly during partial bromination in some reaction mixtures than in others. This suggested that some bromine might be being consumed before it can react with the support allyl groups. As a result, two sets of experiments were carried out to determine bromine loss caused by the presence of sodium acetate. Reaction solvents with the same compositions as those being used in partial brominations, with or without viscosity enhancers and sodium acetate, were rapidly mixed with bromine to obtain a starting concentration of 200 mM bromine. Samples were taken

every 30 s to measure A_{410} in the first 900 s and were taken every 300 s until a total time of 0.5 h elapsed. The results revealed that sodium acetate causes a drastic reduction in bromine concentration in 80% (w/v) sucrose solution (Fig. 2.5). To determine whether the loss of bromine resulted from pH change caused by sodium acetate addition, a second experiment was conducted. NaOH was used to adjust the pH of 80% (w/v) sucrose solution to 7.84, similar to the pH obtained from sodium acetate addition. From this result, it was proven that pH was not the main cause of bromine loss (Fig. 2.5) but the reaction between bromine and sucrose took responsibility. Andersson, et al., (1980) reported that sucrose can be oxidised by bromine in aqueous solution at pH 7 and room temperature (Andersson et al., 1980).

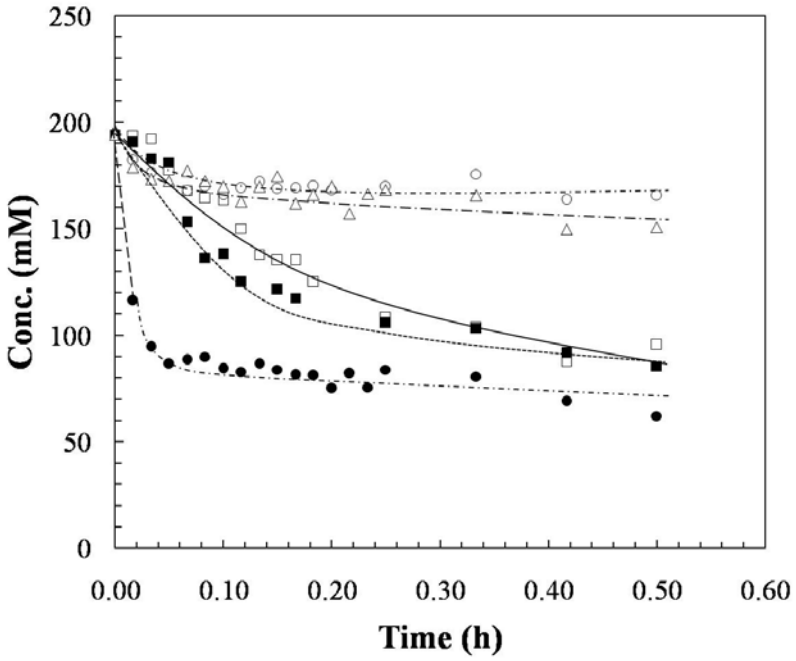

Fig.2.5 Bromine stability in water at room temperature. Symbols; □ : water; ■ : water with sodium acetate addition; ○ : 80% (w/v) sucrose; ● : 80% (w/v) sucrose with sodium acetate addition and; Δ : 80% (w/v) sucrose with NaOH addition.

Similar experiments were conducted on 85% (v/v) DMSO system (Fig. 2.6). In the presence of sodium acetate, bromine concentration rapidly dropped to zero in both 85% (v/v)

DMSO and 40% (w/v) sucrose in 85% (v/v) DMSO, indicating that the presence of sodium acetate has a strong effect on bromine loss. Supporting literature reported that DMSO can be halogenated with chlorine or bromine in the presence of a base (Iriuchijima and Tsuchihashi, 1970).

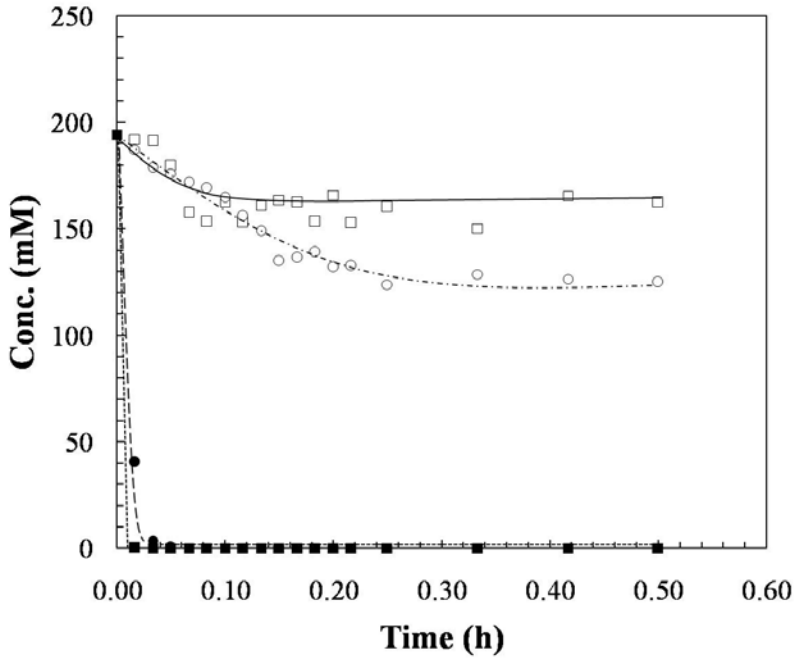

Fig.2.6 Bromine stability in 85% (v/v) DMSO at room temperature. Symbols; □ : 85% (v/v) DMSO; ■ : 85% (v/v) DMSO with sodium acetate addition; ○ : (40% w/v) sucrose in 85% (v/v) DMSO and ; ● : (40% w/v) sucrose in 85% (v/v) DMSO with sodium acetate addition.

2.3.2 Effects of viscosity and temperature

Sucrose was selected as the preferable viscosity enhancer for further studies. Room temperature (21^{o}C) partial bromination reactions were carried out in sucrose solutions with viscosities ranging from 0.001-0.104 Pa.s. Cold partial brominations (2^{o}C) were performed by placing the reaction vessels in iced water, allowing operating viscosities of 0.001-0.451 Pa.s to be achieved. Increased reaction solution viscosities led to dramatic increase of %reductions

in pDNA binding and increased SI values for the resulting SEC/IEC supports at viscosities lower than 0.032 Pa.s and the declines were observed at higher viscosity (Fig. 2.8 and 2.9). However, at viscosities above 0.1 Pa.s a plateau for % reduction in pDNA binding capacity was observed (Fig. 2.7). The data plots at different temperatures seemed to collapse on the same lines suggesting that temperature may not affect the bromination reaction in this system. It was again observed that sodium acetate has disruptive effect on partial bromination reaction, and use of sodium acetate was avoided in subsequent experiments.

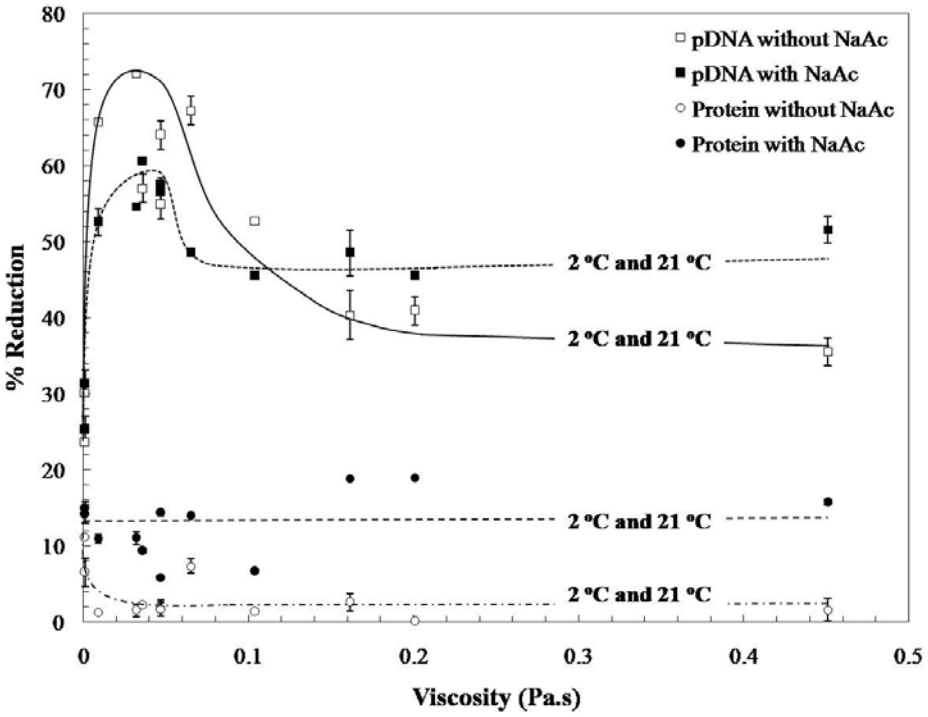

Fig.2.7 Effects of viscosity and temperature on % reduction in binding capacity of pDNA and protein. Symbols; □ : pDNA without sodium acetate addition; ■ : pDNA with sodium acetate addition; ○ : protein without sodium acetate addition and ; ● : protein with sodium acetate addition.

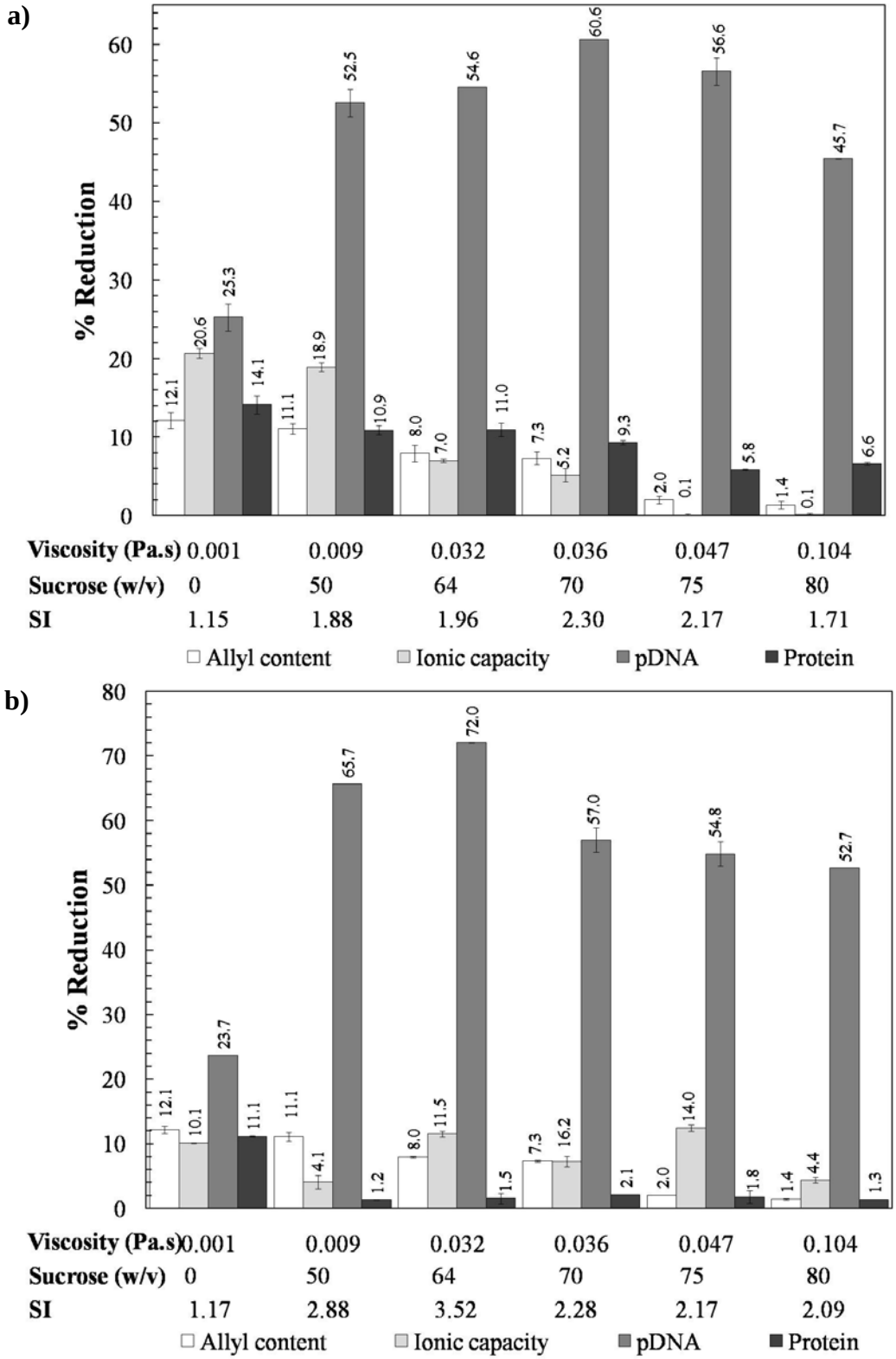

Fig.2.8 Chemical and biochemical characteristics of SEC/IEC supports produced by 10% partial bromination at room temperature (21^{o}C), a) with; b) without addition of 0.75% (w/v) sodium acetate.

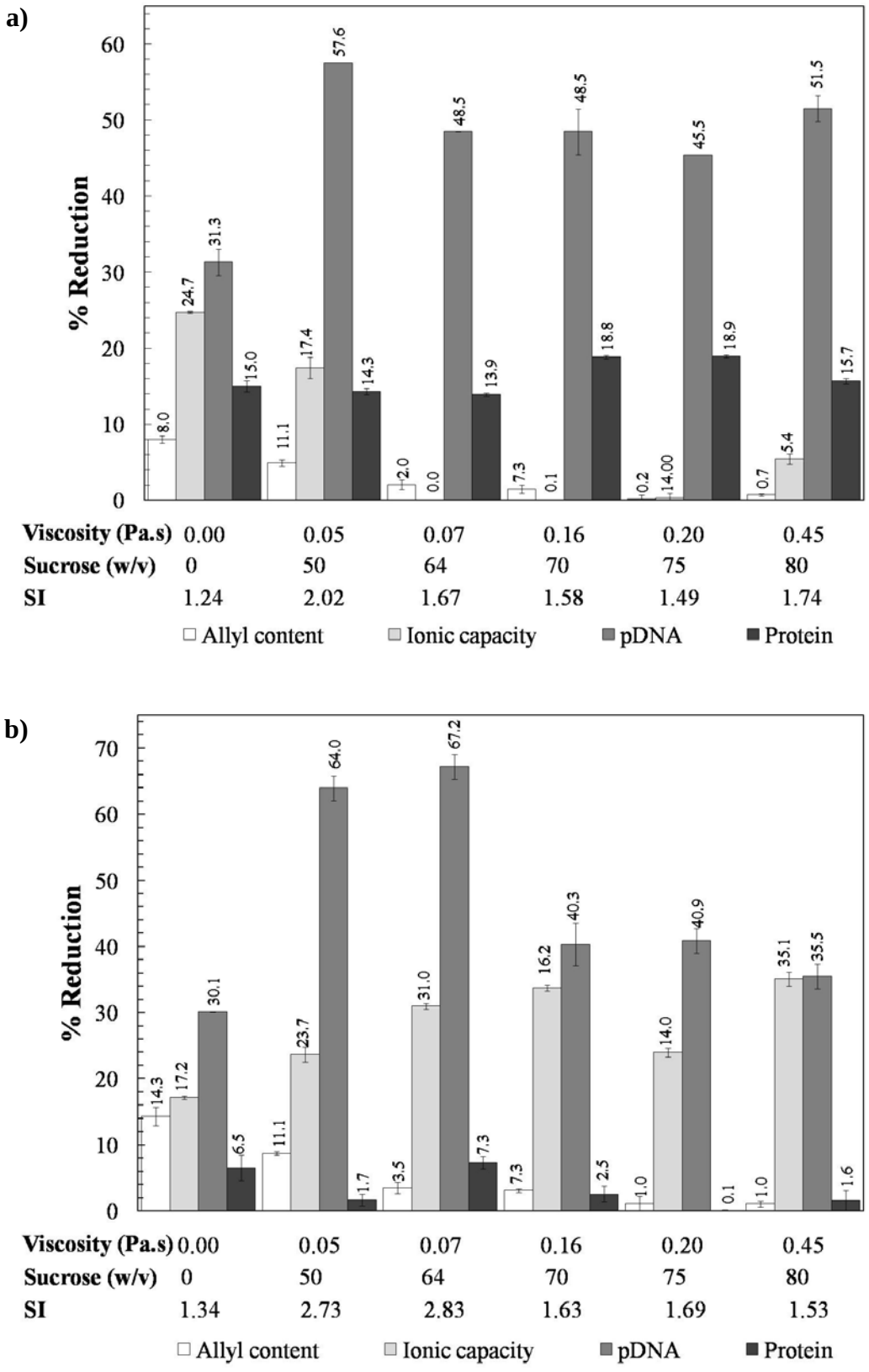

Fig.2.9 Chemical and biochemical characteristics of SEC/IEC supports produced by 10% partial bromination at 2°C; a) with; b) without addition of 0.75% (w/v) sodium acetate.

2.3.3 Effects of different degrees of partial bromination and numbers of bromination-hydrolysis cycles

Preparations of SEC/IEC supports via single partial bromination/hydrolysis cycle were performed at a range of viscosities at room temperature as described in section 2.2.3, without the addition of sodium acetate. For 20% single bromination/hydrolysis, support modification was carried out in the same fashion as described in section2.2.3 except that the quantity of bromine added was now aimed at achieving 20% elimination of allyl content on AGE activated supports. Double partial bromination/hydrolysis cycles were performed by completing a first partial bromination and hydrolysis process, thoroughly washing the supports and then applying a second partial bromination/hydrolysis cycle followed by Q-coupling. When the degree of partial bromination was increased from 10 to 20% in single bromination, improvement of % reduction in pDNA binding was observed at viscosities lower than 0.032 Pa.s. Again, the % reduction in pDNA binding in both cases were declined at the viscosity above 0.032 Pa.s and reached a plateau at viscosities higher than 0.047 Pa.s (Fig. 2.10). The possible explanation is that when sucrose concentration exceed a certain point, the viscosity dominated the reaction rate and prevented the access of bromine to the allyl group and resulted in the oxidation of sucrose by bromine competing with the bromination of allyl groups (Trombotto et al., 2004). In contrast, no decline was observed in supports produced by double 10% bromination (Fig. 2.10). A plateau was reached at viscosities higher than 0.047 Pa.s, giving a wider operation window for production compared to those seen for single bromination processes. The absence of decrease in % reduction in pDNA binding may be caused by the reinforcements of surface elimination of allyl groups on AGE activated supports when bromine can attack surface allyl groups twice. Second partial bromination allows bromine to attack leftover allyl group again from the outermost site of support particles

while in single 20% bromination, large amount of bromine allowed the further diffusion into support's pores, causing core elimination instead of surface elimination.

Although double 10% partial bromination gave higher maximum % reduction in pDNA binding (81.25%) and higher maximum SI (4.48) compared to supports prepared with single 10% partial bromination (Fig. 2.11). Single bromination possesses an advantage over double bromination in terms of simpler process scaling-up. It was concluded that the most suitable condition for SEC/IEC Sepharose CL-6B production was single 10% partial bromination at room temperature in 64% (w/v) aqueous sucrose (giving a viscosity of 0.032 Pa.s) without sodium acetate addition.

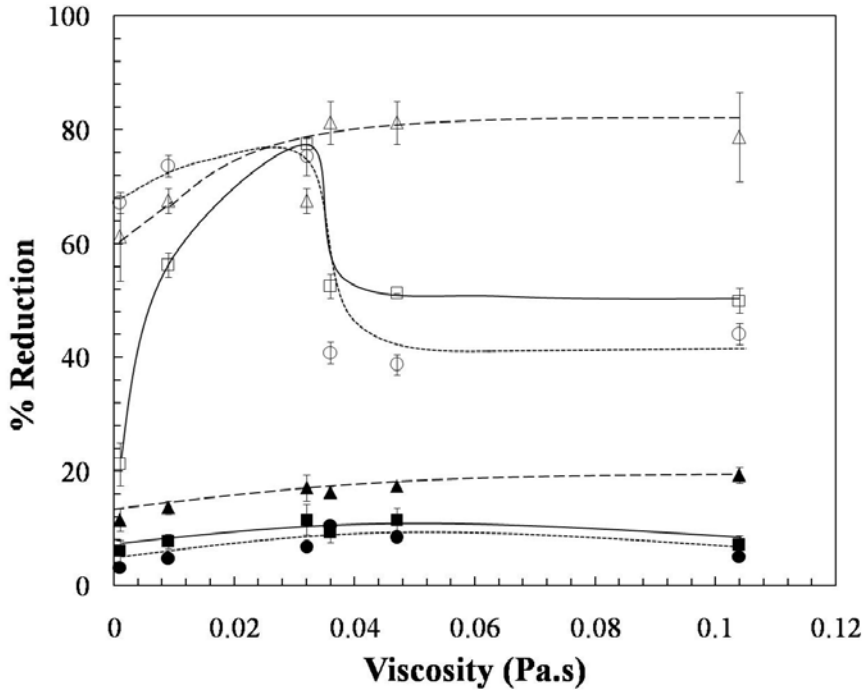

Fig.2.10 Effects of different degrees of partial bromination and numbers of bromination-hydrolysis (PB/Hy) cycles on % reduction in binding capacity of pDNA and protein at room temperature (21^{o}C). Symbols; □ : pDNA, single 10% PB/Hy; ○ : pDNA, single 20% PB/Hy; △ : pDNA double 10+10% PB/Hy; ■ : protein, single 10% PB/Hy; ● : protein, single 20% PB/Hy; ▲ : protein, double 10+10% PB/Hy.

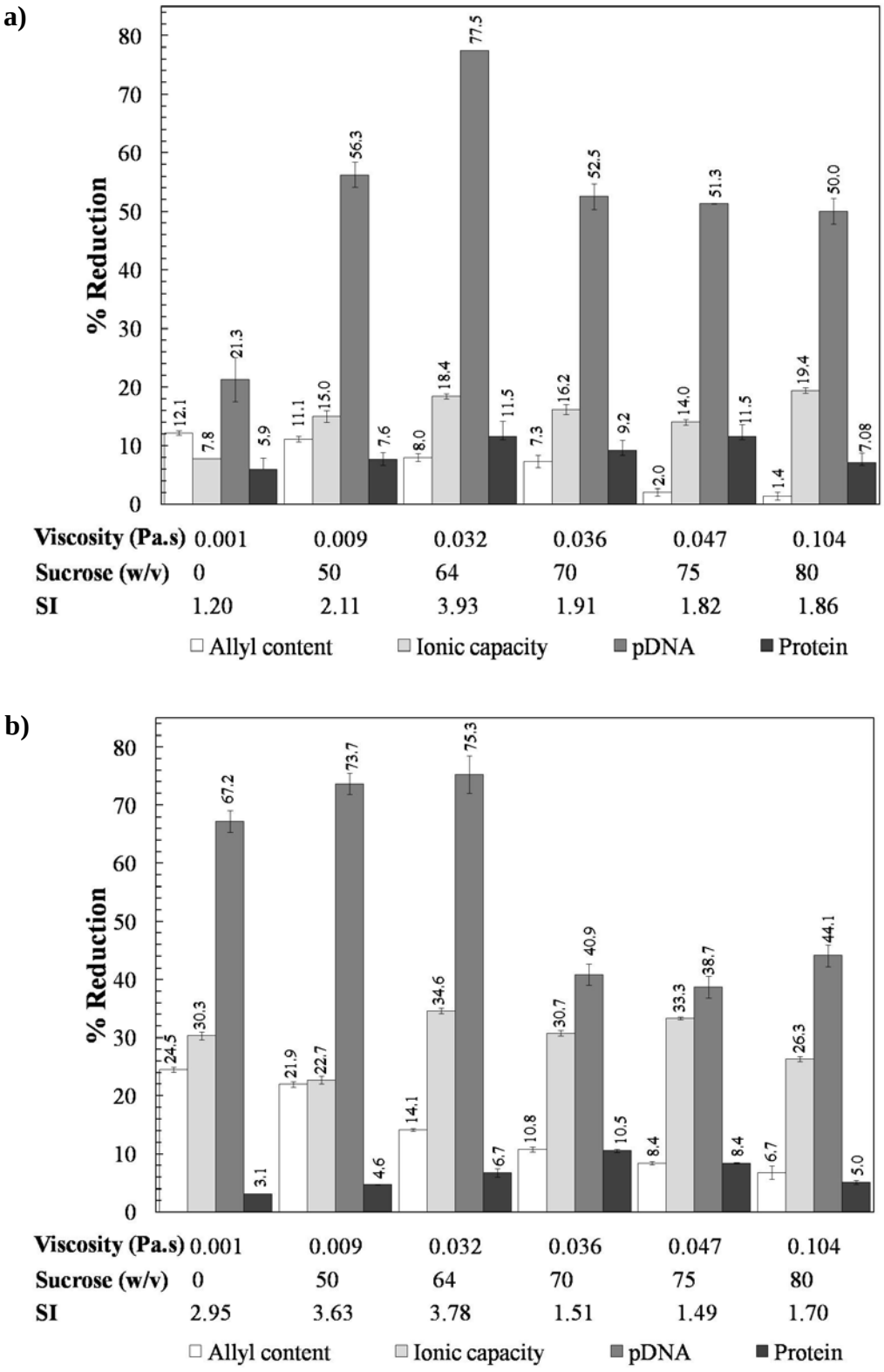

Fig.2.11 Chemical and biochemical characteristics of SEC/IEC supports produced by; a) single 10%, b) single 20% and c) double 10+10%, PB/Hy cycle at room temperature (21^{o}C) without sodium acetate addition.

c)

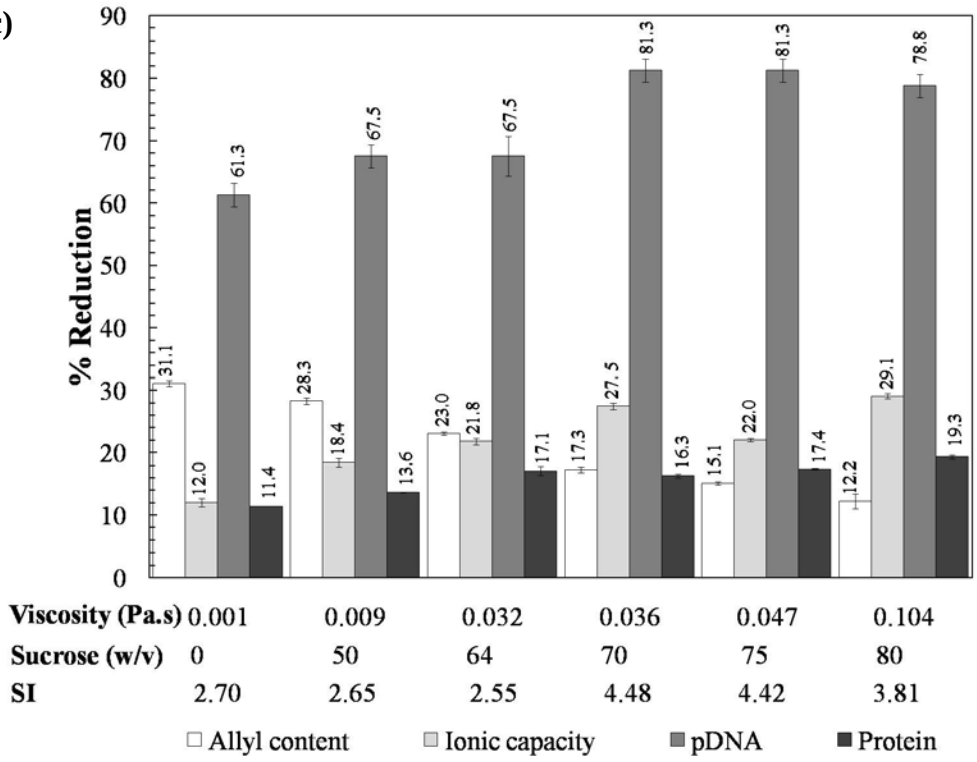

Fig.2.11 (continued) Chemical and biochemical characteristics of SEC/IEC supports produced by; a) single 10%, b) single 20% and c) double 10+10%, PB/Hy cycle at room temperature (21^{o}C) without sodium acetate addition.

2.4 Conclusions

Viscosity enhancement was found to slow down the diffusion rate of bromine into support's pore, resulting in the condensed charge elimination restricted on the outermost surface of support's particle. It was observed during partial bromination reactions that bromine colour took long time to completely disappear when viscosity enhancers were applied (without presence of sodium acetate), especially sucrose which confirmed the slower access of bromine to allyl group. However, without direct visualization, it was difficult to point out the pattern of charge distribution on support particles after modification based on the binding behaviors alone. Attempts to monitor the changes of surface elements by SEM-EDS technique had been

made but the results were unreliable due to the small size of the element of interest (nitrogen). The direct visualization technique according to Gustavsson's report was applied in an attempt to identify the thickness of the outer layer by viewing the SEC/IEC support dyed with a negative dye, Congo red under a light microscopy. Congo red binds to the positively charged core and leaves the inert outer layer unattached, resulting in a clear halo surrounding the red colour core. However, this method was found to have high chance to yield false positive 'halo' caused by the light refraction under light microscope, even with the fully Q-coupled support. Hence, this visualization was not applied further in this study. Confocal scanning laser microscopy of supports bound with fluorescent-tagged protein in order to visualize the core area was also considered. However, due to the nature of SEC/IEC supports, it is presumably impossible to attach fluorescent probes onto the surface via binding. The confocal scanning laser microscopy, therefore, cannot pick up the fluorescent signals to identify the edge of the inert outer layer. For the future work, the development of visualization techniques to identify the thickness of inert outer layer and the patterns of charge elimination might be one of the aspects worth further investigation.

In single bromination approach, the increase in viscosity resulted in the better performance of SEC/IEC supports until a certain point where the %reduction in DNA binding started to decline. The reason behind this is still unclear but it was believed that when sucrose concentration exceed a certain point, the viscosity dominate the reaction rate and instead, prevent the access of bromine to the allyl group and resulted in the oxidation of sucrose by bromine competing with the bromination of allyl groups (Trombotto et al., 2004). An experiment focused on sucrose oxidation by bromine may help addressing the cause of this behavior. However, this decline was not observe in SEC/IEC supports produced by double bromination which may be resulted from the reinforcements of surface elimination of allyl

groups on AGE activated supports when bromine can attack surface allyl groups twice. Second partial bromination allows bromine to attack leftover allyl group again from the outermost site of support particles without the deeper diffusion of bromine into support pores compared to 20% single bromination. The improved performances of SEC/IEC supports seemed to be mostly caused by the effect of viscosity regardless the reaction temperature.

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Chapter 3

Evaluation of SEC/IEC supports prepared by viscosity enhanced, reaction-diffusion balancing for the separation of RNA from *E. coli* neutralised lysates containing pDNA

Abstract

SEC/IEC supports produced via VE-RD approach was proven to effectively separate pDNA from the major contaminant, RNA, in the 'one column-one bead' purification of pDNA from *E. coli* cleared lysate feedstock. Commercially available, underivatised base matrices modified by the AGE activation-partial bromination route prepared in chapter 2 were further evaluated by applying to packed bed chromatography system in order to purify target pDNA; pITT3 (27379 bp), from neutralized *E.coli* cleared lysates. SEC/IEC Sepharose CL-6B exhibited a better performance compared to Sephacryl S-400 HR with the pDNA breakthrough readily observed, confirming its pDNA exclusion property due to the successful elimination of surface charges during the preparation while Sephacryl S-400 HR showed the complete adsorption of pDNA for the first 20 CV of loading and a breakthrough was gradually observed in 30 CV suggesting the remain of surface charges. SEC/IEC Sepharose-CL 6B also exhibited the more efficient RNA removal with the higher RNA breakthrough volume. SEC/IEC Sepharose-CL 6B modified by 10% single partial bromination at room temperature in 64% (w/v) sucrose was examine for the effects of loading conductivity, linear flow rate and target plasmid DNA size. The RNA breakthrough volume was seen to decrease

with the increased conductivity (23-54 mS/cm) and no RNA binding was observed at the highest conductivity of 54 mS/cm while the pDNA breakthrough curves reached plateau at C/C_o of ~ 1 at all conductivities applied except for at 23 mS/cm where breakthrough curves reached a C/C_o plateau of ~ 0.8. Breakthrough volume of RNA and pDNA showed the decrease when linear flow rate was increased from 30 to 90 cm/h. RNA breakthrough curves at 30 and 60 cm/h were almost completely overlapped and pDNA breakthrough curves achieved maximum at the same CV of 15, suggesting flexibility on flow rate application at 30-60 cm/h. Two plasmid species (pITT3; 27379 bp and pORT3a-Bam2k; 4109 bp) loaded gave the similar pattern of pDNA breakthrough curves. RNA breakthrough was slightly steeper for pITT3 compared to pORT3a-BAM2k. After column purification, no alteration of plasmid forms was observed. ~

3.1 Introduction

Since the success of the first approved trial on a four year-old girl suffering from severe combined immunodeficiency (SCID) in 1990 (Blaese et al., 1995, Sheridan, 2011), the interest in gene therapy was skyrocketed. Although therapeutic gene can be transported by several types of viral vectors (Mountain, 2000, Bergen and Schaffer, 2011, Cearley and Wolfe, 2009, Coura and Nardi, 2008) the safety issues involving gene transfer vectors especially viruses has been widely concerned and was emphasized by the death of an 18 year-old patient caused by viral vector-associated toxicity in 1999 (Sheridan, 2011). The viral vector was also reported to cause insertion oncogenesis in patients after the treatment using Murine leukaemia-based retroviral vector in 2008 (Hacein-Bey-Abina et al., 2008). Unlike viral vectors, plasmid vectors are considered to be a safer choice with no toxicity observed in host. The increased use of pDNA vectors in recent pre-clinical and clinical trials of gene

therapy and DNA vaccination has resulted in an increased demand for high-purity therapeutic or pharmaceutical grade supercoiled-form pDNA. In comparison to viral vectors, pDNA vectors are considered to be safer, simpler to use and easier to produce on a large-scale at reasonable cost (Diogo et al., 2005, Wolf et al., 2009), typically via *E. coli* fermentation. However, production of pDNA in *E. coli* cells poses a number of challenges for purification. *E. coli* lysates are usually heavily contaminated with anionic species of very similar structure, charge and physical behaviour (chromosomal DNA, RNA and endotoxin) (Varley et al., 1999) which may result in co-purification alongside the target pDNA (Prazeres and Ferreira, 2004, Wicks et al., 1995). Regarding product safety issues, each of these contaminants needs to be minimised according to the guidelines stated by regulatory organizations, such as the World Health Organization (WHO), Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA) (Prazeres et al., 2001, Stadler et al., 2004). This can be achieved by employing effective processes of purification.

Liquid chromatography has been a potent workhorse for pDNA purification due to its resolution, scalability and ability to provide the product purity required (Han et al., 2009, Tiainen et al., 2007b). Amongst the various chromatography modes available for the purification of pDNA, ion exchange (IEC) and size exclusion (SEC) chromatography have been the most widely used (Ferreira et al., 2000, Gustavsson et al., 2004). However, each approach holds some disadvantages. Ion exchange supports are designed for capturing proteins (2–10 nm largest diameter) (Thwaites et al., 2002, Theodossiou et al., 2000a) while larger plasmid species are unable to enter the support's pores to bind (Theodossiou et al., 2000b, Gustavsson et al., 2004). Therefore, pDNA binding is restricted to the outermost surface of support particles, resulting in a reduced of binding capacity which is 2-3 order of magnitude compared to proteins (Chandra et al., 1992, Prazeres et al., 1999, Theodossiou et

al., 2001). Moreover, similarity of charge properties between major impurities and target pDNA leads to a low selectivity where impurity species are bound and eluted alongside the targets (Diogo et al., 2005). Apart from impurities, the separation of the undesirable pDNA isoforms to achieve high purity supercoiled plasmid is also essential (Urthaler et al., 2005a, Sousa et al., 2008).

Despite the fact that size exclusion chromatography separates biomolecules based on difference in sizes, regardless of charges, problems still exist due to its comparatively limited capacity (< 2% column volume) (Diogo et al., 2005), reduced resolution at higher loading and the dilution of the plasmid product which consequently requires an extra step for concentration. Furthermore, few SEC resins available commercially have limited selectivity toward different plasmid isoforms resulting in co-purification of the other pDNA species along with the most desirable form, supercoiled pDNA (Gómez-Márquez et al., 1987, Latulippe and Zydney, 2009, Prazeres and Ferreira, 2004).

To overcome these drawbacks underlying in both IEC and SEC supports, the idea of bilayered SEC/IEC supports which combine the strengths of SEC and IEC, possessing a functionalized core to capture small impurities and inert, non-stick surface to exclude large charged micromolecules becomes more attractive. Existing approaches, mainly focused on EBA system, to produce bilayered SEC/IEC supports in order to exclude large biomolecules include; laminating IEX base matrices with a non- charged polymer layer i.e. agarose and cross-linked agarose (Dainiak et al., 2002, Jahanshahi et al., 2008, Viloria-Cols et al., 2004). However, the compromisation of bed expansion properties, hydrodynamics and intraparticle mass transfer were normally observed. The lamination of support particles with gel forming polymer naturally possesses a difficulty in casting sufficiently thin, uniform, mechanically

robust layers around core particles. (Arpanaei et al., 2010). Another approach to created bilayered SEC/IEC supports is applying low temperature plasma discharge treatment to either: (i) shave off the surface charges from the support particles (plasma etching): or (ii) coat the support particles with nano-thin polymer (plasma polymerization) (Arpanaei et al., 2010). However, these existing approaches are based on EBA systems while only few researches were published on packed bed column chromatography systems. In 2004, Gustavsson et al. introduced a 'lid bead' produced by chemically functionalisation of commercially available SEC matrix, Sephacryl S500 HR via AGE activation-partial bromination approach based on Bergstorm's methods (Bergstrom, 2002). Conclusively, this process consists of (i) introduction of allyl groups (CH_2=CH-CH_2-) throughout the structure of supports by reaction of allyl glycidyl ether (AGE) with hydroxyl groups on the support; (ii) partial bromination of allyl groups on the surface of each particle to create an outer layer via addition reaction; (iii) hydrolysis of the resulting outer layer of bromo-alkyl groups to create an inert outer layer; (iv) full bromination of the remaining allyl groups on support's core; and finally (v) coupling of a quaternary amine ligand, trimethylamine (Q), to the core. This lid bead was further evaluated by applying to an integrated process for purification of plasmid from a clarified *E. coli* lysate as the polishing step (Kepka et al., 2004a, Gustavsson et al., 2004). However, in tests with clarified alkaline lysate feedstocks at high ionic strength, in order to prevent pDNA binding, it was necessary to sacrifice over 30% of the support's RNA binding capacity. This indicates the lower than ideal definition between the inert outer layer and charged core due to the lack of ability to control the thickness and inertness of the outer size excluding layer during production process which clearly, needs improvements.

In order to improve the SEC/IEC support preparation via AGE activation-partial bromination method, the idea of viscosity enhancement to facilitate reaction-diffusion rate (VE-RD)

balancing, explained in chapter 2, was applied to partial bromination step in order to achieve a thin brominated outer layer to minimize compromisation of core charge by deeper penetration of bromine into the supports. Fine-tuning of viscosity and temperature of the solution used in partial bromination reactions can aid in balancing of diffusion and reaction rate for creating an SEC/IEC support structure with a thin inert outer layer to exclude pDNA without compromising the core binding capacity.

In chapter 2, processes for SEC/IEC supports production by VE-RD balancing approach were optimized. In this chapter, selected SEC/IEC supports from chapter 2 were further evaluated by 'one column-one bead' applications in chromatography systems for pDNA purification to separate the main contaminant, RNA, from neutralized *E. coli* cleared lysate. Variations of parameters such as pDNA sizes, bed volumes, bed heights, flow rates, ionic strengths and dilution factors of *E. coli* cleared lysate as well as different support produced were investigated. Chromatography fractions were monitored for pDNA, RNA and protein contents using UV traces, agarose gel electrophoresis and chemical assays.

3.2 Materials and methods

3.2.1 Materials

The SEC base matrices, Sepharose CL-6B and Sephacryl S400 HR, were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden).

E. coli DH5α containing the 27379 bp plasmid pITT3 was kindly provided by Dr. Eirini Theodosiou, Department of Chemical Engineering, Loughborough University, UK.

This plasmid is a pPR633-based high copy number plasmid (4579 bp) and containing a 22800 bp insert from *Saccharomyces cerevisiae* chromosome III at BamHI site.

The plasmid pORT3a-BAM2k (4109 bp) was provided by Dr. Rocky Cranenburgh, Molecular Biology, Recipharm Cobra Biologics, UK. Competent *E. coli* JM109 cells prepared using a protocol demonstrated by Nishimura et al. (Nishimura et al., 1990) were kindly provided by Mr. Chia-Chang Hsu, School of Chemical Engineering, University of Birmingham.

Luria Bertani (LB) broth, LB agar, D-glucose, ampicillin, kanamycin, prolypropylene glycol (PPG) antifoam used for culture and fermentation and transformation were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). QIAfilter Plasmid Giga Kits and QIAprep Spin MiniPrep Kits for plasmid purification were purchased from Qiagen GmbH (Hilden, Germany).H_2SO_4 and sodium hydroxide were purchased from Fisher Scientific (Loughborough, UK). EDTA, sodium dodecyl sulphate (SDS), 3 M potassium acetate pH 5.5 used for cell lysis and loading buffer preparation were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

Tricorn 5/50 column was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). All chromatography was performed on an ÄKTA Explorer 100 system controlled by UNICORN 4.11 software (GE Healthcare, Uppsala, Sweden). Sodium chloride used for buffer preparation was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

Diphenylamine, perchloric acid, acetaldehyde, glacial acetic acid and calf thymus DNA used for diphenylamine assay was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Pierce® BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL, USA). Orcinol, $FeCl_3$, ribonucleic acid from baker's yeast (*S. cerevisiae*) used in Orcinol assay were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

For phenol-chloroform extraction of nucleic acids and agarose gel electrophoresis, Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution, 0.2 µm filtered 3 M sodium acetate pH 7.0, 100x TE buffer, agarose, Lambda- HinDIII marker, 6x gel loading dye and 10x TBE buffer were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Kb DNA ladder (250 bp-12 kbp) was purchased from Agilent Technologies (Santa Clara, CA, USA). SYBR® safe DNA gel stain (Invitrogen, CA, USA) was purchased from Life Technologies Ltd (Paisley, UK). Restriction endonuclease, Bam HI and topoisomerase I from vaccinia virus were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

Distilled water was used in all experiment unless stated otherwise.

3.2.2 Transformation of plasmid pORT3a-BAM2k

Plasmid pORT3a-BAM2k was introduced into competent *E. coli* JM109 cells via heat shock transformation as described by Nishimura et al. (Nishimura et al., 1990). Competent cells were thawed on ice, 50 µL of thawed cells were transferred to a microcentrifuge tube containing 5 µL (or ~100 pg) of plasmid DNA and incubated on ice up to 0.5 h. A 60 s heat pulse at 42^{o}C was applied to induce the cells to take in plasmids. The tubes were then immediately chilled on ice for 120 s followed by addition of 0.5 mL of 42^{o}C prewarmed LB broth. Resulting transformants were grown overnight at 37^{o}C on LB agar containing 50 µg/mL kanamycin. The cells were extracted for plasmid DNA using QIAprep Spin MiniPrep Kit to check the effectiveness of transformations. Extracted pDNA was visualised using agarose gel electrophoresis as mentioned in section 3.2.6.5.

In order to prepare cell banks, a single colony was transferred to 8 mL LB broth containing 50 µg/mL kanamycin and incubated overnight on an incubator shaker (New Brunswick Scientific, New Jersey, USA) at 37^{o}C, 200 rpm. Two millilitres of sterile glycerol

was added and mixed thoroughly. An aliquot of 0.5 mL was transferred to a cryotube vial and stored at -80^{o}C.

3.2.3 Production of plasmid–containing cells

Preparation of starting cultures and fermentations of plasmid containing *E. coli* cells containing either pITT3 or pORT3a-BAM2k were carried out in the same manners as described in section 2.2.4, chapter 2 but with 50 µg/mL kanamycin being used for pORT3a-BAM2k cultures instead of 100 µg/mL ampicillin. Cells were harvested by centrifugation in a J2-21 centrifuge (Beckman, High Wycombe, UK) operated at 10,000 g and 4^{o}C for 0.25 h. The cell paste was weighed and stored at -20^{o}C.

3.2.4 Cell lysis

Cell lysis was performed using alkaline lysis as described by Gustavsson et al. (Gustavsson et al., 2004). Five grams of cell paste was thawed and resuspended in 36 mL of 10 mM Tris-HCl, pH 8 containing 61 mM glucose and 50 mM EDTA by vortex mixing in a 500 mL screw cap glass bottle. Subsequently, 78 mL of 0.2 M NaOH containing 1% SDS was added followed by 4-6 times inversion and left at room temperature for 600 s. After this incubation period, 59 mL of cold (5^{o}C) 3 M potassium acetate, pH 5.5 was added followed by gently mixing by 4-6 times inversion. White precipitate (floc) containing potassium dodecyl sulfate, genomic DNA and cell debris was removed by centrifugation at 10,000 g, 4^{o}C for 0.5 h. The supernatant was then transferred to a clean container and stored at -20^{o}C. DNA and RNA contents of lysates prepared from pITT3 containing cells were 22.94 $\pm$ 0.75 µg/mL and 1069.69 $\pm$ 47.26 µg/mL, respectively (n=8). For the lysate prepared from pORT3a-BAM2k

containing cells, DNA and RNA contents were 16.44 µg/mL and 843.00 µg/mL, respectively (n=1).

3.2.5 Chromatography

All chromatography was performed on an ÄKTA Explorer 100 system controlled by UNICORN 4.11 software (GE Healthcare, Uppsala, Sweden) at room temperature (~ 21^{o}C). SEC/IEC Sepharose CL-6B and Sephacryl S-400 HR were produced by employing a VE-RD balancing approach as described and discussed in chapter 2. Buffer A used for equilibration, loading and washing was prepared to mimic the compositions of clarified neutralised alkaline lysate by mixing 36 mL of 10 mM Tris-HCl, pH 8 containing 61 mM glucose and 50 mM EDTA with 78 mL of 0.2 M NaOH containing 1% SDS. Subsequently, 59 mL of cold (5^{o}C) 3 M potassium acetate, pH 5.5 was added followed by 4-6 times inversion. White precipitate was removed by filtration through a 0.2 µm membrane filter.

SEC/IEC supports being evaluated were packed into Tricorn 5/50 column (GE Healthcare, Uppsala, Sweden). A bed volume of 1 mL was applied in order to obtain a 5 cm bed height. Chromatographic runs were performed as follows. A packed column was equilibrated with 5 column volumes of diluted buffer A (dilution factor corresponding to the dilution factor applied to *E. coli* lysate for feedstock preparation) at a flow rate of 30 cm/h. The loading feedstock was prepared by diluting clarified neutralised *E. coli* lysate with distilled water to obtain the conductivity required for the loading phase. A specified volume (35-80 CV) of diluted lysate was loaded onto column using P-960 sample pump at a determined flow rate (30-90 cm/h). After the loading phase was complete, the column was washed with 4 CV diluted buffer A used for equilibration. The column was then eluted with a linear salt gradient

(gradient length of 20 CV) of 2 M NaCl, 1 mM EDTA, 25 mM Tris–HCl, pH 8. Finally, stripping was performed using 25 CV of a 0.2 M NaOH, 2 M NaCl solution. The chromatography runs were monitored for UV absorbance at 260 and 280 nm. The flowthrough, washed and eluted fractions were subsequently analysed by agarose gel electrophoresis, chemical assays for DNA, RNA and protein contents. Restriction enzyme digestions and topoisomerase reactions were performed to identify plasmid topology.

Variation of loading conductivity was conducted by dilution of buffer A as followed;

23 mS/cm : 1 : 4 dilution of buffer A by water

33 mS/cm : 1 : 2 dilution of buffer A by water

44 mS/cm: 1 : 1 dilution of buffer A by water

54 mS/cm: 2 : 1 dilution of buffer A by water

3.2.6 Analysis

3.2.6.1 Diphenylamine (DPA) assay

DPA assay for DNA content was performed as described in section 2.2.5.5, chapter 2.

3.2.6.2 Orcinol assay (Almog and Shirey, 1978)

Orcinol assay for RNA content used in this study was modified from the method reported by (Almog and Shirey, 1978). Working reagent was prepared by mixing 70 mL of solution A with 2.45 mL of solution B. Solution A was prepared by dissolving 100 mg of $FeCl_3$ in 100 mL concentrated HCl. Solution B was prepared by dissolving 300 mg of

orcinol in 5 mL H_2O. Samples were diluted 1:2 with water, 200 µL of each diluted sample was transferred to a 1.5 mL microcentrifuge tube followed by addition of 300 µL freshly prepared working reagent. The tubes containing samples and working reagent were mixed by a vortex mixer and incubated in a water bath at 95^{o}C for 1200 s. After incubation, the tubes were left to cool at room temperature and centrifuged at 600 rpm for 0.25 h. The supernatants were measured for A_{665} using a UVIKON 922 spectrophotometer (KONTRON Instruments, Bletchley, UK). Standard curves were constructed using ribonucleic acid from baker's yeast (*S. cerevisiae*) at a concentration range of 0-100 µg/mL. Water was used as blank.

3.2.6.3 Bicinchoninic acid (BCA) assay

BCA assay for protein was carried out in a 96-well microplate using a Pierce® BCA Protein Assay Kit in accordance with the assay kit manual. A working reagent was prepared by mixing 50 parts of reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of reagent B (containing 4% cupric sulfate). To perform the assay, 200 µL of working reagent along with 25 µL of sample was added to each well. The tubes were then incubated in a DTS-2 Microplate Thermoshaker (ATR, Laurel, MD, USA) at 37^{o}C, 200 rpm for 0.5 h. After cooling down to room temperature, samples were measured for absorbance at 562 nm using a Modulus II Microplate Multimode Reader (Turner Biosystems, Sunnyvale, CA, USA). A standard curve was constructed as described in the assay kit manual using standard 2 mg/mL BSA solution provided with the kit.

3.2.6.4 Phenol chloroform extraction of nucleic acids

Before applying to agarose gel electrophoresis, nucleic acid species in chromatography fractions were extracted using a phenol-chloroform extraction method. Two-hundred microlitres of chromatography fraction was mixed with 200 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution then centrifuged at 10,000g for 600 s. The top aqueous phase was transfered to a clean microcentrifuge tube and 20 µL of 0.2 µm filtered 3 M sodium acetate pH 7.0 was added followed by addition of 0.5 mL ice cold absolute ethanol. The tubes were mixed gently by inverting 7 times, then left at -20 ^{o}C overnight and centrifuged at 10,000g for 600 s. The pellet was washed with ice cold 70% (v/v) ethanol and centrifuged at 10,000g for 600 s. The pellet was then air dried for 0.5 h and resuspended in 20 µL of Tris EDTA pH 8. The extracted nucleic acid solution was stored at -20 ^{o}C.

3.2.6.5 Agarose gel electrophoresis

Agarose gel at a concentration of 0.8 % (w/v) was pre-stained using SYBR safe stain as follows; 10 µL of SYBR safe stain was mixed with 100 mL of 1x TBE buffer prior to addition of 0.8 g agarose powder. After melting the agarose by heating in a microwave oven, the gel was casted on a casting box equipped with an 18 µL, 26-well comb. Agarose gel electrophoresis and imaging were performed in similar manners as described in section 2.2.5.7, chapter 2 but the gel was processed directly to visualization step without ethidium bromide staining after electrophoresis.

3.2.6.6 Restriction enzyme digestions

Restriction endonuclease, Bam HI was used for digest pITT3 at insertion points. Digestion was performed by mixing 10 µL of Bam HI (containing 100 U of enzyme

activity) with 3µL of 10x digestion buffer SB and 3 µL of water. The mixture was then added with 14 µL (~ 1 µg DNA) of phenol-chloroform extracted flowthrough fraction. Enzyme-sample mixture was then incubated at 37^{o}C for 2.5 h. The enzyme was inactivated by incubation at 65^{o}C for 0.5 h. Resulting cut fractions were visualised by agarose gel electrophoresis as described in section 3.2.6.5.

3.2.6.7 Topoisomerase reaction

One microlitre (~ 10 units) of topoisomerase I from vaccinia virus was added to 14 µL (~ 1 µg DNA) of phenol-chloroform extracted flowthrough fraction. The reaction was preceded at 37^{o}C for 1 h. Resulting relaxed plasmid samples were visualised by agarose gel electrophoresis as described in section 3.2.6.5.

3.3 Results and discussion

Commercially available, underivatised base matrices modified by the AGE activation-partial bromination route using VE-RD approach prepared in chapter 2 were further evaluated by applying to packed bed chromatography system in order to purify target pDNA; pITT3, from neutralized *E.coli* cleared lysates. Plasmid DNA, RNA and protein contents were monitored by UV traces and chemical assays as well as agarose gel electrophoresis. Plasmid topology of pDNA in the flowthrough was identified using restriction enzyme digestions topoisomerase reactions.

Bam HI digestion of the flowthrough fraction containing pITT3 yielded a 22800 bp fragment of *Saccharomyces cerevisiae* chromosome III and a 4579 bp fragment of pPR633 plasmid vector as demonstrated in Fig. 3.1a, confirming the circular form of plasmid in the

flowthrough which can be either supercoiled (SC) or open circular (OC) forms. This result indicated that there was no contamination of linear pDNA after column purification, suggested the mild purification property of SEC/IEC support without damaging the pDNA. Result from topoisomerase relaxation of pDNA in the flowthrough showed that supercoiled (SC) band migrated faster on the gel compared to the open circular form. After relaxation by topoisomerase, supercoil pDNA was unwound to yield one band representing the open circular (OC) form (Fig. 3.1b).

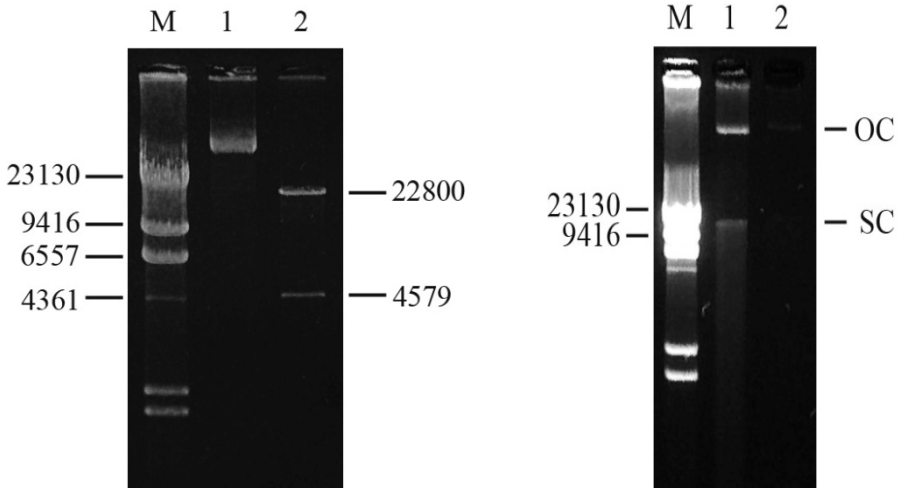


Fig. 3.1 Agarose gel electrophoresis images of; a) BamHI digestion of pITT3 in flowthrough fraction after applying to SEC/IEC column. Lanes; M: Lambda HinDIII marker, 1: uncut pITT3, 2: pITT3 cut by BamHI. b) Topoisomerase relaxation of pITT3 in flowthrough fraction after applying to SEC/IEC column. Lanes; M: Lambda HinDIII marker, 1: native pITT3, 2: pITT3 after topoisomerase reaction.

3.3.1 Effects of different base matrices

SEC/IEC supports based on Sepharose CL-6B and Sephacryl S-400 HR modified by 20% single partial bromination at room temperature in 80% (w/v) sucrose were loaded with 60 column volumes of neutralized *E.coli* lysate with a conductivity of 33 mS/cm at a linear flow rate of 30 cm/h. DNA and high molecular weight RNA (HM-RNA) breakthrough curves

(Fig. 3.2) show a better performance for Sepharose CL-6B than Sephacryl S400 HR, as indicated by a shallower slope for RNA and a steeper curve for pDNA. Shallower RNA breakthrough curve of Sepharose CL-6B suggests a higher RNA binding capacity which is confirmed by the UV_{260} trace on the chromatogram and total RNA content measured by orcinol assay (Fig. 3.3) as well as agarose gel electrophoresis (Fig.3.4). A steeper pDNA breakthrough indicates a lower pDNA binding capacity on Sepharose CL-6B compared to Sephacryl S-400 HR. Moreover, pDNA breakthrough was observed readily after loading, confirming pDNA exclusion due to successful elimination of surface charges during support preparation. In contrast, pDNA content (Fig 3.3) and agarose gel electrophoresis showed a complete adsorption of pDNA on Sephacryl S-400 HR for the first 20 CV of loading and a breakthrough was gradually observed in 30 CV. Plasmid adsorption on this support was evident by significant amounts of pDNA in elution fractions (Fig. 3.3 and 3.4). The different performance on pDNA binding was thought to be caused by difference in pore sizes between Sepharose CL-6B and Sephacryl S-400 HR (24 nm and 31 nm, respectively) (Hagel et al., 1996). However, with large biomolecules such as plasmids which normally are larger than 0.2 µm (Fishman and Patterson, 1996) and much larger the pore sizes of these two supports. Conclusively, the difference in pore sizes of supports does not cause the difference in absorption behaviour of pDNA in this case. Therefore, it is possible that modified Sephacryl S400 HR tested still possesses surface charges, high enough to still be presence at high conductivity applied and resulting in a later breakthrough compared to modified Sepharose CL-6B.

Protein contents measured by BCA assay from each chromatography runs were stable during loading phase and declined after washing (Fig. 3.3). However, BCA assay results did not correspond to UV_{280} traces. This may be caused by the low selectivity of UV_{260} and UV_{280} adsorption signals between protein and nucleic acid.

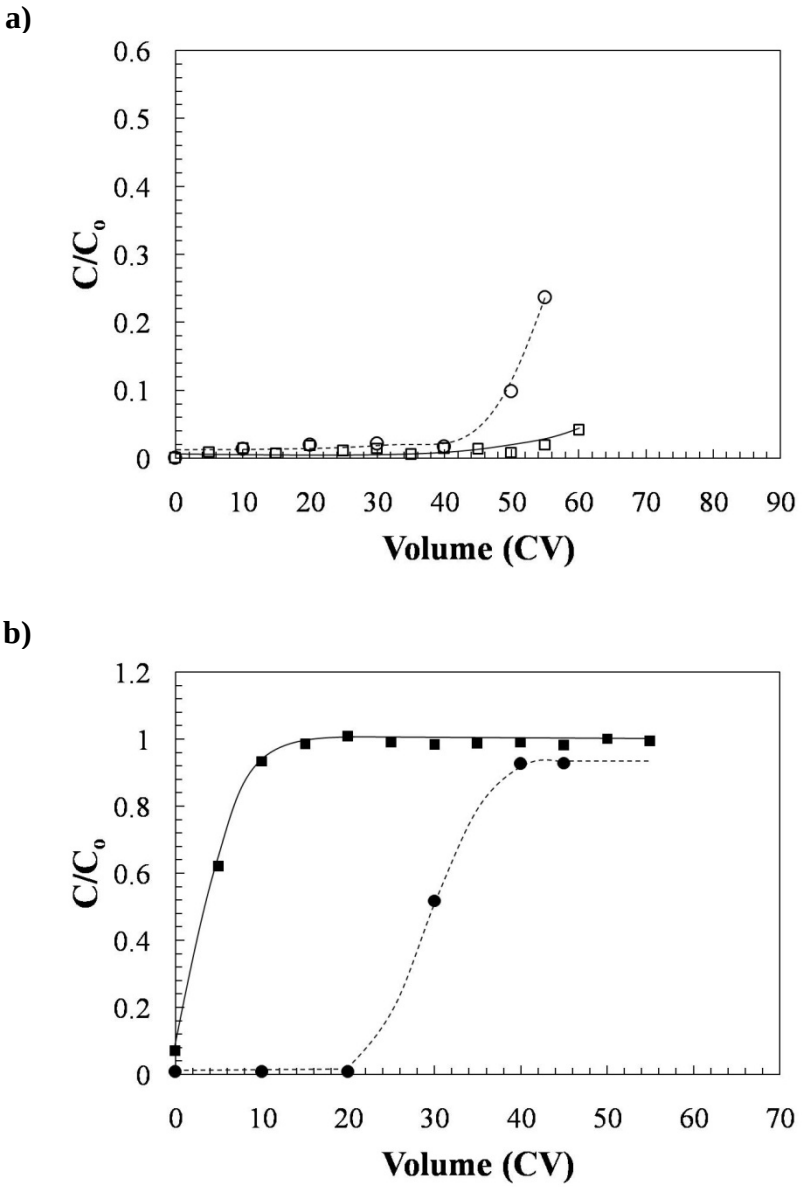


Fig. 3.2 Influence of different base matrices on breakthrough curves for; a) HM-RNA (open symbols) and b) pDNA (closed symbols) binding in packed bed chromatography. Symbols; Squares : Sepharose CL-6B; Circles : Sephacryl S400 HR. Chromatography runs were performed at a conductivity of 33 mS/cm, linear flow rate of 30 cm/h.

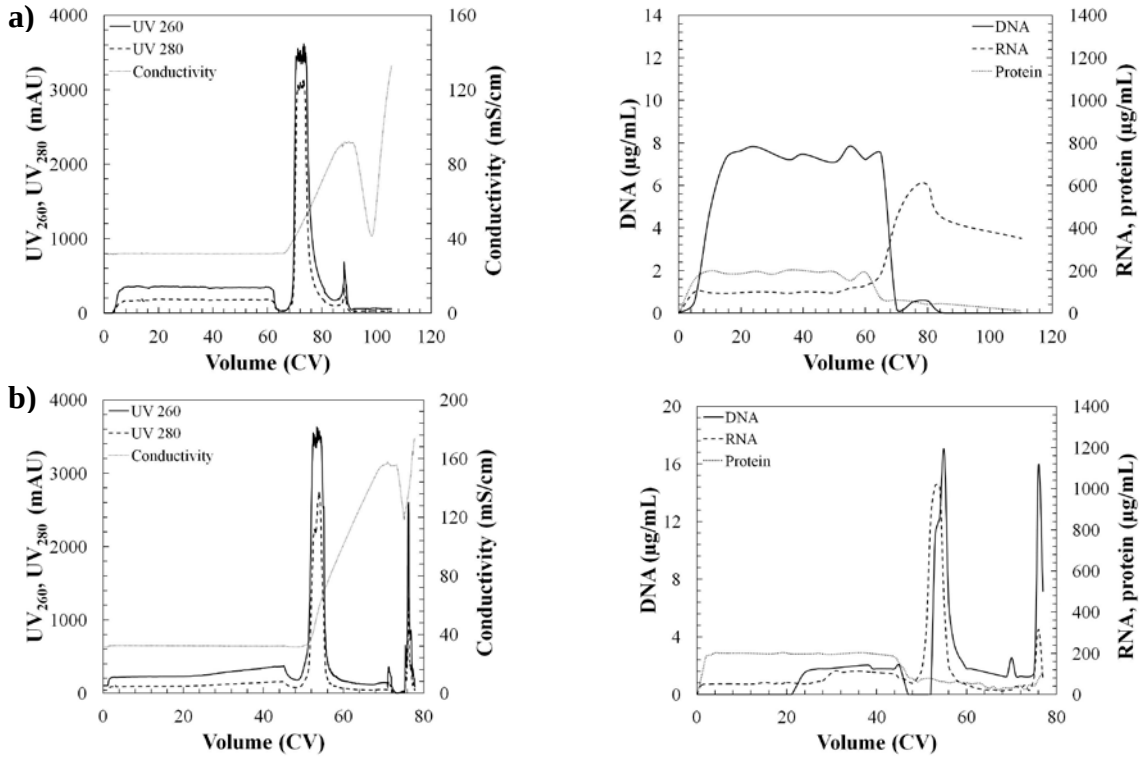


Fig. 3.3 Chromatograms (left) and chemical measurements of chromatographic fractions (right) obtained from packed bed chromatography of a) Sepharose CL-6B and b) Sephacryl S-400 HR. Chromatography runs were performed at a conductivity of 33 mS/cm, linear flow rate of 30 cm/h.

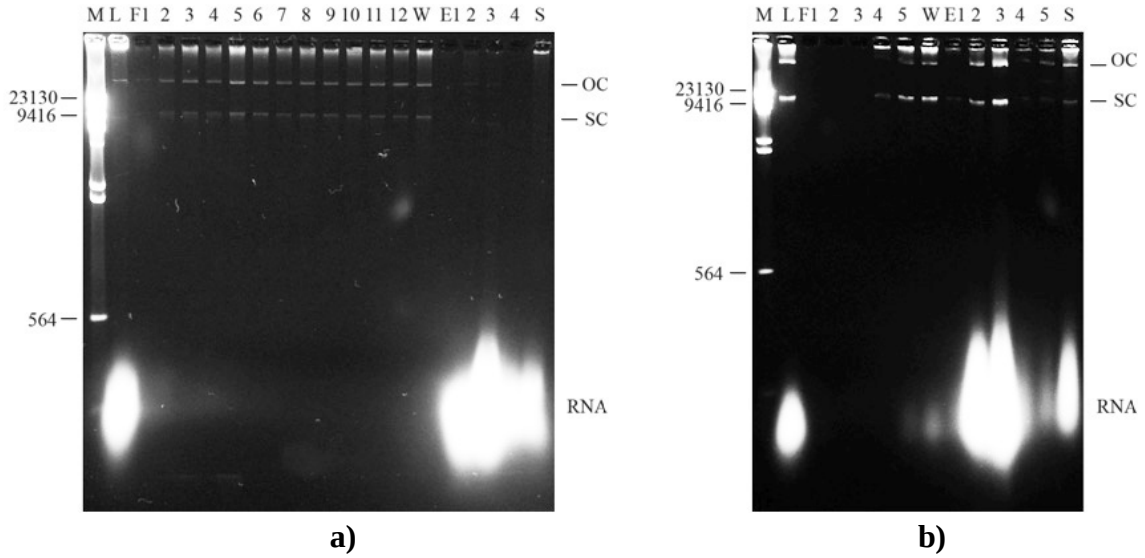


Fig. 3.4 Agarose gel eletrophoresis of chromatographic fractions obtained from a) Sepharose CL-6B and b) Sephacryl S-400 HR. Fraction sizes were 5 CV for the run performed on Sepharose CL-6B and 10 CV for the run performed on Sephacryl S-400 HR. Abbreviations; M : Lambda HindIII marker, L : cleared lysate fed onto column, F : flowthrough, W : wash fraction, E : elution fractions and S : stripped fraction.

3.3.2 Effects of loading conductivities

Feedstock with loading conductivities of 23-54 mS/cm were applied to chromatography runs using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose. 1 mL of supports was packed in a Tricorn 5/50 column to obtain a bed height of 5 cm. Chromatography runs were operated at a flow rate of 30 cm/h. Breakthrough curves of HM-RNA and pDNA are shown in Fig. 3.5. RNA breakthrough increased when conductivities were decreased. Small amount of RNA in elution and stripped fractions indicated that almost no binding was observed when the conductivity was increased to 54 mS/cm (Fig. 3.6 and 3.7). It was reported that under conditions of relatively high ionic strength (> 50 mS/cm), almost no RNA binding occur and most of the RNA elutes in the flow through (Stadler et al., 2004). Plasmid DNA breakthroughs showed similar trends where pDNA binding increased when conductivity was decreased. At all conductivities applied, pDNA breakthrough curves reached plateau at C/C_o of ~ 1 except for at 23 mS/cm where breakthrough curves reached a C/C_o plateau of ~ 0.8. This observation suggested pDNA loss during loading phase via binding, which was promoted by low conductivity. These findings on various conductivities applied are supported by chromatograms and chemical measurements as shown in Fig. 3.6 as well as gel electrophoresis results shown in Fig. 3.7. Protein contents measured by BCA assay from each chromatography runs were stable during loading phase and declined after washing (Fig. 3.6).

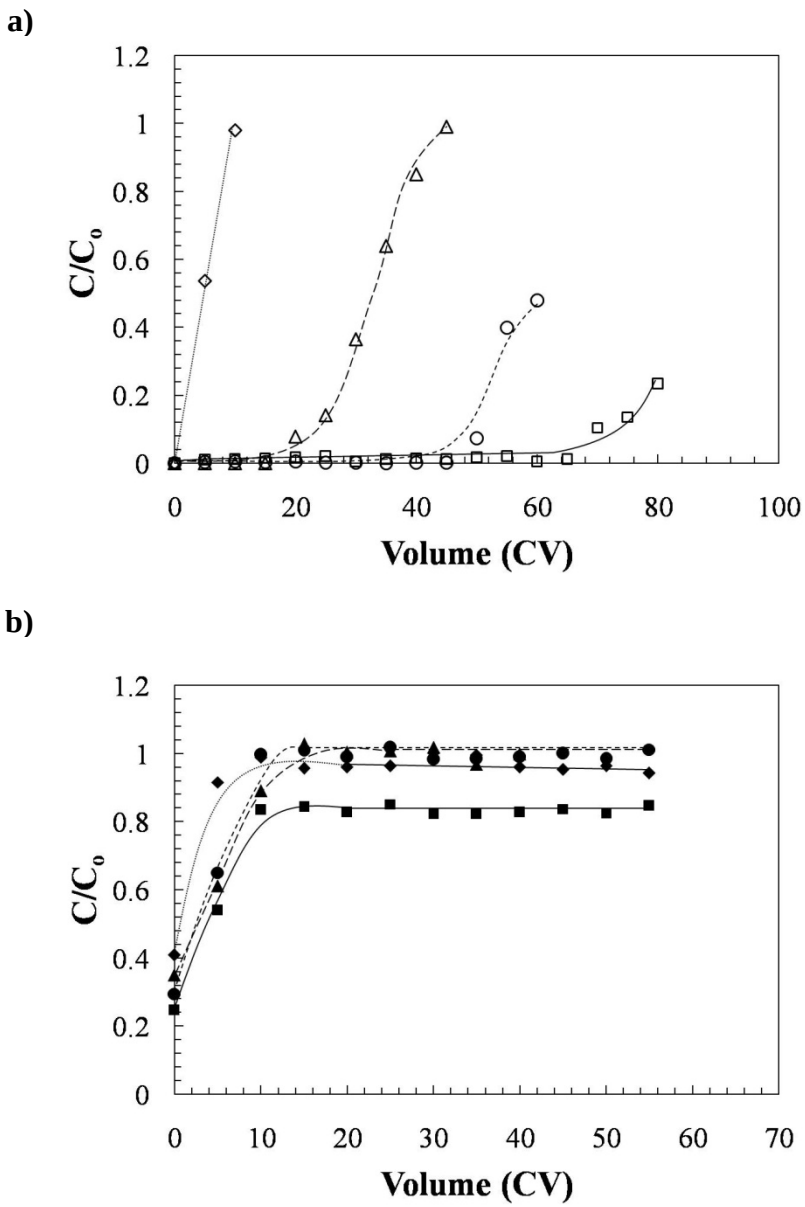


Fig. 3.5 Influence of different loading conductivities on breakthrough curves for; a) HM-RNA (open symbols) and b) pDNA (closed symbols) in packed bed chromatography. Symbols; squares : 23 mS/cm; circles : 33 mS/cm; up-triangles : 44 mS/cm and diamonds : 54 mS/cm. Chromatography runs were operated at a flow rate of 30 cm/h using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

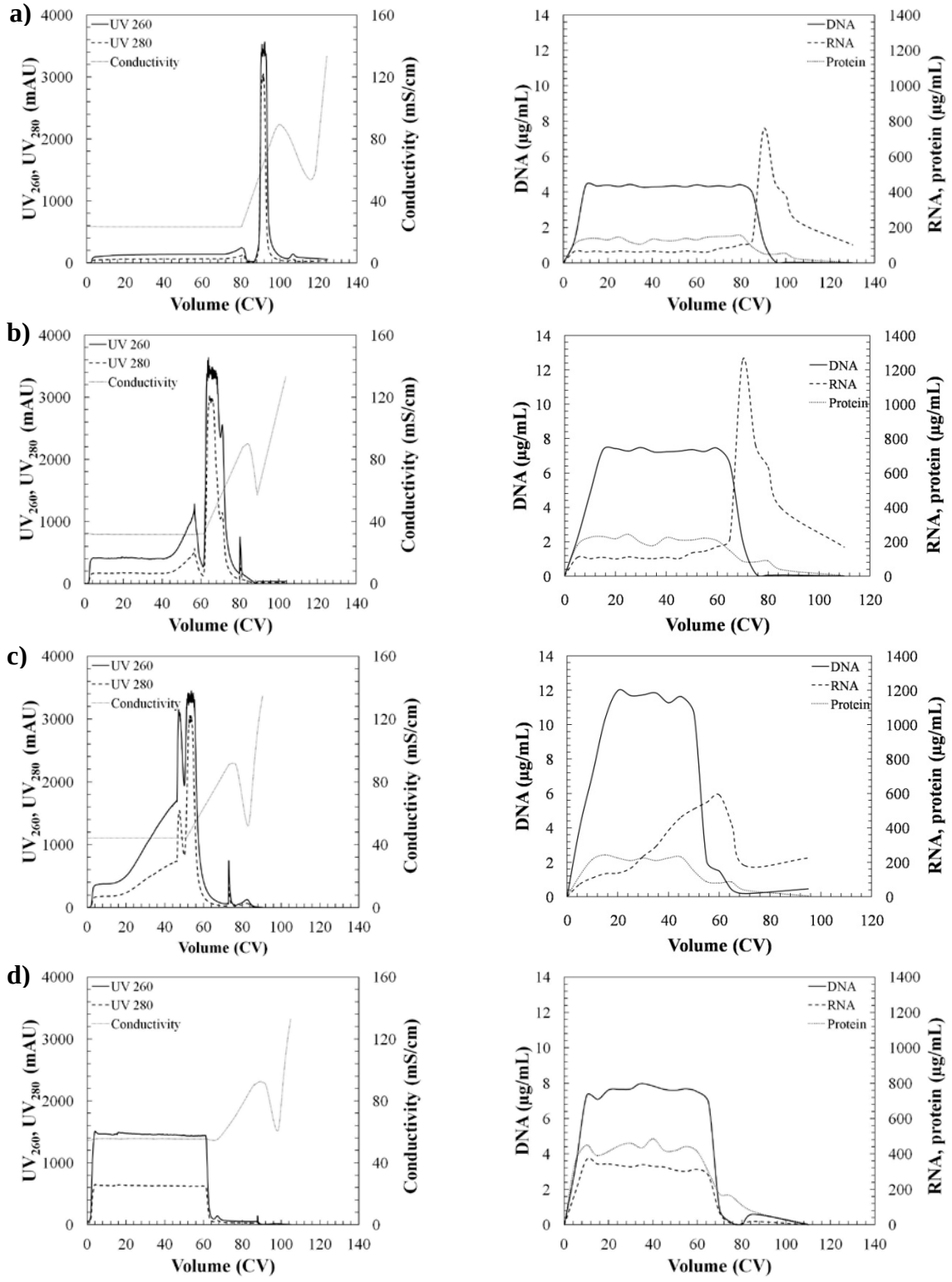


Fig. 3.6 Chromatograms (left) and chemical measurements of chromatographic fractions (right) obtained from packed bed chromatography at different loading conductivities of a) 23 mS/cm, b) 33 mS/cm, c) 44 mS/cm and d) 54 mS/cm. Chromatography runs were operated at a flow rate of 30 cm/h using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

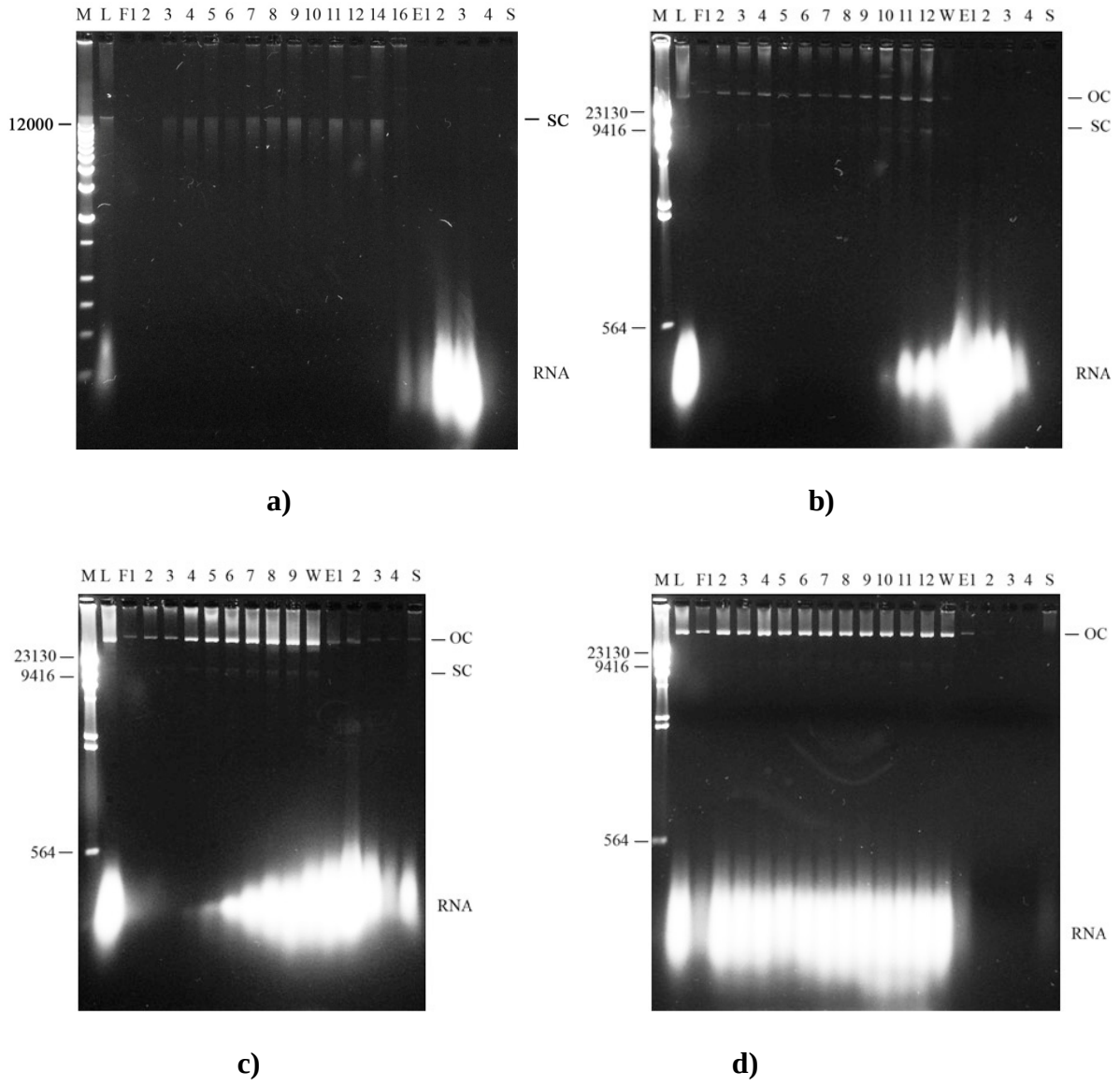


Fig. 3.7 Agarose gel eletrophoresis of chromatographic fractions obtained from packed bed chromatography at different loading conductivities of a) 23 mS/cm, b) 33 mS/cm, c) 44 mS/cm and d) 54 mS/cm. Fraction size was 5 CV. Abbreviations; M : Kb DNA ladder (250 bp-12kbp), (gel a) or Lambda HindIII marker (gel b-d), L : cleared lysate fed onto column, F : flowthrough, W : wash fraction, E : elution fractions and S : stripped fraction.

3.3.3 Effects of linear flow rates

Various linear flow rates of 30-90 cm/h were applied to chromatography runs using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose. Loading conductivity of 33 mS/cm was obtained by 3 times dilution of

neutralized *E.coli* cleared lysates or loading buffer with water. One millilitre of support was packed into a Tricorn 5/50 column to obtain a bed height of 5 cm. Breakthrough curves of HM-RNA and pDNA bindings showed a reduction in both pDNA and RNA binding capacities when linear flow rate was increased (Fig. 3.8). Plasmid DNA breakthrough curves of all flow rates reached the maximum of $C/C_0 = 1$. Interestingly, RNA breakthrough curves at 60 cm/h almost overlapped with breakthrough curve at 30 cm/h and pDNA breakthrough curves achieved maximum at the same CV of 10, suggesting flexibility on flow rate application at 30-60 cm/h. While the flow rate of 90 cm/h showed earlier RNA breakthrough at 30-35 CV. Agarose gel images presented in Fig 3.10 confirmed the results observed in breakthrough curves and suggested the same trends.

Protein contents measured by BCA assay from each chromatography runs were stable during loading phase and were decreased after washing (Fig. 3.9).

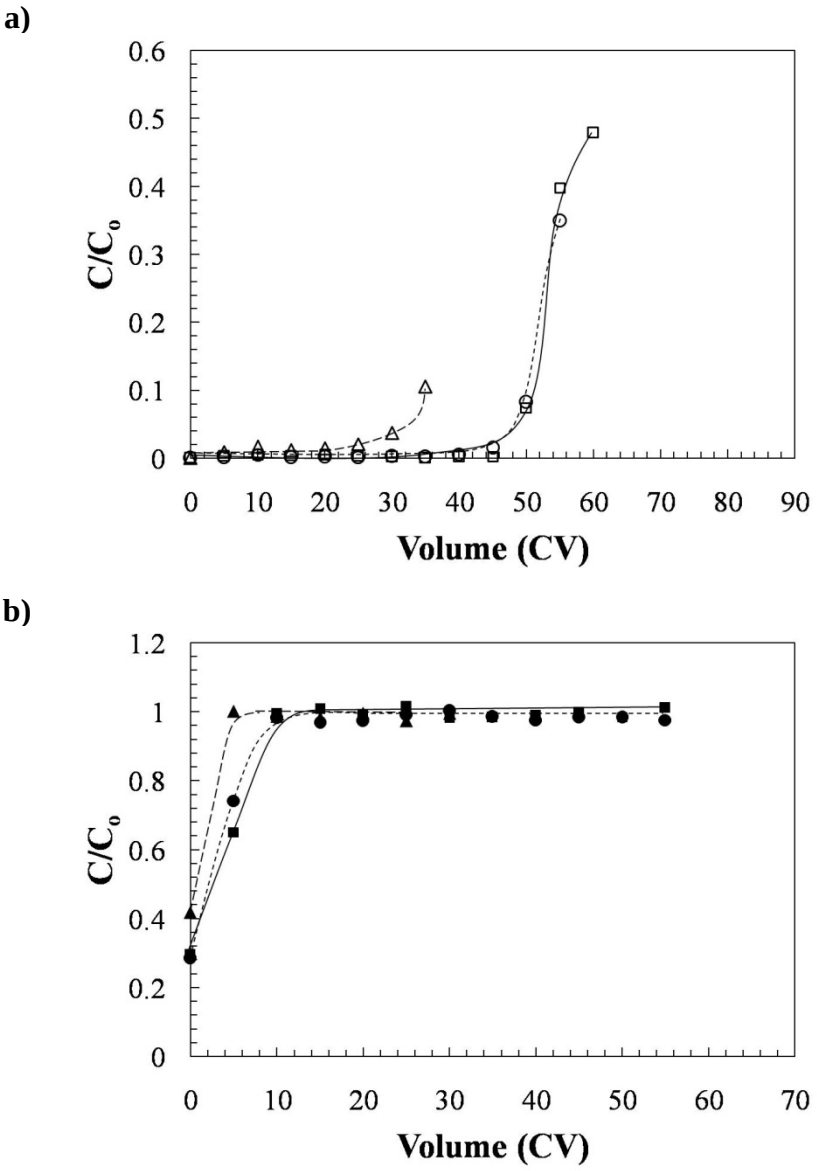


Fig. 3.8 Influence of different linear flow rates on breakthrough curves for; a) HM-RNA (open symbols) and b) pDNA (closed symbols) in packed bed chromatography. Symbols; squares: 30 cm/h; circles: 60 cm/h; and up-triangles: 90 cm/h. Chromatography runs were operated at a loading conductivity of 33 mS/cm using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

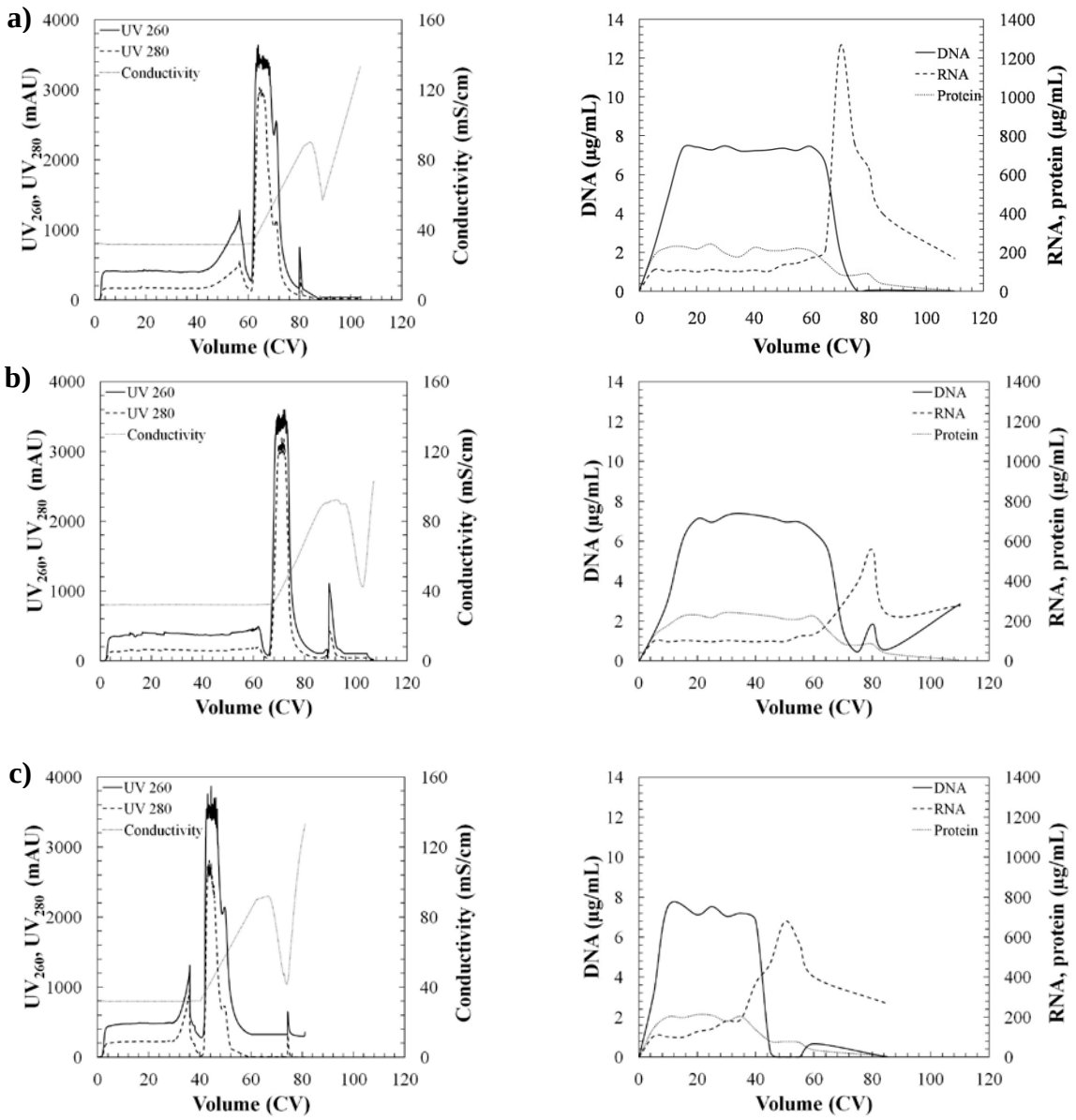


Fig. 3.9 Chromatograms (left) and chemical measurements of chromatographic fractions (right) obtained from packed bed chromatography at linear flow rates of a) 30 cm/h, b) 60 cm/h, c) 90 cm/h. Chromatography runs were operated at a loading conductivity of 33 mS/cm using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

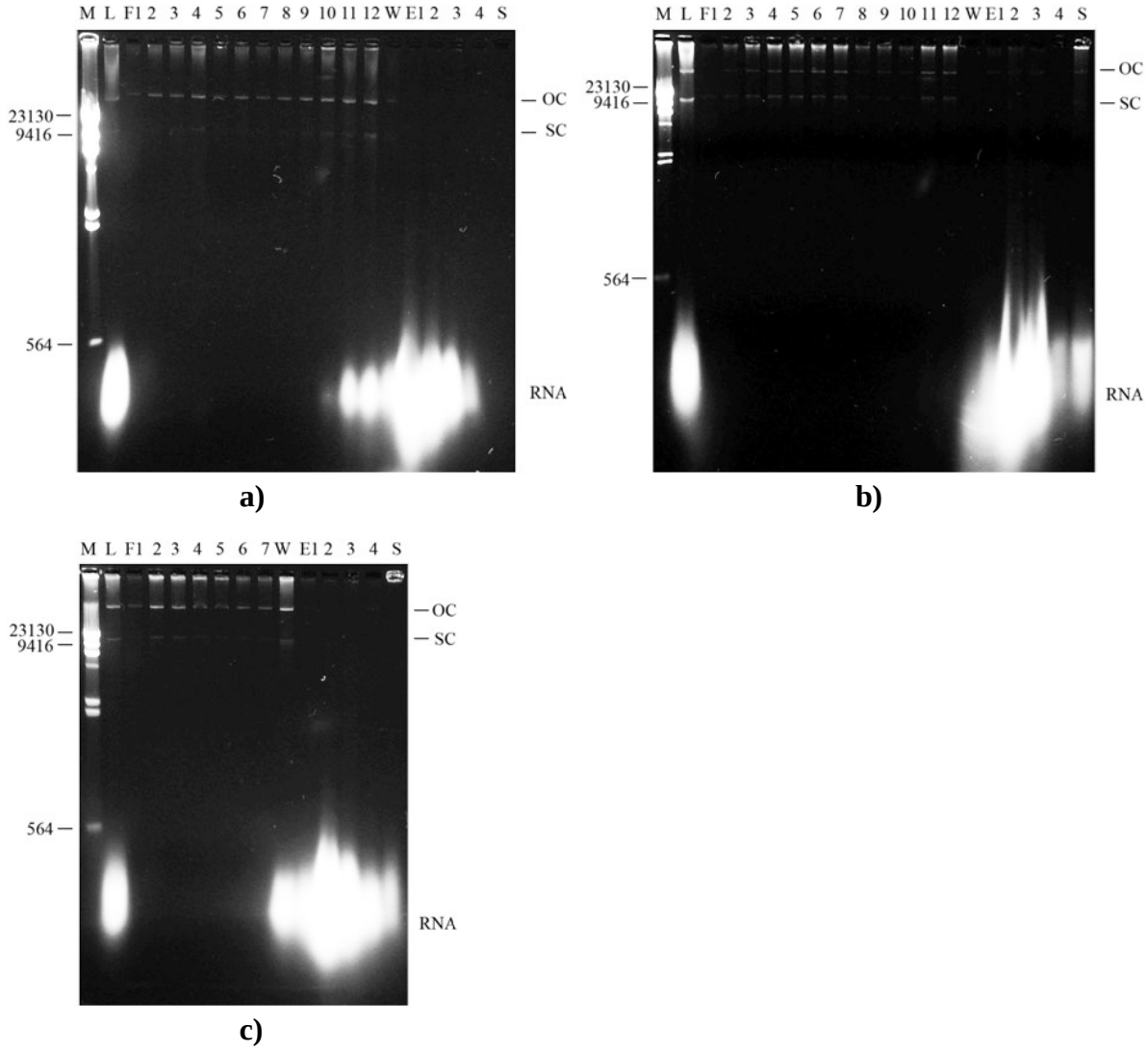


Fig. 3.10 Agarose gel eletrophoresis of chromatographic fractions obtained from packed bed chromatography at linear flow rates of; a) 30 cm/h, b) 60 cm/h and c) 90 cm/h. Fraction size was 5 CV. Abbreviations; M: Lambda HindIII marker, L: cleared lysate applied onto column, F: flowthrough, W: wash fraction, E: elution fractions and S: stripped fraction.

3.3.4 Performances on different plasmids

To further assess the pDNA exclusion properties of SEC/IEC supports, neutralized *E. coli* cleared lysate containing a smaller plasmid; pORT3a-BAM2k (4109 bp) was applied to a Tricorn 5/50 column packed with 1 mL SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose to obtain a 5 cm bed height. Loading

conductivity of 33 mS/cm was obtained by 3 times dilution of neutralized *E.coli* cleared lysates or loading buffer with water. Diluted lysate was loaded onto the column at a linear flow rate of 30 cm/h. Breakthrough curves for pDNA showed similar pattern for both plasmid species despite the fact that the curve representing pORT3a-BAM2k reached a plateau at a $C/C_0 < 1$ (Fig. 3.11). This observation is confirmed by pDNA content measured by DPA assay (Fig. 3.12) and agarose gel electrophoresis result of pORT3a-BAM2k (Fig. 3.13) showing pDNA in elution and stripped fractions, especially the bands located at high molecular weights region on gel. The higher molecular weight pDNA are generally bind more tighly with anion ligands on support. HM-RNA breakthrough was slightly steeper for pITT3 and RNA appeared in the flowthrough earlier at 50 CV on agarose gel while pORT3a-BAM2k appeared later on the gel at 55 CV (Fig. 3.11 and 3.13). The possible cause of various molecular weights for pORT3a-BAM2k will be discussed later on.

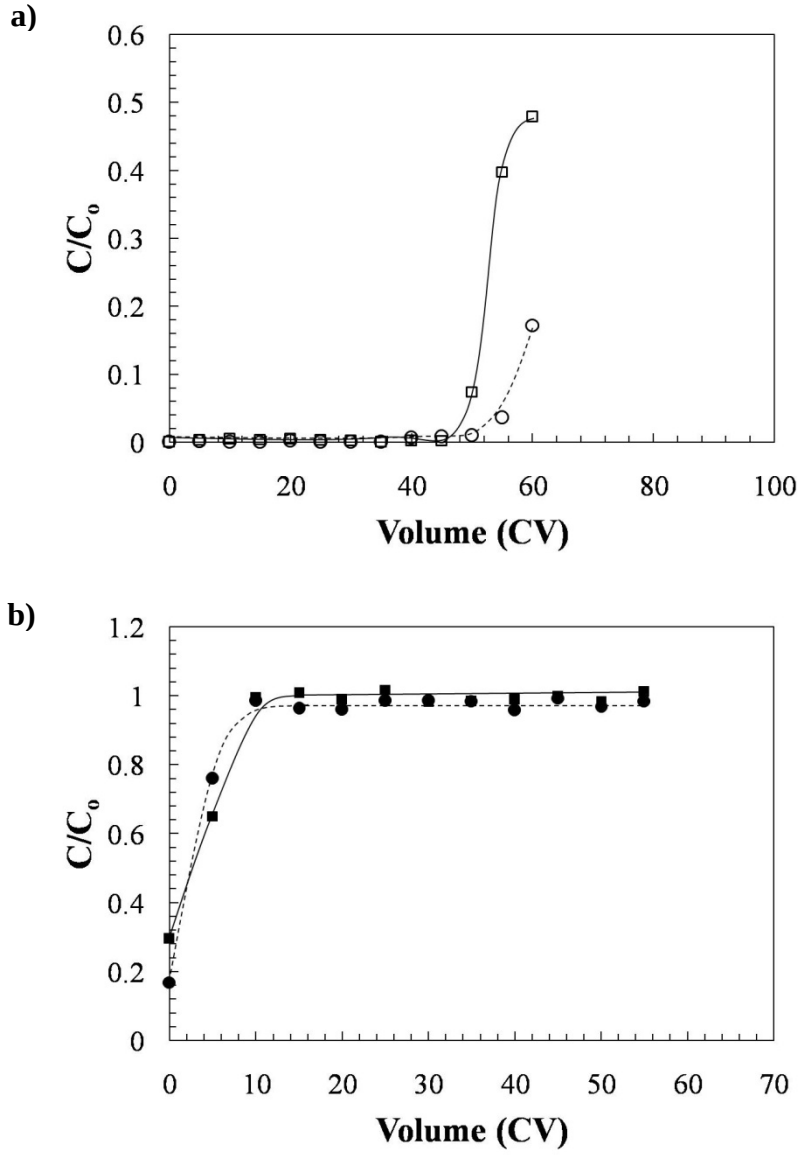


Fig. 3.11 Influence of different plasmid species on breakthrough curves for; a) HM-RNA (open symbols) and b) pDNA (closed symbols) in packed bed chromatography. Symbols; squares: clarified *E. coli* lysate containing pITT3 (27379 bp); circles: clarified *E. coli* lysate containing pORT3a-BAM2k (4109 bp). Chromatography runs were performed at a loading conductivity of 33 mS/cm using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

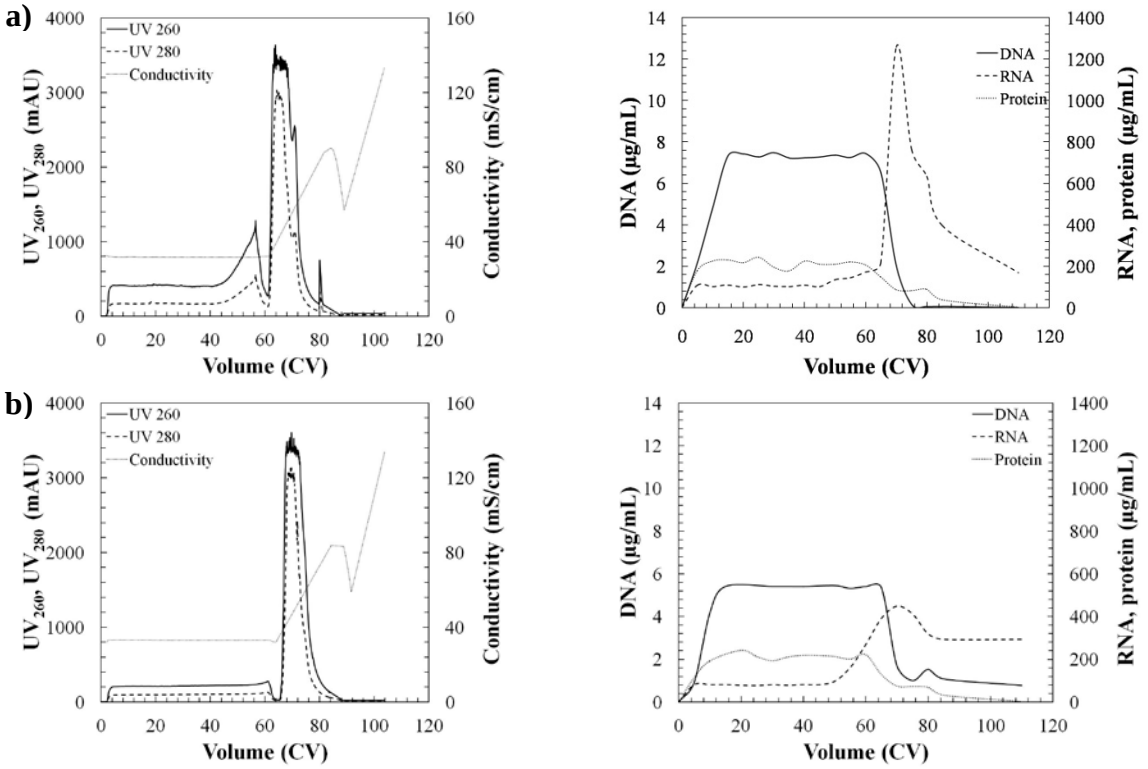
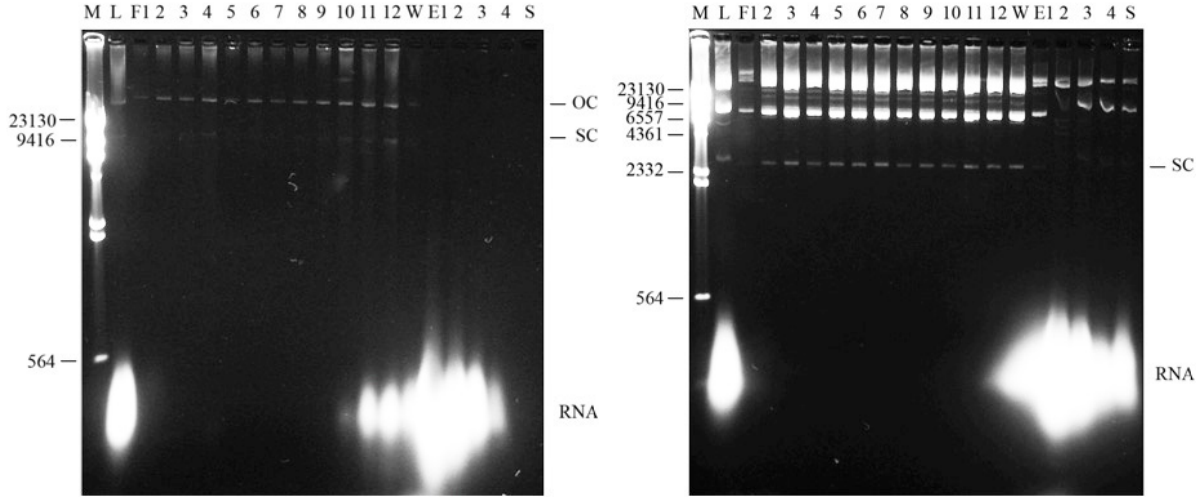


Fig. 3.12 Chromatograms (left) and chemical measurements of chromatographic fractions (right) obtained from packed bed chromatography applied with lysates containing different species of plasmid; a) pITT3 and b) pORT3a-BAM2k. Chromatography runs were performed at a loading conductivity of 33 mS/cm using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.



a) **b)**

Fig. 3.13 Agarose gel eletrophoresis of chromatographic fractions obtained from packed bed chromatography applied with lysates containing different species of plasmid; a) pITT3 and b) pORT3a-BAM2k. Fraction size was 5 CV. Abbreviations; M: Lambda HindIII marker, L: cleared lysate fed onto column, F: flowthrough, W: wash fraction, E: elution fractions and S: stripped fraction.

It was also noted that the different forms of pORT3a-BAM2k remain unaltered throughout the loading phase (Fig. 3.13). The pDNA bands of various molecular weights other than SC and OC bands are possibly caused by catenanes formation.

Generally, it is likely that two or more circular DNA molecules can interlink. Circular DNAs that link together one or more times are called catenanes (Mirkin, 2001, Levene et al., 1995, Laurie et al., 1998) as shown in Fig. 3.14. Catenanes are routinely detected inside living cells and are believed to form at the late stages of DNA replication and can be subsequently resolved by topoisomerases (Mirkin, 2001).

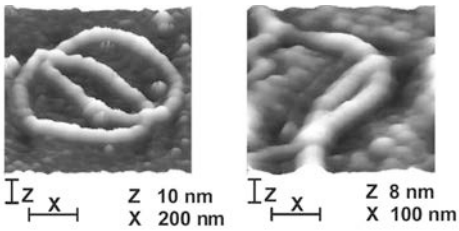


Fig. 3.14 Crossover points between fully catenated DNA molecules are clearly demonstrated using Atomic force microscopy (AFM) in a publication by Harmon et al. In order to aid visualization, DNA molecules in the catenane sample were coated with RecA protein. AFM provides height information (*Z*), in addition to length and width measurements (X). (Harmon et al., 2003)

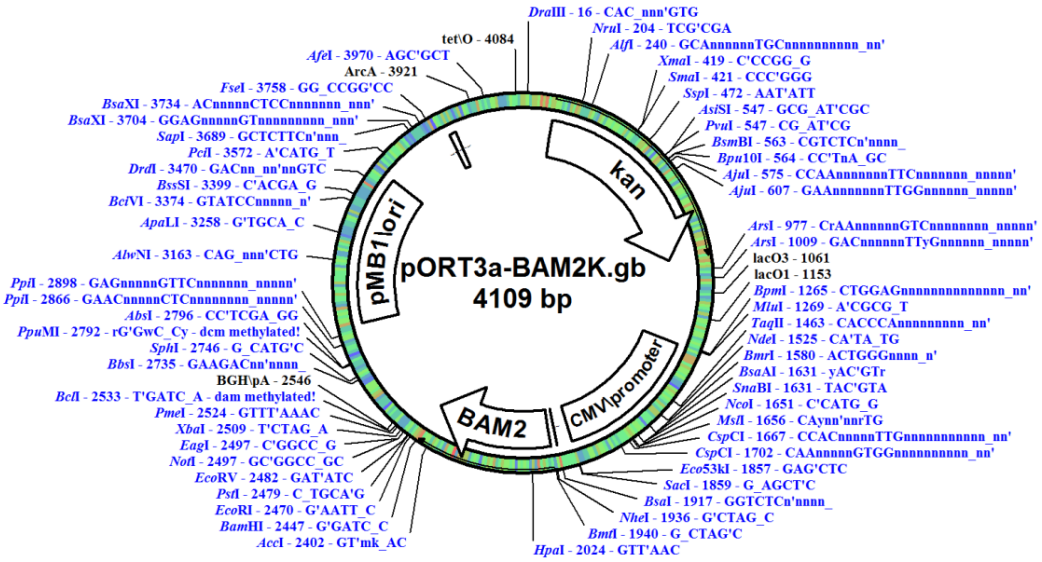


Fig. 3.15 Restriction map of pORT3a-BAM2k, constructed by using computational processing of DNA sequence by artemis software (Wellcome Trust Sanger Institution) followed by pDRAW32 1.0 (revision 1.1.112, Accaclone software).

In order to confirm catananes formations, one-site cutting by Bam HI was applied to pORT3a-BAM2k plasmid. From plasmid restriction map, pORT3a-BAM2K only contains 1 restriction site for Bam HI (Fig. 3.15). Therefore, cutting catananes rings of this plasmid specie should result in 1 band of 4109 bp linear DNA, not various bands with assorted size which may result from contamination. As expected, the result reveals that pDNA bands that are larger than normal plasmid size are likely to be caused by catananes formations (Fig. 3.16).

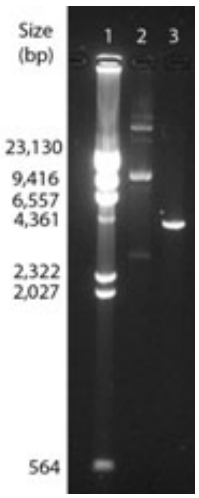


Fig. 3.16 One-site cutting of pORT3a-Bam2K using Bam HI. Lane 1: Lamba HinD III marker; Lane 2: pORT3a-Bam2K before cutting; Lane 3: pORT3a-Bam2K after cutting, showing 1 band of 4109 bp linear DNA.

Protein contents, as measured by BCA assay, were stable during the loading phase and were decreased after washing (Fig. 3.12).

3.3.5 Performances of different SEC/IEC Sepharose CL-6B

Three SEC/IEC supports based on Sepharose CL-6B, produced at room temperature using the VE-RD balancing approach described in chapter 2, were packed into a Tricorn 5/50 column at a bed volume of 1mL and bed height of 5 cm (Table 3.1). Neutralized *E. coli* cleared lysate was diluted three times with water (~ conductivity of 33 mS/cm) and applied to

the column at a flow rate of 30 cm/h. Preparation conditions, % reduction in binding capacities and selectivity indices for the supports are shown in Table 3.1.

Table 3.1 Characteristics of SEC/IEC supports applied to column in section 4.3.5

Support	% (w/v) sucrose	% Bromination	% Reduction in DNA binding	% Reduction in protein binding	SI
64s10	64	10	77.50	11.49	3.93
70d10	70	10+10	81.25	16.31	4.46
80s20	80	20	92.45	17.23	10.96

From existing data, HM-RNA breakthrough curves (Fig. 3.17a) suggested that RNA binding capacity followed SI values, except for 70d10 support which showed earliest RNA breakthrough while having 2nd highest SI. The 70d10 supports also show distinct pDNA breakthrough behaviour (Fig. 3.17b), with a more delayed pDNA breakthrough, whereas the breakthrough curves for 64s10 and 80s20 largely overlap one another. Distinctive pDNA breakthrough behaviour of 70d10 support is also evidenced by the DPA assay shown in Fig. 3.18 and agarose gel electrophoresis image shown in Fig. 3.19 and may be caused by the different bromination mechanism of double bromination applied for this support preparation while the other two supports were prepared by single bromination. Double bromination also exhibited different surface binding behaviour with the increased viscosity used for support preparation compared to single bromination (see chapter 2).

SI value were used to evaluate the effectiveness of bilayering methods in the creation of SEC/IEC supports and in this respect SI might be expected to correlate with a supports ability to discriminate between DNA and RNA binding during chromatographic operation. However, the static binding studies employed for SI calculations were performed in relatively low ionic strength buffer, 0.05 mM Tris-HCl pH 8.0 (~ 4.8 mS/cm) compared to loading

buffer used for column experiment. High conductivity compromises binding capacity of target biomolecules (Harinarayan et al., 2006) and therefore, nucleic acids binding behaviour might be quite different to that expected from SI values.

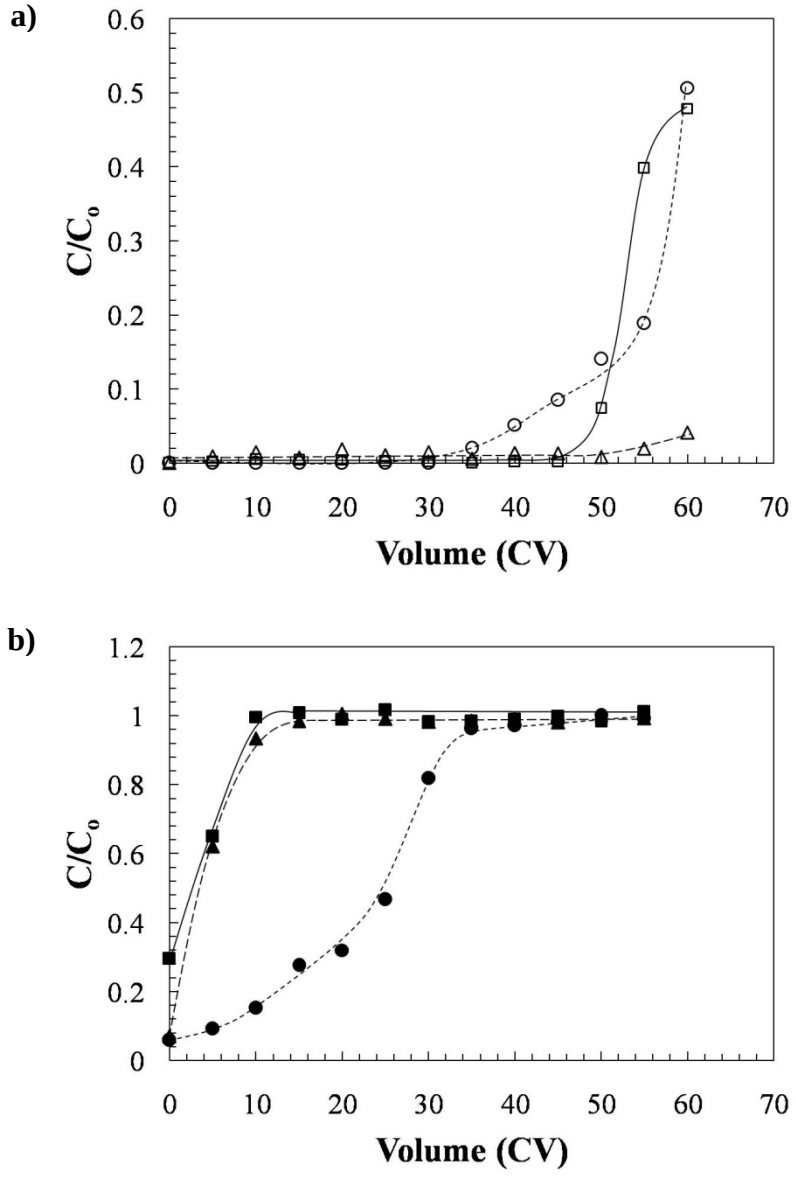


Fig. 3.17 Influence of different SEC/IEC Sepharose CL-6B on breakthrough curves for; a) HM-RNA (open symbols) and b) pDNA (closed symbols) in packed bed chromatography. Symbols; squares: 64s10; circles: 70d10; and up-triangle: 80s20. Chromatography runs were performed at a loading conductivity of 33 mS/cm using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

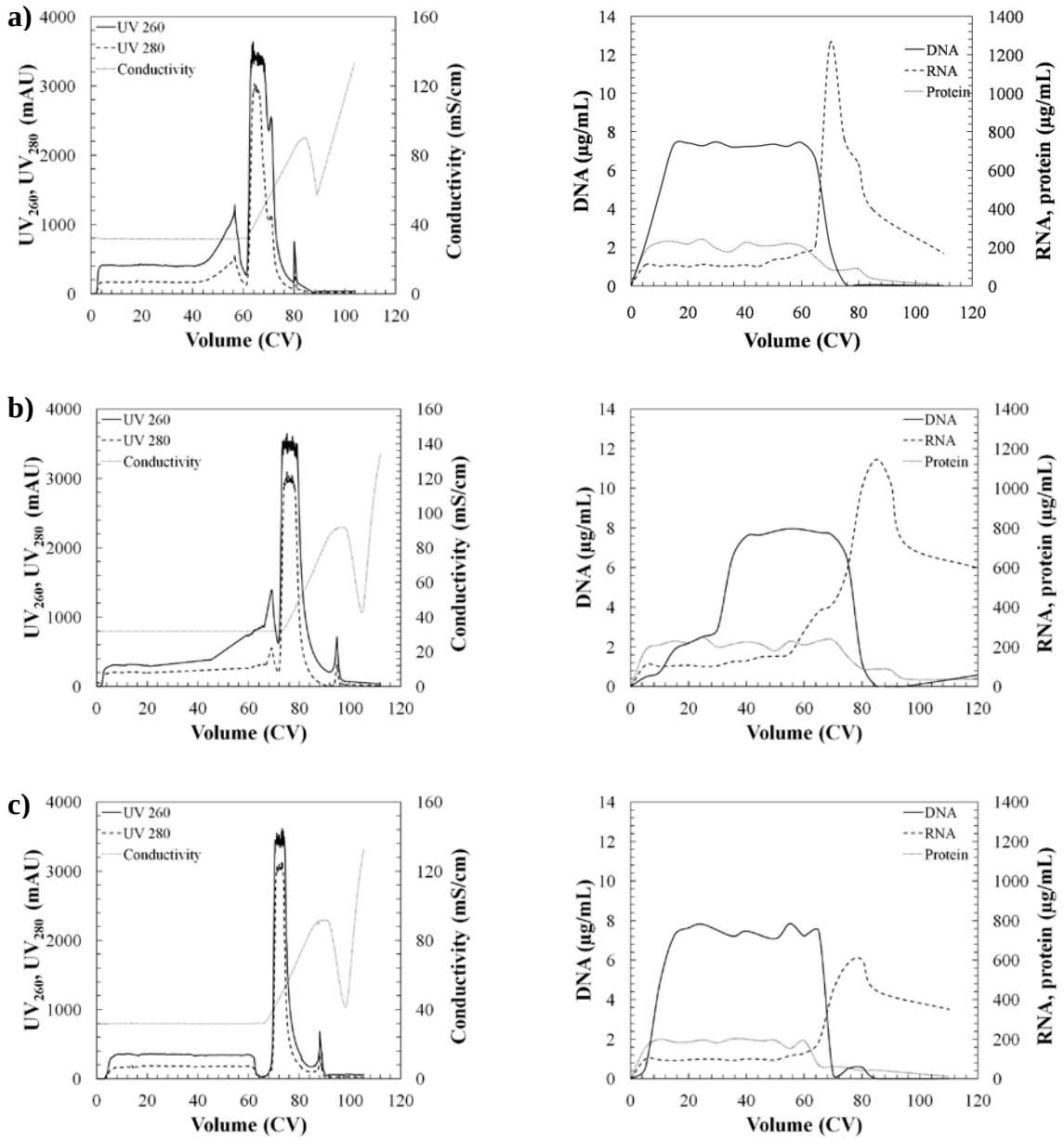


Fig. 3.18 Chromatograms (left) and chemical measurements of chromatographic fractions (right) obtained from packed bed chromatography using different SEC/IEC Sepharose CL-6B ; a) 64s10, b) 70d10 and c) 80s20. Chromatography runs were performed at a loading conductivity of 33 mS/cm using SEC/IEC Sepharose CL-6B modified by 10% partial bromination at room temperature in 64% (w/v) sucrose.

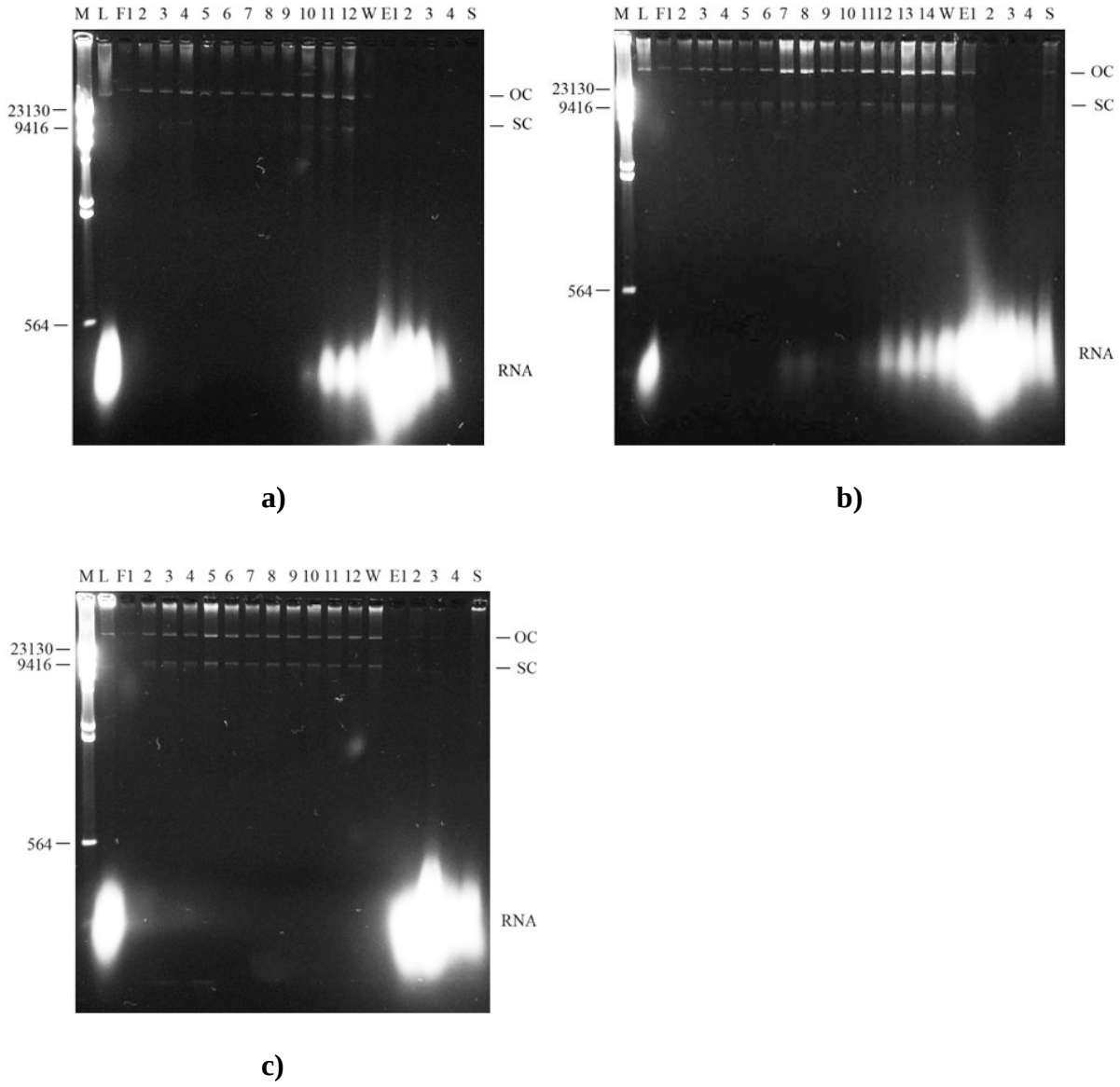


Fig. 3.19 Agarose gel eletrophoresis of chromatographic fractions obtained from packed bed chromatography using SEC/IEC supports based on Sepharose CL-6B; a) 64s10, b) 70d10 and c) 80s20. Fraction size was 5 CV. Abbreviations; M: Lambda HindIII marker, L: cleared lysate applied onto column, F: flowthrough, W: wash fraction, E: elution fractions and S: stripped fraction.

3.4 Conclusions

Generally, RNA is the main contaminant to be focused on since it can escape cell lysis and possesses similar characteristic as pDNA. RNA can be reduced by addition of RNA-digesting enzymes, such as RNase. However, this approach cannot be used for the purification of

therapeutic pDNA since the application of animal-derived materials, such as commercially available RNases, is prohibited by regulatory organizations (Sousa et al., 2008) In this chapter, the selected SEC/IEC supports produced in chapter 2 were evaluated in column chromatography system to separate RNA from *E. coli* cleared lysate. SEC/IEC Sepharose CL-6B modified by 10% single bromination exhibited a better performance especially on pDNA exclusion compared to SEC/IEC Sephacryl S400 HR. The pore sizes of both supports are considerably similar and are small enough to exclude plasmid pITT3 (27379 bp) used, suggesting that the binding of pDNA on SEC/IEC Sephacryl S400 HR, evident by large amount of pDNA in eluted fractions, may be caused by the remaining surface charge. Variations of operating parameters (conductivity, linear flow rate, plasmid size) were tested using SEC/IEC Sepharose CL-6B. The increase in conductivity resulted in the reduced binding capacity for both RNA and pDNA. Ideally, a successful pDNA-RNA separation requires minimum pDNA binding and maximum RNA binding. From this result, it is possible to fine-tune the conductivity to assist the separation. A flexible operating flow rate range of 30-60 cm/h was observed which means that the chromatography can be operated at twice the standard flow rate (30 cm/h) which helps shorten operation period. The smaller plasmid (4109 bp) show similar pattern during chromatography proved the flexibility of this support towards different plasmid sizes. The SEC/IEC Sepharose CL-6B modified by double partial bromination/hydrolysis was seen to have different binding behaviours especially for RNA compared to those modified by single bromination (10 or 20%). It is worth mentioning that no change in plasmid form was observed in every chromatography runs using SEC/IEC supports.

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Chapter 4

Microwave assisted reaction-diffusion balancing for the preparation of SEC/IEC supports to separate RNA from *E. coli* neutralised lysates containing pDNA

Abstract

Microwave heating provided a fast, effective and controlled process for production of bilayered SEC/IEC supports containing non-charged surfaces and anionic cores for pDNA purification via AGE activation – partial bromination route. Partial bromination reactions on allylated supports were heated up by microwave irradiation in a monomodal microwave reactor to the target temperatures of 70-90^{o}C in 55-70 s which were much faster than conventional heatings. SEC/IEC Sepharose CL-6B supports produced at 80-90^{o}C with 10% partial bromination showed almost complete surface charge eliminations indicated by high values of % reduction in pDNA binding capacity of 95-98% and a highest selectivity index (SI) value of 57.4 was achieved at 80^{o}C. Also, SEC/IEC Sephacryl S400 HR produced at 70-90^{o}C with 10% partial bromination exhibited high values of % reduction in pDNA binding capacity of 69-83% and a highest SI value of 10.2 was achieved at 70^{o}C. These high SI values indicated efficient bilayer creations on both base matrices, resulted from the effect of increased temperature on reaction rate as described by Arrhenius equation. The relationship between reaction/diffusion rate ratio (k/D) and temperature for this bromination reaction suggested that a dramatic increase in reaction rate can be achieved when the temperature is raised to above 60^{o}C and that ratio of reaction vs. that of diffusion (k/D) increase approximately fourfold per 10^{o}C increase in temperature. These results proved the effectiveness of microwave heating on the production of bilayered SEC/IEC supports from

underivatised base matrices. The best SEC/IEC support, Sepharose CL-6B modified by 10% bromination at 83^{o}C was further evaluated in a packed bed chromatography system using a Tricorn 5/50 column to purify a 6336 bp plasmid from *E. coli* cleared lysate. This support showed the high core binding capacity suggested by the absence of RNA breakthrough even a large volume of lysate (90 CV) was loaded. However, a delayed pDNA was also observed which may due to the remained surface charge.

4.1 Introduction

An increase in demand for high-purity therapeutic supercoiled-form plasmid DNA has arisen due to the increase application in pre-clinical and clinical trials for gene therapy and DNA vaccination. Therapeutic pDNA is widely produced in large scale by *E. coli* fermentation which possesses a number of challenges during purification process due to its high level of anionic contaminant with similar properties (i.e. charge, size and physical behaviours) to target pDNA such as chromosomal DNA, RNA and endotoxin (Varley et al., 1999). Moreover, for clinical trials where therapeutic DNA products are administered to patients, extremely high purity pDNA is required. This requirement leads to attempts to achieve an effective purification process.

Ion exchange (IEC) and size exclusion (SEC) chromatography have been the most widely used for pDNA purification (Ferreira et al., 2000, Gustavsson et al., 2004). Still, each technique possesses some disadvantages. Ion exchange chromatography suffers from low pDNA binding capacity since most of the IEC supports available commercially were designed for protein purification (2–10 nm diameter) (Theodossiou et al., 2000a, Thwaites et al., 2002) while large target biomolecules such as pDNA are too large to enter the pores and bind only at the support's surface (Eon-Duval and Burke, 2004, Prazeres et al., 1999,

Gustavsson et al., 2004, Tiainen et al., 2007b, Mao et al., 1993, Ljunglöf et al., 1999, Grunwald and Shields, 2001). Moreover, similarity of charge properties between major impurities and target pDNA resulting in binding and co-elution of impurity species alongside the targets (Diogo et al., 2005). Apart from impurities, the separation of the undesirable pDNA isoforms to achieve high purity supercoiled plasmid is also essential (Urthaler et al., 2005a, Sousa et al., 2008). In contrast, size exclusion chromatography can tackle the problems caused by similar charge property by separating large biomolecules from smaller impurities based on their size differences, regardless of charges. However, problems still exist due to the comparatively low selectivity of the technique and limitations of the feedstock volume that can be processed (Lemmens et al., 2003).

In order to overcome these issues, the idea of a bifunctional restricted access SEC/IEC support consists of a positively charged core and an inert outer layer for 'one column- one bead' purification has become more and more attractive. SEC/IEC supports combine the strengths of IEC and SEC by having charged core which absorbs negatively charged small impurities while inert outer layer excludes pDNA, resulting in an effective separation where impurities are captured inside the beads and target plasmids come out in the flowthrough.

Existing approaches to produce bilayered SEC/IEC supports are mainly focused in EBA system. The attempts to develop SEC/IEC supports in order to exclude large biomolecules include; coating IEX base matrices with a non- charged polymer i.e. agarose and cross-linked agarose (Viloria-Cols et al., 2004, Jahanshahi et al., 2008). Low temperature glow discharge plasma treatment was applied on functionalized base matrices to either (i) shave off the surface charges from the support particles (plasma etching): or (ii) coat the support particles with nano-thin polymer (plasma polymerization) (Arpanaei et al., 2010). However, these approaches mentioned are based on EBA systems while only few researches were published based on packed bed column chromatography systems. In 2004, Gustavsson et al. introduced

a SEC/IEC matrix called ‘lid bead’ produced by chemically functionalisation of commercially available SEC matrix, Sephacryl S500 HR via AGE activation-partial bromination approach. Conclusively, this process consists of (i) introduction of allyl groups (CH_2=CH-CH_2-) throughout the structure of supports by reaction of allyl glycidyl ether (AGE) with hydroxyl groups on the support; (ii) partial bromination of allyl groups on the surface of each particle to create an outer layer via addition reaction; (iii) hydrolysis of the resulting outer layer of bromo-alkyl groups to create an inert outer layer; (iv) full bromination of the remaining allyl groups on support’s core; and finally (v) coupling of a quaternary amine ligand, trimethylamine (Q), to the core. This lid bead was further evaluated by applying to polishing step in an integrated process for pDNA purification from a clarified *E. coli* lysate. (Kepka et al., 2004a, Gustavsson et al., 2004). However, these bi-functional supports seemed to lack the sharp definition between the inert outer layer and the charged core. For example, in tests with clarified alkaline lysate feedstocks at high ionic strength, over 30% of the support’s RNA binding capacity needed to be sacrificed in order to prevent pDNA binding. This problem appears to caused by the imperfect ability to control the thickness and inertness of the outer size excluding layer during the production process in which therefore, needs to be improved.

It has been realized that in order to obtain the maximum elimination of surface pDNA binding while maintain the maximum core binding for impurities for AGE activation- partial bromination approach, the inert outer layer needs to be very thin where charge elimination occurs densely and restricted on the outermost surface of support particle therefore, core binding is not compromised. This can be achieved by employing ‘reaction-diffusion balancing’ approach which involves increasing the reaction rate of the outer layer defining step, partial bromination, by increasing temperature (according to the Arrhenius equation) to

be fast enough that the reaction occurs only at the outermost part of adsorbent particle with minimal diffusion of reactant (bromine) deeper into support's interior.

The aim of this chapter is to optimise the conditions used for SEC/IEC supports preparation using microwave-assisted, reaction-diffusion balancing approaches on a commercially available underivatised chromatographic supports, Sepharose CL-6B and Sephacryl S400 HR for application on 'one column-one bead' pDNA purification. Microwave heating by a bench top monomodal microwave reactor was applied during partial bromination step prior to addition of bromine in order to rapidly ramp up the reaction rate to be much faster than diffusion rate. The effects of various temperatures during partial bromination reaction on the properties of SEC/IEC support produced were studied. The resulting SEC/IEC adsorbents were also characterised physically, chemically and biologically by means of scanning electron microscopy (SEM), chemical assays and binding studies with pDNA and bovine serum albumin (BSA) to identify the changes in surface and core binding capacity, respectively. The best SEC/IEC support was further evaluated in a packed bed chromatography system in order to purify pDNA from *E. coli* cleared lysate.

4.2 Microwave principles

Microwave heating possesses a number of advantages over conventional heating including non-contact heating, transfer of energy instead of heat (Fig. 4.1), higher heating rate (> 400 ^{o}C/min (Agrawal, 1998)), rapid start-up and stopping of the heating, uniform heating with minimal energy loss (almost 100% conversion of electromagnetic energy into heat (Agrawal, 1998), selective heating properties, reverse heating profile (heating starting from the interior of the material body) which eliminates limitations cased by thermal conductivity of the vessel (Mallakpour and Rafiee, 2011, Çalışkan et al., 2012), reduction in the amount of

solvent required (Koroskenyi and McCarthy, 2002), energy savings and improved product yield results in a reduction in manufacturing cost (Pääkkönen et al., 2010). Due to these advantages, microwaves are widely used in various organic synthesis to heat up different types of reactions from library syntheses (Gelens et al., 2006), drug discoveries and syntheses (Frank et al., 2007, Alexandre et al., 2003), ceramic syntheses (Agrawal, 1998, Victor and Kumar, 2008, Naik et al., 2004), polymer syntheses and grafting (Koroskenyi and McCarthy, 2002, Wiesbrock et al., 2004b, Singh et al., 2012, Wan et al., 2011) to regeneration of activated carbon (Çalışkan et al., 2012, Ania et al., 2005, Menéndez et al., 2010) and so on. The first published reports on the application of microwave irradiation to carry out organic chemical transformation were produced by the groups of Gedye (1986) and Giguere (1986) (Gedye et al., 1986, Giguere et al., 1986).

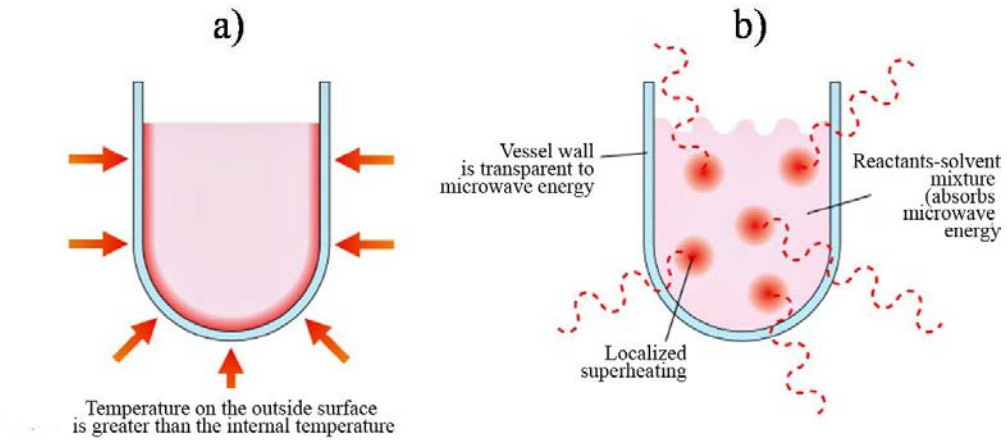

Fig. 4.1 Schematic diagram of sample heating by a) conventional heating; and b) microwave irradiation (Hayes, 2002).

Microwaves are nonionising electromagnetic radiation (Banik et al., 2003) having the frequency range of 0.3-300 GHz, corresponding to wavelength of 0.001 – 1 m, which lies in the electromagnetic spectrum between infrared radiation and radio frequency (Mallakpour and Rafiee, 2011, Menéndez et al., 2010). Generally, microwave heating is usually applied at 0.915 GHz (0.896 GHz in the UK) and 2.450 GHz which are the most popular of the frequencies allowed for ISM (industrial, scientific and medical) applications, set aside for

non-communication proposes (Das et al., 2009). Operating frequency of 2·45 GHz, corresponding to a wavelength of 12.2 cm and energy of 1.02×10−5 eV (Jones et al., 2002), is mostly used for domestic and laboratory scale microwave ovens while 0.915-2.45 can be found in industrial microwave ovens (Das et al., 2009, Nóbrega et al., 2002).

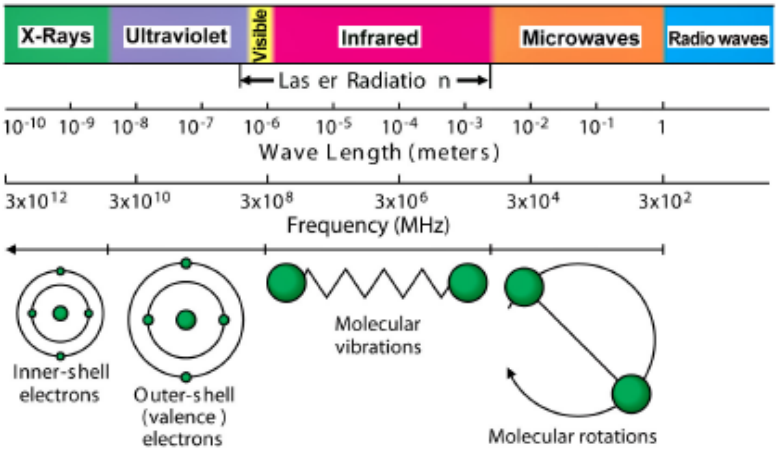

Fig. 4.2 A typical spectrum of common electromagnetic radiation (Hayes, 2002).

Microwave energy is transferred to the material by interaction of the electromagnetic field at the molecular level. Microwave heating is based on two fundamental mechanisms; dipole rotation and ionic conduction. Dipole rotation is an interaction of polar molecules trying to align in the alternating electric field, resulting in an energy loss in the form of heat through molecular friction and dielectric loss. The amount of heat generated depends on nature of the dipole (shape, size, dielectric constant, etc.) and frequency of the radiation applied. Under low frequency irradiation, dipole rotates in synchronized fashion with electric field but the motion is not enough to generate heat. On the other hand, under high frequency electric field the dipole does not have enough time to respond to the oscillation field and does not rotate. In both cases, no heating occurs. The assigned frequency of 2.45 GHz used in commercial systems lies between these two extremes cases, and gives the molecular dipole time to align in the field but not to follow the alternating field precisely. Under such conditions, rapid heating of chemical reaction mixtures to high temperatures can be obtained (Thostenson and

Chou, 1999, Das et al., 2009, Kappe and Dallinger, 2006, Figueiredo et al., 2011, Mallakpour and Rafiee, 2011, Lidström et al., 2001). Alternatively, ionic conduction occurs with the presence of free ions or ionic species, even a single isolated ion with hydrogen bond cluster, in substance being heated. The electric field generates ionic motion as the molecules try to orient themselves to the rapidly changing field, leads to an increased collision rate and finally, converts the kinetic energy to heat. The temperature of material affects ionic conduction by enhancing energy transfer efficiency when the temperature increased (Lidström et al., 2001, Hayes, 2002). Considering their heat generating capacity, ionic conductivity mechanism is a much stronger interaction than dipole rotation mechanism. Both mechanisms cooperate each other synergistically, resulting in an enhanced heat production (Lidström et al., 2001). Schematic diagrams of dipole rotation and ionic conduction during microwave irradiation are shown in Fig. 4.3 and 4.4, respectively.

Fig. 4.3 Dipole rotation mechanism. Dipolar molecules tries to align with an oscillating electric field (Lidström et al., 2001).

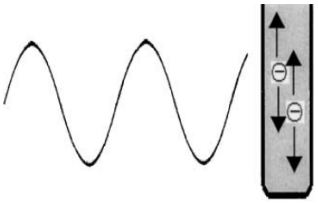

Fig. 4.4 Ionic conduction mechanism. Charged particles in a solution follows the applied electric field (Lidström et al., 2001).

Materials can be classified into three categories based on their interaction with microwaves (Fig. 4.5): (i) materials that reflect microwaves which are called ‘conductor’, example of

these materials are bulk metals and alloys, e.g. copper, graphite and highly graphitized carbons; (ii) materials that are transparent to microwaves, namely 'insulator', typified by fused quartz, several glasses, ceramics, Teflon, etc.; and (iii) materials that absorb microwaves as known as 'absorber' which constitute the most important class of materials for microwave synthesis, e.g. aqueous solution, polar solvent, etc (Li and Yang, 2008, Menéndez et al., 2010, Jones et al., 2002).

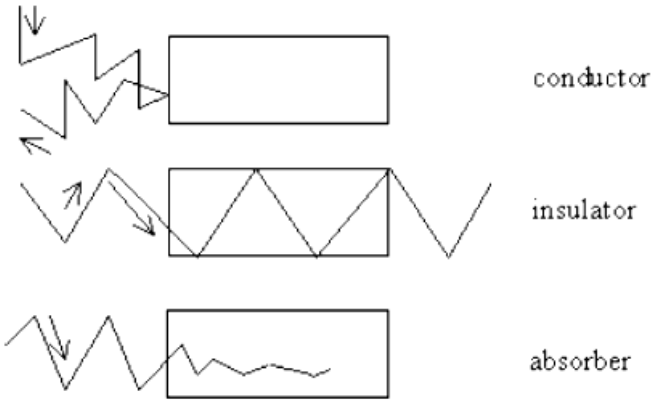

Fig. 4.5 Microwave absorption characteristic of conductor, insulator and absorber (Jones et al., 2002).

The ability of a material to be heated in the presence of a microwave field is defined by its dielectric loss tangent (also known as dissipation factor); tan δ which is normally used to predict the behaviours of materials under a microwave field. The microwave absorption ability of a material is directly proportional to its tan δ value (Li and Yang, 2008).

$$\tan \delta = \varepsilon'' / \varepsilon' \qquad \text{(Eq. 4.1)}$$

The dielectric loss tangent is composed of two parameters, the dielectric constant (or real permittivity); ε′, and the dielectric loss factor (or imaginary permittivity); ε″. The dielectric constant (ε′) indicates how much of microwave energy is reflected and how much is absorbed, while the dielectric loss factor (ε″) represents the dissipation of electric energy in form of heat within the material. To achieve an optimum microwave energy coupling, a combination of moderate value of ε′ and a high value of ε″ (yielding a high tanδ value) is

required to convert microwave energy into thermal energy effectively. This explains the heating property of some microwave transparent materials which do not possess a sufficiently high loss factor to allow dielectric heating while other materials such as some inorganic oxides and most carbon materials are excellent microwave absorbers. In general, materials can be classified as high (tan $\delta > 0.5$), medium (tan $\delta = 0.1$-0.5), and low microwave absorbing (tan $\delta < 0.1$) (Kappe, 2004). Dielectric constants and loss tangents of solvents commonly used in organic synthesis is shown in Table 4.1.

Table 4.1 Dielectric constants and loss tangents of solvents commonly used in organic synthesis (Lidström et al., 2001).

Solvent	**Dielectric constant (ε')[a]**	**Loss tangent (tan δ)[b]**
Hexane	1.9	
Benzene	2.3	
Carbon tetrachloride	2.2	
Chloroform	4.8	
Acetic acid	6.1	0.091
Ethyl acetate	6.2	0.174
THF	7.6	0.059
Methylene chloride	9.1	0.047
Acetone	20.6	0.042
Ethanol	24.6	0.054
Methanol	32.7	0.941
Acetronitrile	36	0.659
Dimethylformamide	36.7	0.062
DMSO	47	0.161
Formic acid	58	0.722
Water	80.4	0.123

[a] Dielectric constant (ε') at room temperature and under the influence of a static electric field.

[b] Values determined at 2.45 GHz and room temperature.

The three dimensional stationary pattern of standing waves generated by the reflections of the wave within a cavity is called ‘mode’. In domestic microwave ovens, the cavities are designed to have typically 3-6 different modes in order to provide a uniform heating pattern (Lidström et al., 2001). In the very first chemical synthesis studies conducted on microwaves, most of the chemical reactions were performed using domestic microwave ovens (Yu et al., 1988, Engelhart, 1990, Molina et al., 1993, Prasad et al., 2010, Banik et al., 1992, Kalyani et al., 2003, Dabirmanesh and Roberts, 1993, Giguere et al., 1986, Gedye et al., 1986, Bram et al., 1990, Ali et al., 1989, Baptistella et al., 1993, Banik et al., 1993, Komarneni et al., 1992, Liu et al., 2001) where generally the irradiation power was controlled by on/off cycles of the magnetron, the high-power generator of microwave power (Stuerga, 2008). These unmodified multimode microwave ovens were popular due to their easy accessibility and low cost. However, their use was not much encouraged due to the safety concerns, insufficient control over the reaction temperature and pressure as well as the lack of efficiency to process samples in small volume (Singh et al., 2012, Lidström et al., 2001, Bond et al., 1993, Kappe et al., 2009). ‘Hot and cold spots’ issue due to poor distribution of high and low field strength which resulting in a drastic variation of heating efficiency between different positions of samples, is also observed. In order to overcome these problems, several modifications to domestic microwave ovens for laboratory scale synthesis were made (Kappe, 2004, Ge and Luo, 2005, Huacai et al., 2006). Nowadays, all the specialized commercially available microwave reactors designed for chemical synthesis featured built-in magnetic stirrers, fibre-optic probes or IR sensors for direct temperature control of the reaction mixture and software for on-line temperature/pressure control by regulation of microwave power output (Mingos, 2009, Kappe and Dallinger, 2006, Singh et al., 2012). Currently, two different widely used microwave reactor designs are multimode and monomode (single mode) reactors. In the multimode reactors (employing similar concept to a domestic oven), the microwaves that

enter the typically large cavity (~ 40-50 L) are reflected by the walls of the cavity, and therefore interact with the sample in a chaotic manner (Kappe and Dallinger, 2006). The smaller monomodal reactors possess much smaller cavities where only one mode is present. Electromagnetic irradiation is directed through an accurately designed rectangular or circular wave guide onto the reaction vessel mounted at a fixed distance from the radiation source to creatie a standing wave (Kappe, 2004, Kappe and Dallinger, 2006). These features of a monomodal microwave reactor promote accurate temperature and pressure control with improved reliability, leading to better reproducibility of the results and also facilitating the scale-up of the reactions (Wiesbrock et al., 2004a, Huacai et al., 2006).

.4.3 Materials and methods

4.3.1 Materials

The SEC base matrix, Sepharose CL-6B and Sephacryl S400 HR, were purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Allyl glycidyl ether (AGE), 50% (w/v) sodium hydroxide solution, sodium hydroxide pellets, sodium borohydride ($NaBH_4$, 99%), bromine, sodium chloride and trimethylamine hydrochloride (Q) used in SEC/IEC supports preparations were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Ethanol was purchased from Fisher Scientific (Loughborough, UK).

The monomodal microwave reactor used in this experiment was CEM Discover S-Class (CEM Corporation, Matthews, NC, USA).

E. coli DH5α containing the 27379 bp plasmid pITT3 was kindly provided by Dr. Eirini Theodosiou, Department of Chemical Engineering, Loughborough University, UK.

This plasmid is a pPR633-based high copy number plasmid (4579 bp) and containing a 22800 bp insert from *Saccharomyces cerevisiae* chromosome III at BamHI site.

E. coli DH1 carrying the 6335 bp plasmid pORT.mpt64 was provided by Dr. Rocky Cranenburgh, Molecular Biology, Recipharm Cobra Biologics, UK.

Luria Bertani (LB) broth, LB agar, D-glucose, ampicillin, kanamycin, prolypropylene glycol (PPG) antifoam used for culture and fermentation were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). QIAfilter Plasmid Giga Kits for plasmid purification were purchased from Qiagen GmbH (Hilden, Germany).

For bromine assay, standard potassium bromate solution (0.1 M) was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). H_2SO_4 was purchased from Fisher Scientific (Loughborough, UK). For ionic capacity assay, mercury II thiocyanate was purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA) and ammonium iron III sulphate was purchased from Fisher Scientific (Loughborough, UK). For binding studies, bovine serum albumin (BSA), Tris HCl and Trizma base were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). For diphenylamine assay, diphenylamine, perchloric acid, acetaldehyde, glacial acetic acid and calf thymus DNA were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Pierce® BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL, USA). Orcinol, $FeCl_3$, ribonucleic acid from baker's yeast (*S. cerevisiae*) used in Orcinol assay were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

Tricorn 5/50 column was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). All chromatography was performed on an ÄKTA Explorer 100 system controlled by UNICORN 4.11 software (GE Healthcare, Uppsala, Sweden). EDTA, sodium dodecyl

sulphate (SDS), 3 M potassium acetate pH 5.5 used for cell lysis and loading buffer preparation were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA).

For phenol-chloroform extraction of nucleic acids and agarose gel electrophoresis, Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution, 0.2 µm filtered 3 M sodium acetate pH 7.0, 100x TE buffer, agarose, 6x gel loading dye and 10x TBE buffer were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). Kb DNA ladder (250 bp-12 kbp) was purchased from Agilent Technologies (Santa Clara, CA, USA). SYBR® safe DNA gel stain (Invitrogen) was purchased from Life Technologies Ltd (Paisley, UK).

Distilled water was used in all experiment unless stated otherwise.

4.3.2 Viscosity measurement

Rheological behaviour of water was measured using an Advanced Rheometer AR1000 from TA Instruments (New Castle, DE, USA) equipped with a 40 mm, 2^o angle stainless steel cone geometry. Viscosity changes were measured during the increasing temperature from 2 ^oC to 90 ^oC (applying a temperature ramp rate of 4^oC/min) at a shear rate of 10 s^{-1}.

4.3.3 Preparation of SEC-IEC supports

SEC/IEC supports can be produced by employing an AGE activation/ partial bromination route, briefly described in Fig. 4.6. The detailed procedures are also presented hereby.

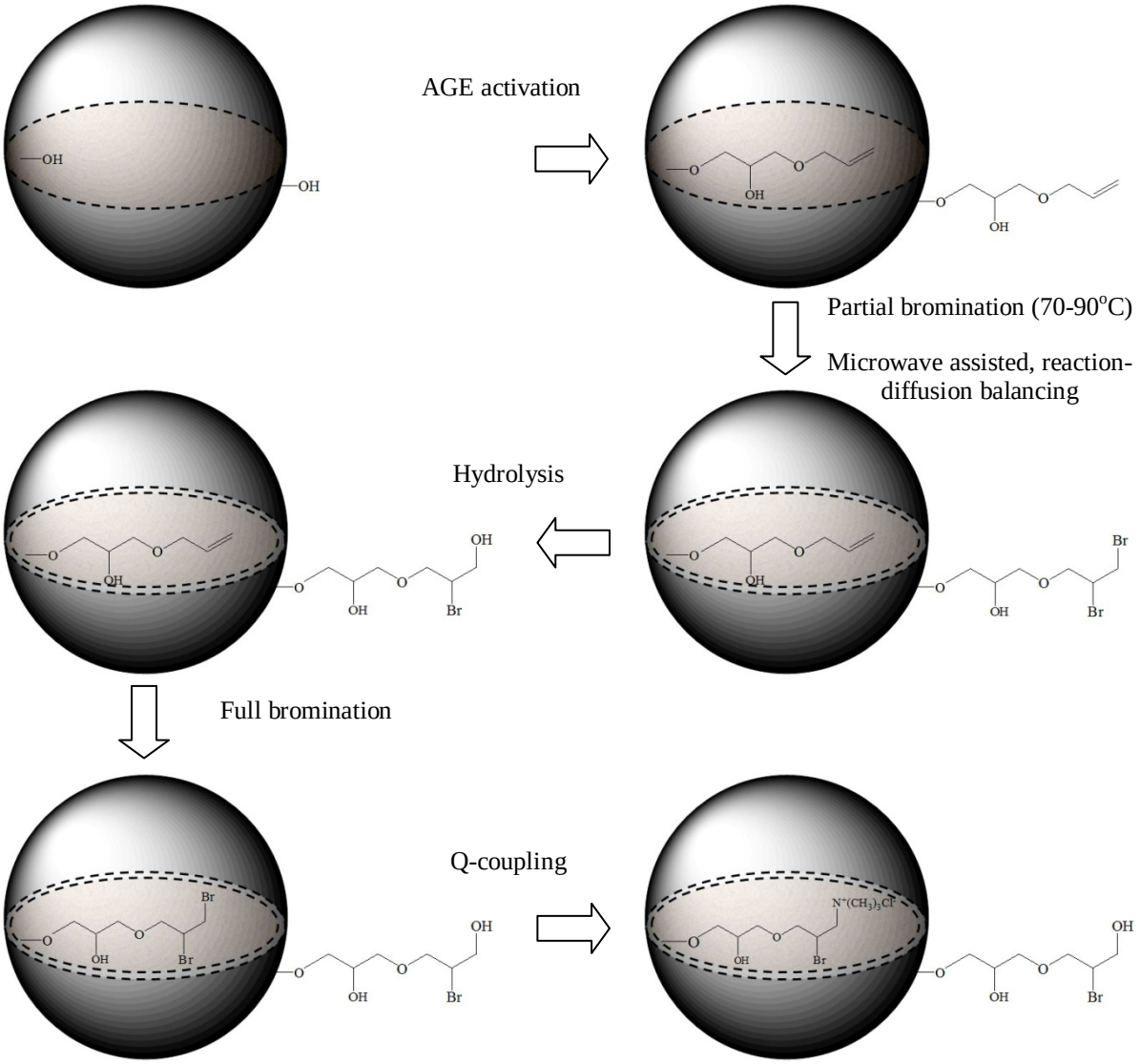

Fig.4.6 Schematic diagram of SEC/IEC supports preparation via microwave-assisted AGE activation-partial bromination route. Trimethylamine chloride (Q) was used for ligand coupling in this experiment.

4.3.3.1 AGE activation

AGE activation (Adapted from Gustavsson, 2004) was carried out in the same manner as described in section 2.2.3.1, chapter 2. The suction drained support was assayed for allyl groups content by the bromine assay. The AGE activated support was stored in 20% ethanol at 4°C. Allyl contents of AGE activated supports were 199 µg/mL supports for Sepharose CL-6B and 189 µg/mL supports for Sephacryl S400 HR.

4.3.3.2 Partial bromination

A 30 mL portion of allylated supports was equilibrated 3 times in water. The equilibrated supports were then suction dried, added to a round bottom flask. Additional water (20 mL) was added to this flask, being used to rinse all support from the sides of the flask to ensure that all supports were collected at the bottom of the flask. A magnetic stirrer flea was added to the suspension and the flask was then placed in the CEM Discover microwave cavity (see Fig. 4.7).

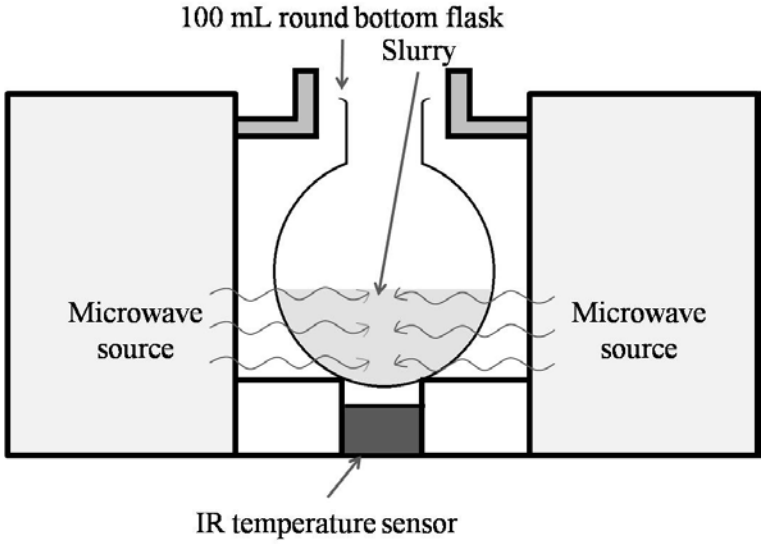

Fig. 4.7 Setup for microwave heating of chromatography support slurry in 100 mL round bottom flask.

Microwave heating for partial bromination reactions was performed using the CEM Discover under 'Dynamic' heating mode. In this mode, a target temperature can be set by the user and the system uses temperature feedback data to control the amount of power supplied to allow the target temperature to be reached and maintained in a controlled fashion. The support slurries containing 60% support in water were heated using the dynamic mode,

with mixing by a magnetic stirrer. A calculated amount of bromine, aiming for 10% or 20% elimination of total allyl content, was added as soon as the target temperature had been reached. The target temperature was held for a further 60 s, with continued mixing. The flask was then removed from microwave apparatus and leave to cool down at room temperature. The brominated supports were washed 3 times with 60 mL of water (60 mL x 3) on a sintered glass filter funnel under vacuum. A sample of support (0.2 g) was removed for analysis the remaining allyl content by bromine assay.

4.3.3.3 Hydrolysis of the partially brominated supports

The remaining of partially brominated supports were suction dried and added to a 100 mL screw cap bottle containing 25 mL of 1 M NaOH followed by addition of 0.08 g sodium borohydride. The bottle was sealed immediately and left to be shaken overnight at 40^{o}C in an orbital shaker incubator (New Brunswick Scientific, New Jersey, USA). After time elapse, hydrolysed supports were washed thoroughly with water.

4.3.3.4 Full bromination of hydrolysed supports and coupling of trimethylamine chloride (Q)

A whole portion of hydrolysed supports from hydrolysis step were transferred to a 100 mL screw cap bottle containing 13 mL of water. After mixing, 1.46 g of sodium acetate was added and dissolved. Bromine was added to the mixture until a permanent yellow colour was obtained. The supports were then washed thoroughly with water and transferred to a clean 100 mL screw cap bottle containing 13 mL of water. A 9.79 mL portion of 50% (w/v) sodium hydroxide was added to the mixture followed by 0.13 g of sodium borohydride. After mixing, 19 mL of 65% (w/v) trimethylamine chloride was added. The bottle was sealed

immediately and left to be shaken overnight at room temperature on a blood tube rotator SB1 (Stuart Scientific, Staffordshire, UK). The supports were then washed with water, 1 M NaCl and water once again. Modified supports were stored at 4^{o}C in 20% (v/v) ethanol.

4.3.4 Production of plasmid–containing cells

Preparation of starting cultures and fermentations of plasmid containing *E. coli* cells containing pITT3 (27379 bp) or pORT.mpt64 (6336 bp) were carried out in the same manners as described in section 2.2.4, chapter 2. For pITT3, 100 µg/mL ampicillin was used while 50 µg/mL kanamycin was added in case of pORT.mpt64. Cells were harvested by centrifugation in a J2-21 centrifuge (Beckman, High Wycombe, UK) operated at 10,000 g and 4^{o}C for 0.25 h. The cell paste was weighed and stored at -20^{o}C.

Cells containing pITT3 were extracted for plasmid using QIAfilter Plasmid Giga Kits (Hilden, Germany) to be used for static binding study.

4.3.5 Chromatography

4.3.5.1 Cell lysis

Cell lysis of *E. coli* DH1 containing pORT.mpt64 was performed using alkaline lysis method as described in section 3.2.4, chapter 3. DNA and RNA contents of lysate were 34.32 µg/mL and 661.47 µg/mL, respectively.

4.3.5.2 Column operation

The chromatography was performed on an ÄKTA Explorer 100 system controlled by UNICORN 4.11 software (GE Healthcare, Uppsala, Sweden) at room temperature (~ 21^{o}C). SEC/IEC Sepharose CL-6B modified by 10% bromination at 90^{o}C was packed into Tricorn 5/50 column (GE Healthcare, Uppsala, Sweden). A bed volume of 1 mL was applied in order to obtain a 5 cm bed height. Buffer A used for equilibration, loading and washing was prepared to mimic the compositions of clarified neutralised alkaline lysate by mixing 36 mL of 10 mM Tris-HCl, pH 8 containing 61 mM glucose and 50 mM EDTA with 78 mL of 0.2 M NaOH containing 1% SDS. Subsequently, 59 mL of cold (5^{o}C) 3 M potassium acetate, pH 5.5 was added followed by 4-6 times inversion. White precipitate was removed by filtration through a 0.2 µm membrane filter.

Chromatographic run was performed as follows. A packed column was equilibrated with 5 column volumes of diluted buffer A (dilution factor corresponding to the dilution factor applied to *E. coli* lysate for feedstock preparation) at a flow rate of 30 cm/h. The loading feedstock was prepared by three-fold dilution of clarified neutralised *E. coli* lysate with distilled water to obtain a conductivity of ~ 33 mS/cm. Ninety CV of diluted lysate was loaded onto column using P-960 sample pump at a flow rate of 30cm/h. After the loading phase was complete, the column was washed with 4 CV diluted buffer A used for equilibration. The column was then eluted with a linear salt gradient (gradient length of 20 CV) of 1 M NaCl, 1 mM EDTA, 25 mM Tris-HCl, pH 8. Finally, stripping was performed using 25 CV of a 0.2 M NaOH, 2 M NaCl solution. The chromatography run was monitored for UV absorbance at 260 and 280 nm. The flowthrough, washed and eluted fractions were subsequently analysed by agarose gel electrophoresis, chemical assays for DNA and RNA contents.

4.3.6 Analysis

4.3.6.1 Environmental scanning electron microscopy (ESEM)

ESEM visualization of SEC/IEC supports was kindly assisted by Mrs Theresa morris, School of Metallurgy and Materials, University of Birmingham. Samples were prepared by dehydrating in ethanol followed by critical point drying. Imaging of samples was carried out on Philips XL-30 FEG Environmental SEM (FEI Company, OR, USA).

4.3.6.2 Bromine assay

Bromine assay was performed in the same manner as stated in section 2.2.5.2, chapter 2.

4.3.6.3 Ionic capacity assay (Theodossiou and Thomas, 2002)

Ionic capacity of supports was measured by the method adapted from Theodossiou and Thomas (2002). The assay was performed as mentioned in section 2.2.5.3, chapter 2.

4.3.6.4 Static binding studies

The cell paste containing pITT3 obtained from fermentation was purified by QIAfilter Plasmid Giga Kit according to the product manual provided. Purified plasmid was visualised by agarose gel electrophoresis and measured for pDNA content using diphenylamine (DPA) assay.

DNA and protein binding studies were performed in the same fashion as described in section 2.2.5.4, chapter 2.

4.3.6.5 Diphenylamine (DPA) assay

DPA assay for DNA content was performed as described in section 2.2.5.5, chapter 2.

4.3.6.6 Orcinol assay (Almog and Shirey, 1978)

Orcinol assay for RNA content used in this study was modified from the method reported by Almog and Shirey (1978) as described in section 3.2.6.2, chapter 3.

4.3.6.7 Bicinchoninic acid (BCA) assay

BCA assay for protein content was carried out using a Pierce® BCA Protein Assay Kit (Pierce, USA) in accordance with the assay kit manual. A working reagent was prepared by mixing 50 parts of reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with 1 part of reagent B (containing 4% cupric sulfate). The assay was performed as stated in section 2.2.5.6, chapter 2.

4.3.6.8 Phenol chloroform extraction of nucleic acids

Before applying to agarose gel electrophoresis, nucleic acid species in chromatography fractions were extracted using a phenol-chloroform extraction method as described in section 3.2.6.4, chapter 3.

4.3.6.9 Agarose gel electrophoresis

Agarose gel at a concentration of 0.8 % (w/v) was pre-stained using SYBR safe stain as follows; 10 µL of SYBR safe stain was mixed with 100 mL of 1x TBE buffer prior to addition of 0.8 g agarose powder. After melting the agarose by heating in a microwave oven, the gel was casted on a casting box equipped with an 18 µL, 26-well comb. Agarose gel electrophoresis and imaging were performed in similar manners as described in section 2.2.5.7, chapter 2 but the gel was processed directly to visualization step without ethidium bromide staining after electrophoresis.

4.4 Results and discussion

Bilayered SEC/IEC supports were prepared by modification of two commercially available SEC supports, Sepharose CL-6B and Sephacryl S400 HR, via AGE activation-partial bromination route. Conclusively, this process consists of (i) introduction of allyl groups (CH_2=CH-CH_2-) throughout the structure of Sepharose CL-6B by reaction of allyl glycidyl ether (AGE) with hydroxyl groups on the support; (ii) partial bromination of allyl groups on the surface of each particle to create an outer layer via addition reaction; (iii) hydrolysis of the resulting outer layer of bromo-alkyl groups to create an inert outer layer; (iv) full

bromination of the remaining allyl groups on support's core; and finally (v) coupling of a quaternary amine ligand, trimethylamine (Q), to the core.

Amongst all steps, partial bromination is the most crucial step to determine the sharp discrimination between inert outer layer and charged core. Essentially, inert outer layer is preferred to be as thinnest as possible in order to minimize compromisation of core binding capacity. Thin inert outer layer can be achieved by minimizing bromine diffusion into support's pores and simultaneously increase reaction rate between bromine and double bonds on allylated supports. By balancing bromine diffusion rate and bromine-allyl reaction, it should be possible to improve the separation of the 'inert' and anion-exchanging layers and to create more effective SEC/IEC supports. The idea of increasing temperature to facilitate reaction-diffusion balancing can be explained by the Arrhenius equation and Einstein-Stoke equation as shown below (Eq. 4.2 and 4.3).

Arrhenius equation

$$k = A\, exp^{(-Ea/RT)} \qquad \text{(Eq. 4.2)}$$

Einstein-Stoke equation

$$D = \frac{k_B T}{6\pi\eta r} \qquad \text{(Eq. 4.3)}$$

Where;

k	is the rate coefficient (mol/L.s)
A	is the frequency factor (s^{-1})
E_a	is the activation energy, ~ -120 kJ/mol for bromination of double bond (Lister, 1941, Conn et al., 1938)
R	is the gas constant (8.314 x 10^{-3} kJ/mol/K)
T	is a temperature (K)
D	is the diffusion constant (m^2/s)
k_B	is Bolzmann's constant (1.381 x 10^{-23} J/K)
η	is viscosity (kg/m/s)
r	is the radius of the spherical diffusing species, 228 pm for Br_2 (Mountain, 2000)

Considering these equations, it is clear that temperature (T) plays an important role in both reaction and diffusion. Increasing temperature results in increased reaction and diffusion rates while high reaction rate and low diffusion rate are essential. By adjusting temperature,it is expected that the 'balanced' point can be achieved.

4.4.1 Effect of temperature on reaction/diffusion rates

The viscosity of water was dramatically reduced when the temperature was reduced from 2-60^{o}C and reached plateau after 60^{o}C until the end of measurement at 90^{o}C (Fig. 4.8).

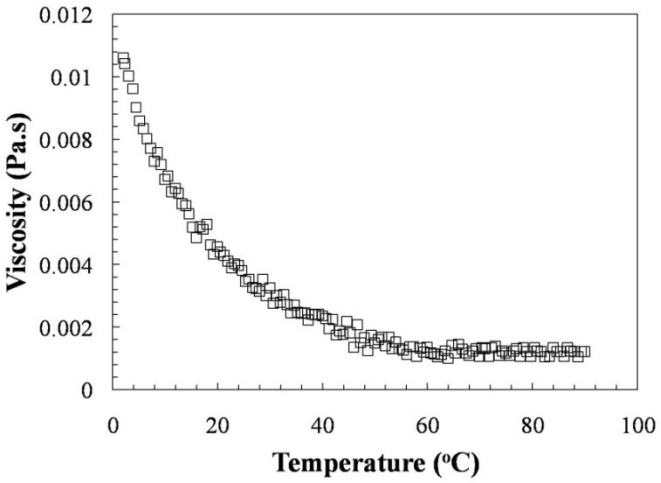

Fig. 4.8 Viscosity-temperature profile of water. Viscosity was measured during the increasing temperature from 2 ^{o}C to 90 ^{o}C (applying a temperature ramp rate of 4^{o}C/min) at a shear rate of 10 s^{-1}.

Water viscosity values (η) at each temperature were used to calculate the reaction/diffusion rate ratio (k/D). k is the reaction rate coefficient, calculated by using the Arrhenius equation (Eq. 4.2) while D is the diffusion rate coefficient calculated by using the Stokes-Einstein equation (Eq. 4.3). The relationship between temperature and k/D is shown in Fig. 4.9.

Increasing temperature leads to an increase in the reaction/diffusion rate ratio (k/D) (Fig. 4.9). Dramatic increase in k/D was observed when the temperature was increased to above 60°C. D was observed to be relatively constant over this temperature range (Fig. 4.8), with the increase in k/D coming from the significant increase in reaction rate seen as temperature is increased. Due to the lack of variation seen in D at the reaction temperatures which the studies in this chapter focus on (> 60°C), the effect of temperature on diffusion rate was neglected.

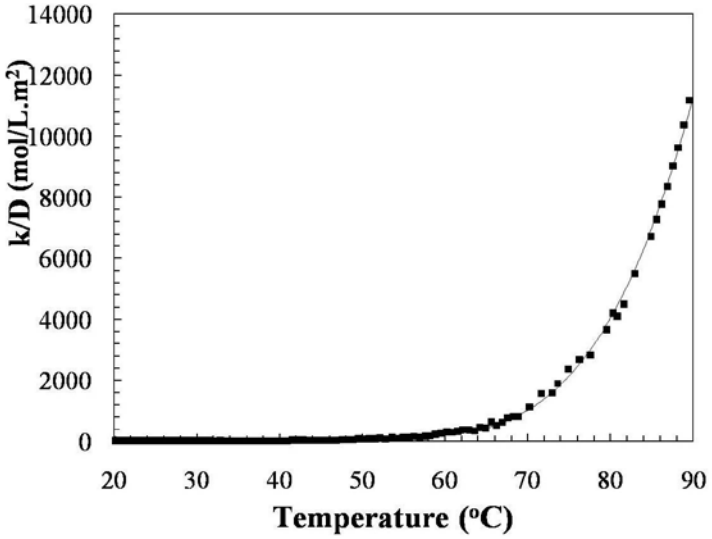

Fig. 4.9 Relationship between reaction/diffusion rate ratio (k/D) and temperature for bromination reaction of double bond {E_a = ~ -120 kJ/mol (Lister, 1941, Conn et al., 1938)}.

As shown in Fig. 4.9, in order to gain the full benefit of heating, reactions should be processed at temperature higher than 60°C. Therefore, temperatures for partial bromination reaction were pre-determined to be 70, 80 and 90°C. At these temperatures, reaction rate between bromine and allyl groups on allylated support was predicted to be very fast. At such high reaction rate, bromine added is expected to be completely reacted before diffusing further into support interior, yielding a SEC/IEC support with a high-definition, thin inert outer layer.

4.4.2 Microwave heating curves during partial bromination reaction

Microwave-assisted partial bromination of Sepharose CL-6B was performed by heating up 50 mL of Sepharose CL-6B slurry (60% v/v support in water) using dynamic mode to 83 and 93 ^{o}C. Heating speeds of microwave irradiation were proved to be extremely fast compared to conventional heating. It has been reported that microwave heats up materials at extremely higher speed compared to conventional heating (Agrawal, 1998, Ania et al., 2005, Mallakpour and Rafiee, 2011). For 10% bromination, microwave heating of Sepharose CL-6B in dynamic mode took 60 s to reach 83^{o}C and 70 s to reach 93^{o}C respectively (Fig. 4.10). Fast heating speeds were also observed during the heating of support slurry for 20% bromination. The slurries took 55 s to reach 71^{o}c, 60 s to reach 81^{o}c and 70 s to reach 92^{o}C, respectively (Fig. 4.11). Bromine was added at these target temperatures and bromine colour was seen to disappear almost immediately, indicating that the reaction between bromine and allyl groups was very rapid. This was as expected, due to the increased reaction rates associated with the increased temperatures used as described by Arrhenius law (Eq. 4.2).

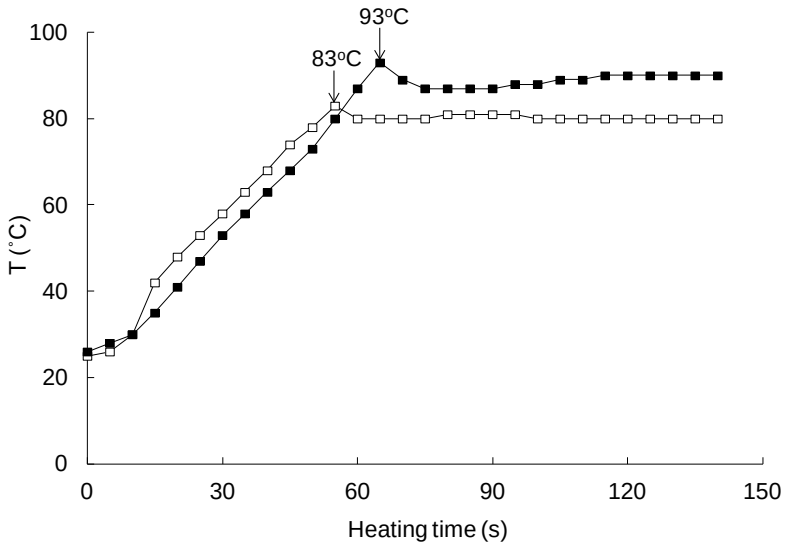

Fig. 4.10 Changes in temperature during microwave heating for 10% bromination of Sepharose CL-6B under 'dynamic mode'. The temperature was held constant after target temperature was reached. Bromine was added at points indicated by arrows.

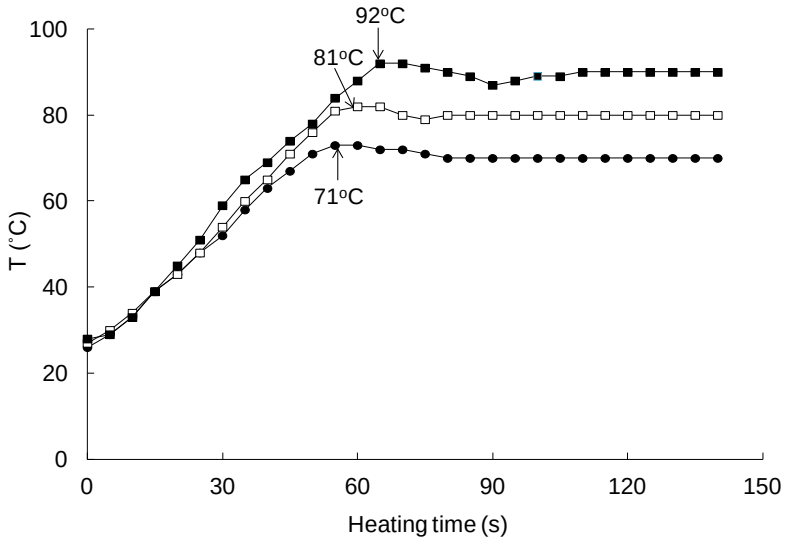

Fig. 4.11 Changes in temperature during microwave heating for 20% bromination of Sepharose CL-6B under 'dynamic mode'. The temperature was held constant after target temperature was reached. Bromine was added at points indicated by arrows.

For Sephacryl S-400 HR, microwave-assisted partial bromination reactions were performed by heating up 50 mL of support slurry (60% v/v support in water) to 79, 85 and 89°C. Microwave heating of Sephacryl S400 HR in dynamic mode took 60 s to reach 79°C, 65 s to reach 85°C and 70 s to reach 89°C respectively (Fig. 4.12). For 20% bromination, microwave heating of Sephacryl S400 HR in dynamic mode took 60 s to reach 80°C, 65 s to reach 85°C and 70 s to reach 91°C respectively (Fig. 4.13). Again, fast heating was observed and bromine colour disappeared almost instantly suggesting high reaction rates following the Arrhenius law.

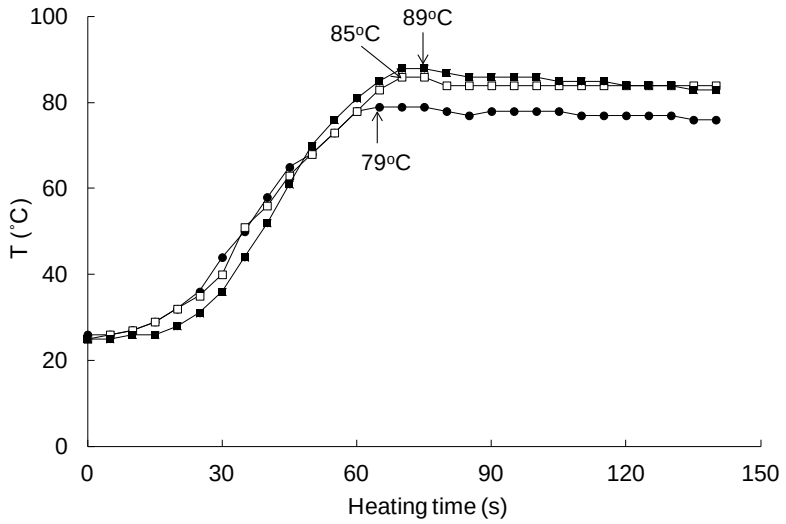

Fig. 4.12 Change in temperature during microwave heating for 10% bromination of Sephacryl S400 HR under 'dynamic mode'. The temperature was held constant after target temperature was reached. Bromine was added at points indicated by arrows.

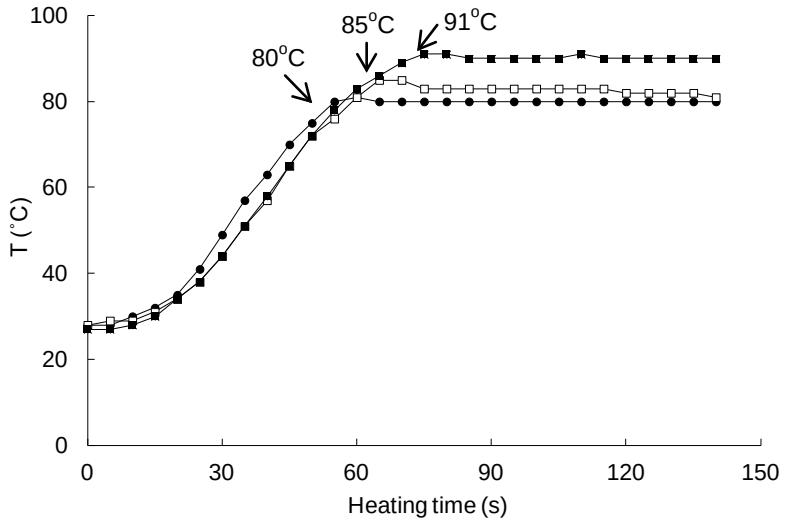

Fig. 4.13 Change in temperature during microwave heating for 20% bromination of Sephacryl S400 HR under 'dynamic mode'. The temperature was held constant after target temperature was reached. Bromine was added at points indicated by arrows.

4.4.3 Characterisations of SEC/IEC supports produced

Sepharose CL-6B and Sephacryl S400 HR were modified by the AGE activation-partial bromination route with reaction-diffusion balancing approach to create SEC/IEC supports. Microwave heating by a monomodal microwave reactor was applied to aid partial bromination reaction. Various temperature and degrees of partial bromination were investigated for their effects on thickness and inertness of supports' outer layer. The supports were analysed at various stages of preparation for allyl contents, ionic binding capacity as well as pDNA and protein binding capacities.

ESEM visualisations of SEC/IEC Sepharose CL-6B and Sephacryl S400 HR confirmed that microwave power does not affect support's structure even at the highest temperature tested (90^{o}C) compared to unmodified supports (Fig. 4.14).

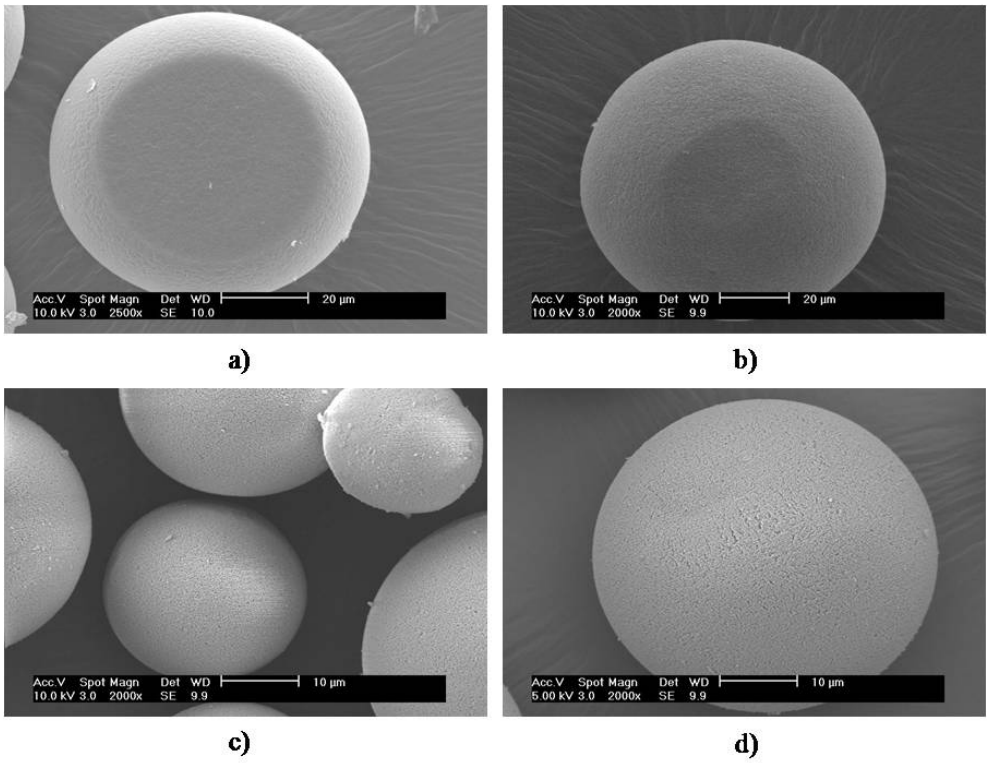

Fig. 4.14 ESEM images of; a) unmodified Sepharose CL-6B, b) SEC/IEC Sepharose CL-6B, modified using microwave heating at 90^{o}C, c) unmodified Sephacryl S400 HR and d) SEC/IEC Sephacryl S400 HR, modified using microwave heating at 90^{o}C, 10% bromination.

SEC/IEC matrices produced were further characterised for allyl contents, ionic capacity as well as pDNA and protein binding capacities. The relative values, % reduction, were used to express the changes in each parameter after modifications as described below;

The reductions in allyl contents were calculated by comparing the allyl content of partially brominated supports to that of the original allylated supports as shown in Eq. 4.4.

$$\%\ Reduction\ in\ allyl\ contents = \frac{(\text{allyl contents of origina l allylated supports}) - (\text{allyl contents of partially brominated supports})}{\text{allyl contents of original allylated supports}} \text{ x } 100\% \quad \text{(Eq. 4.4)}$$

% Reductions in ionic capacity were calculated by comparing ionic capacities of modified supports to fully Q-coupled supports as shown in Eq. 4.5.

$$\%\ Reduction\ in\ ionic\ capacity = \frac{(\text{ionic capacity of fully Q-coupled supports}) - (\text{ionic capacity of sample})}{\text{ionic capacity of fully Q-coupled supports}} \text{ x } 100\% \quad \text{(Eq. 4.5)}$$

Effectiveness of surface or core charge elimination is expressed by % reductions in pDNA or protein binding capacities, respectively. % Reductions in binding capacity were calculated by comparing the binding capacities of modified supports to fully Q-coupled supports as shown in Eq. 4.6.

$$\%\ Reduction\ in\ binding\ capacity = \frac{(\text{binding capacity of fully Q-coupled supports}) - (\text{binding capacity of sample})}{\text{binding capacity of fully Q-coupled supports}} \text{ x } 100\% \quad \text{(Eq. 4.6)}$$

For Eq. 4.5 and 4.6, fully Q-coupled supports were produced by directly applying the full bromination and Q-coupling steps (section 2.2.3.4) to the supports directly after AGE activation.

The effectiveness of the bilayer creations can be indicated by selectivity index (SI) value. SI value was expressed by comparing the remaining protein binding capacity to the remaining DNA binding capacity which therefore, indicates the ratio of residual core charge to the degree of surface charge elimination, respectively. From this aspect, SI can therefore be used to virtually demonstrate the 'thinness' of the inert outer layer as well as the depth of bromine penetration into support's pores. Selectivity indices (SI) were calculated using equation;

$$SI = \frac{100\% - (\%\ \text{reduction in protein binding capacity})}{100\% - (\%\ \text{reduction in pDNA binding capacity})} \qquad \text{(Eq. 4.7)}$$

Initially, microwave heating at 40^{o}C was used to aid partial bromination reaction due to a promising result reported by Liddy (2010) obtained by heating Sepharose CL-6B slurry with a domestic microwave oven (Liddy, 2009). However, in the preliminary test using monomodal microwave reactor, modified Sepharose CL-6B produced at the same temperature of 40^{o}C resulted in a rather disappointing result. Only 3.94% reduction in pDNA binding capacity and a relatively high % reduction in protein binding capacity of 12.86% were observed. This contradiction is believed to be caused by the insufficient temperature control and monitoring in the domestic microwave oven operation used by Liddy, in which heated samples were removed from the microwave oven and the temperatures were measured by a mercury thermometer, leaving a time delay between heating and measurement with a consequent temperature loss. The actual reaction temperature was, therefore, believed to be much higher than 40^{o}C. According to the relationship between k/D and temperature shown in Fig.4.9, the value of k/D approximately doubles for every 5^{o}C change in temperature, so from this it was surmised that higher temperatures would be more suitable for partial bromination, although not so high as to cause significant loss/damage of supports or loss of added bromine.

Hence, the raises in reaction temperatures to 71-93^{o}C were applied later on in both Sepharose CL-6B and Sephacryl S400 HR.

As expected, increasing the reaction temperature to 71-93^{o}C led to reduction in pDNA binding capacity at a range of 85-98% for modified Sepharose CL-6B supports, suggesting almost complete elimination of surface charge (Fig. 4.15). The highest SI value of 48 was obtained when applying 10% partial bromination at 83^{o}C. This corresponds to a reduction in pDNA binding of 98.4% with only 4.3% reduction in protein binding was observed. Increased degree of partial bromination from 10-20% led to a greater decrease in allyl group contents (Fig. 4.15). This decrease in the number of available allyl groups was seen to correlate with a decrease in the protein binding and ionic capacities. Strangely, the reductions in pDNA binding decreased when the degree of partial bromination was increased from 10-20% while an increase in % reduction for pDNA binding was expected. However, reductions in protein binding capacity seemed to follow the trend, along with reductions in allyl content and ionic capacity.

SEC/IEC Sephacryl S400 HR matrices produced were also characterised for allyl contents, ionic binding capacity as well as pDNA and protein binding capacities (Fig 4.16). Again, an increased degree of partial bromination from 10-20% led to a greater decrease in allyl group content, correlating with an increase in the reduction in protein binding and ionic capacities. Reduction in pDNA binding capacity values at a range of 52-83% was achieved. The highest SI value of 5.7 was obtained when applying 10% partial bromination at 79^{o}C. This corresponds to a reduction in pDNA binding of 83% with only 8% reduction in protein binding was observed. Once again, the reductions in pDNA binding decreased when the degree of partial bromination was increased from 10-20% while an increase in % reduction in pDNA binding was expected. Reduction of protein binding seemed to follow the trend, along with reductions in allyl content and ionic capacity.

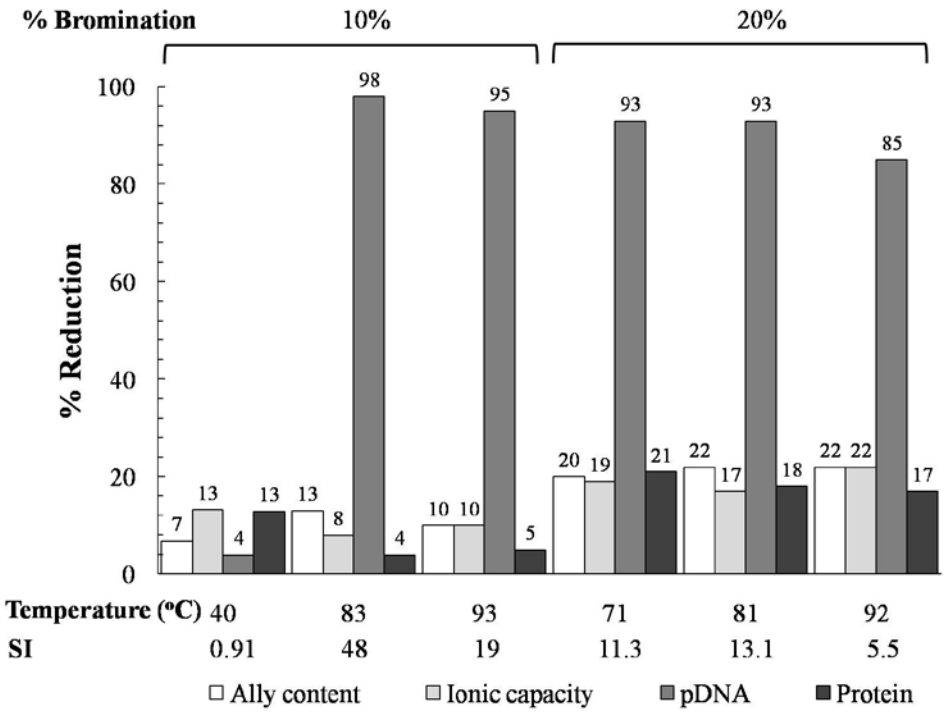

Fig. 4.15 Chemical and biochemical characterisation of SEC/IEC Sepharose CL-6B produced via AGE activation-partial bromination route facilitated by microwave heating at various temperatures and degrees of partial bromination.

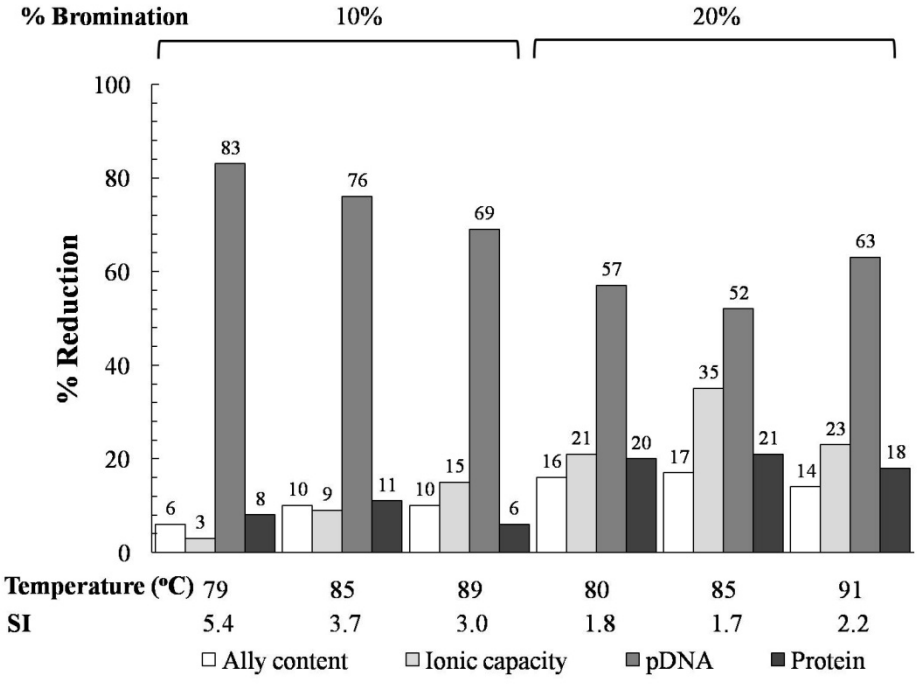

Fig. 4.16 Chemical and biochemical characterisation of SEC/IEC Sephacryl S400 HR produced via AGE activation-partial bromination route facilitated by microwave heating at various temperatures and degrees of partial bromination.

Although high % reductions in DNA binding capacity were seen for the bilayered Sephacryl S400 HR, the associated SI values were not as high as those seen for Sepharose CL-6B in similar conditions due to the lower % reduction in pDNA binding capacity while % reduction in protein binding capacity seemed to be indifferent (Fig. 4.17). The different performances on pDNA binding was thought to be caused by difference in pore sizes between Sepharose CL-6B and Sephacryl S-400 HR (24 nm and 31 nm, respectively) (Kornberg and Baker, 2005). However, with large biomolecules such as plasmids which normally are larger than 0.2 µm (Fishman and Patterson, 1996) and much larger than the pore sizes of these two supports. Conclusively, the difference in pore sizes of supports does not cause the difference in absorption behaviour of pDNA in this case. Therefore, it is possible that modified Sephacryl S400 HR tested still possesses surface charges. Further column experiments on modified Sephacryl S400 HR may be able to help addressing the cause of this difference.

4.4.4 Chromatography

The SEC/IEC support exhibited the highest SI value, Sepharose CL-6B modified by microwave-assisted, 10% bromination at 83^{o}C was chosen to be further evaluated by applying to packed bed chromatography system in order to purify target pDNA; pORT.mpt64 (6336 bp), from neutralized *E.coli* cleared lysates. Plasmid DNA and RNA contents were monitored by UV traces and chemical assays as well as agarose gel electrophoresis.

From the gel electrophoresis and chemical assay results (Fig. 4.18a and 4.19), it was observed that HM-RNA breakthrough was not achieved even after a large loading volume of 90 CV, suggesting the high core binding capacity of this support. After column purification, plasmid forms remain unchanged, reflecting the benefit of negative mode chromatography

where target molecules do not bind to the matrix. However, pDNA breakthrough was observed to be slightly delayed at 10 CV, suggested the possibility that the support still have surface charge. The agarose gel image of eluted and stripped fractions confirmed that a relatively large amount pDNA was bound to this support even at high conductivity. It was noted no change in plasmid forms was observed on agarose gel.

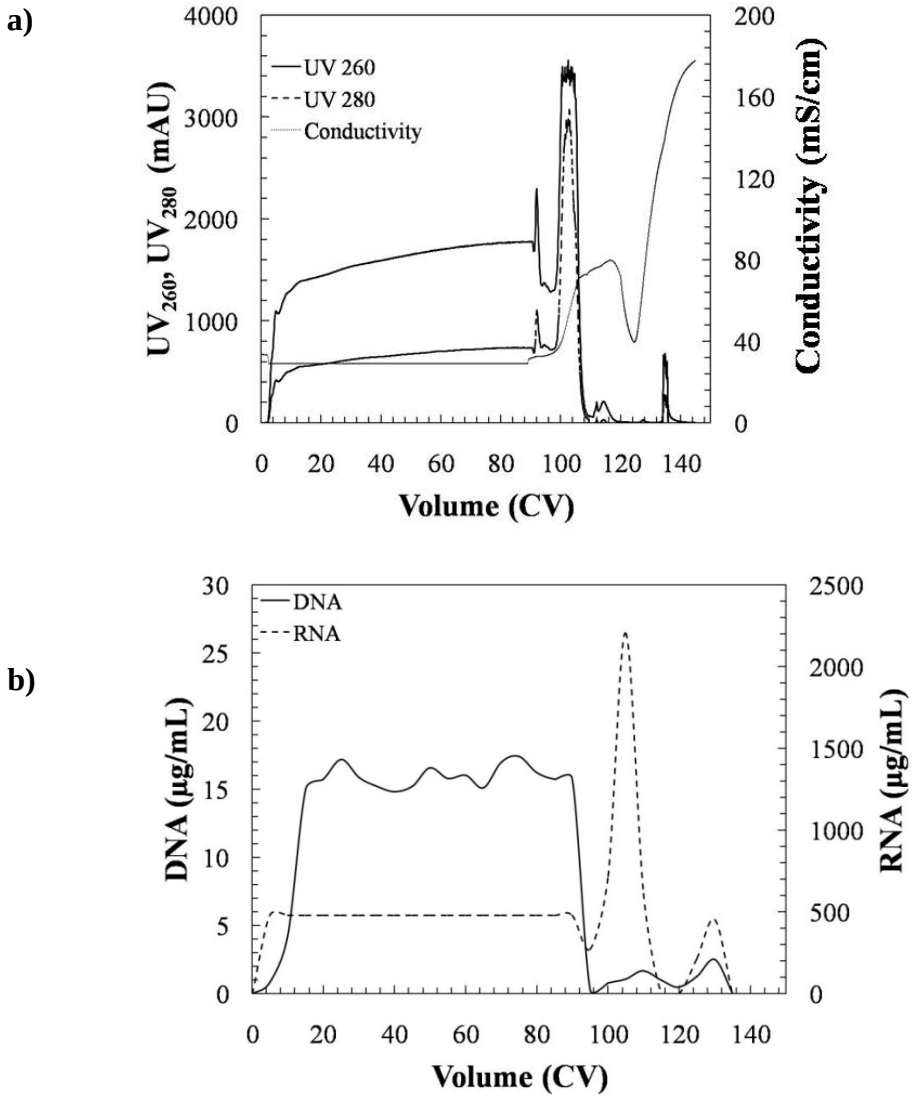

Fig. 4.17 Chromatograms (a) and chemical measurements of chromatographic fractions (b) obtained from packed bed chromatography of Sepharose CL-6B modified by microwave-assisted, 10% bromination at 83^{o}C. Chromatography run was performed at a conductivity of 33 mS/cm, flow rate of 30 cm/h.

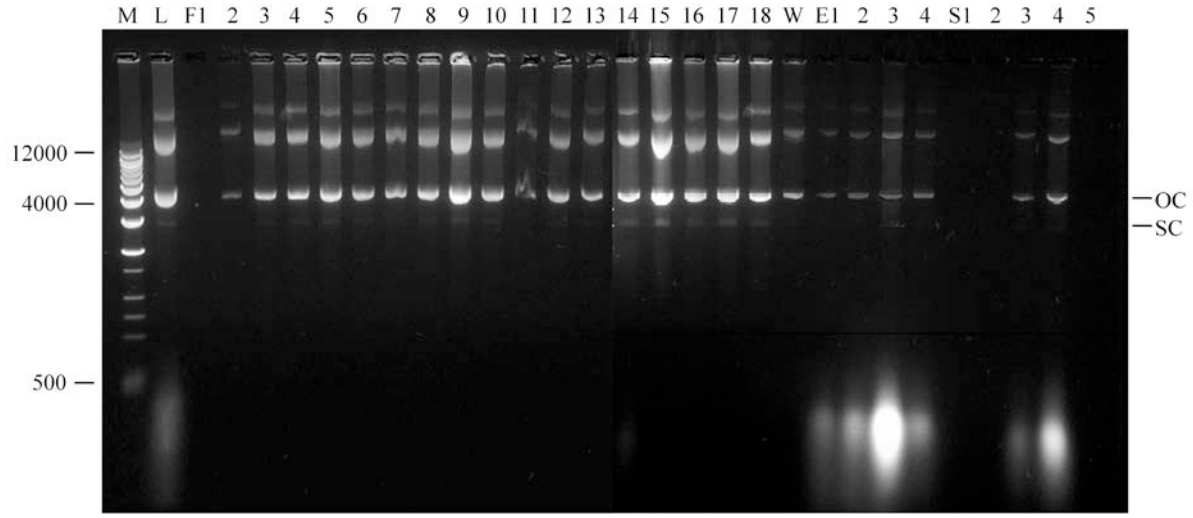

Fig. 4.18 Agarose gel eletrophoresis of chromatographic fractions obtained from packed bed chromatography of Sepharose CL-6B modified by microwave-assisted, 10% bromination at 83^{o}C. Fraction sizes was 5 CV. Abbreviations; M : Kb DNA Ladder (250bp-12Kb), L : cleared lysate fed onto column, F : flowthrough, W : wash fraction, E : elution fractions and S : stripped fraction.

4.5 Conclusions

The microwave-assisted reaction diffusion balancing approach was employed for SEC/IEC supports preparation. The monomodal microwave reactor provided a fast and controlled heating compared to conventional heating without damaging the supports. The high reaction temperature (70-90^{o}C) resulted in almost complete surface charge elimination in SEC/IEC Sepharose CL-6B. The enhancement of reaction rate by the increased temperature can be explained by the Arrhenius equation. The calculation of reaction/diffusion rate ratio (k/D) for bromination reaction suggested that in order to gain the full benefit of heating, reactions should be processed at temperature higher than 60^{o}C and double reaction rate can be achieve every 5^{o}C increase in temperature. The SEC/IEC support with the highest SI was tested in column chromatography system for pDNA purification from *E. coli* cleared lysate. It was noted that the plasmid forms remain unchanged, suggesting the mild purification condition. This support exhibited the high core binding capacity, however, the delayed pDNA

breakthrough and the relatively large amount of pDNA in eluted and striped fractions reflected that the surface charges still remain. Again, it was difficult to point out the pattern of charge distribution on support particles after modification based on the binding behaviors alone. In order to identify the patterns of charge elimination on the support produced, the development of visualization techniques may be worth investigation.

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Chapter 5

Concluding remarks

The worldwide growing demands for gene therapy products, especially for clinical trials where the large doses are required for each administration, has raised the requirement of the processes for the efficient large scale production of clinical grade gene transfer vectors with the high purity compliant with the specifications set by the governmental regulatory agency. For these gene therapy products to be administered safely in human, the purification process becomes the crucial step among the whole process. Generally, packed bed chromatography especially ion exchange chromatography, has been the method of choice for the purification of these gene transfer vectors 'nanoplexes' due to its high resolution, and selectivety. However, some problems still exist due to the similar charge properties between the target nanoplexes and impurities. Moreover, the large size of these nanoplexes led to the restricted binding only on support's surface and the fragility of these molecules requires gentle handling methods. The idea of bilayered SEC/IEC supports can tackle these problems by providing negative mode purification where the charged core captures small impurities while the non-charged surface excludes the large target biomolecules without having chemical interaction therefore considerably more gentle than the general binding-elution approaches. In this thesis, the preparations of SEC/IEC supports from commercially available, underivatised base matrices by AGE activation-partial bromination technique via two different approaches; (i) viscosity enhanced-reaction diffusion (VE-RD) and (ii) microwave-assisted reaction diffusion, were studied and optimised. The selected supports were further evaluated in column chromatography to purify pDNA from *E. coli* cleared lysate.

In chapter 2, preparation of SEC/IEC supports via VE-RD approach was optimised. The viscosity enhancement was found to greatly aid the creation of thin inert outer layer by slowing down the diffusion of bromine into support's pores, resulted in the condensed charge elimination restricted on the outermost surface of support's particle. The improved performances of SEC/IEC supports seemed to be mainly caused by the effect of viscosity regardless the reaction temperature. The decline of support's performance at the sucrose viscosity above 0.032 Pa.s suggested the 'balance' point. After this point, the viscosity became excessive with excessive sucrose concentration which may led to another reaction between bromine and sucrose, competed with the bromination reaction of allyl group and resulted in bromine loss.

In chapter 3, the selected SEC/IEC supports produced in chapter 2 were evaluated in column chromatography system to separate RNA from *E. coli* cleared lysate. SEC/IEC Sepharose CL-6B modified by 10% single bromination exhibited a better performance especially on pDNA exclusion compared to SEC/IEC Sephacryl S400 HR. The pore sizes of both supports are considerably similar and are small enough to exclude plasmid pITT3 (27379 bp) used, suggesting that the binding of pDNA on SEC/IEC Sephacryl S400 HR, evident by large amount of pDNA in eluted fractions, may be caused by the remaining surface charge. Variations of operating parameters (conductivity, linear flow rate, plasmid size) were tested using SEC/IEC Sepharose CL-6B. The increase in conductivity resulted in the reduced binding capacity for both RNA and pDNA. Ideally, a successful pDNA-RNA separation requires minimum pDNA binding and maximum RNA binding. From this result, it is possible to fine-tune the conductivity to assist the separation. A flexible operating flow rate range of 30-60 cm/h was observed which means that the chromatography can be operated at twice the standard flow rate (30 cm/h) which helps shorten operation period. The smaller plasmid (4109 bp) show similar pattern during chromatography proved the flexibility of this support

towards different plasmid sizes. The SEC/IEC Sepharose CL-6B modified by double partial bromination/hydrolysis was seen to have different binding behaviours especially for RNA compared to those modified by single bromination (10 or 20%). It is worth mentioning that no change in plasmid form was observed in every chromatography runs using SEC/IEC supports.

In chapter 4, the microwave-assisted reaction diffusion balancing approach was employed for SEC/IEC supports preparation. The monomodal microwave reactor provided a fast and controlled heating compared to conventional heating without damaging the supports. The high reaction temperature (70-90^{o}C) resulted in almost complete surface charge elimination in SEC/IEC Sepharose CL-6B. The enhancement of reaction rate by the increased temperature can be explained by the Arrhenius Law. The calculation of reaction/diffusion rate ratio (k/D) for bromination reaction suggested that in order to gain the full benefit of heating, reactions should be processed at temperature higher than 60^{o}C and double reaction rate can be achieve every 5^{o}C increase in temperature. The SEC/IEC support with the highest SI was testes in column chromatography system for pDNA purification from *E. coli* cleared lysate. It was noted that the plasmid forms remain unchanged, suggesting the mild purification condition. This support exhibited the high core binding capacity, however, the delayed pDNA breakthrough and the relatively large amount of pDNA in eluted and striped fractions reflected that the surface charge still remain.

Although the binding studies can be used to indirectly demonstrate the charge elimination patterns and the thickness of the inert outer layer. However, without direct visualization, it was difficult to point out the pattern of charge distribution on support particles after modification based on the binding behaviors alone. Attempts to monitor the changes of surface elements by SEM-EDS technique had been made but the results were unreliable due to the small size of the element of interest (nitrogen). The direct visualization technique

according to Gustavsson's report was applied in an attempt to identify the thickness of the outer layer by viewing the SEC/IEC support dyed with a negative dye, Congo red under a light microscopy. Congo red binds to the positively charged core and leaves the inert outer layer unattached, resulting in a clear halo surrounding the red colour core. However, this method was found to have high chance to yield false positive 'halo' caused by the light refraction under light microscope, even with the fully Q-coupled support. Hence, this visualization was not applied further in this study. Confocal scanning laser microscopy of supports bound with fluorescent-tagged protein in order to visualize the core area was also considered. However, due to the nature of SEC/IEC supports, it is presumably impossible to attach fluorescent probes onto the surface via binding. The confocal scanning laser microscopy, therefore, cannot pick up the fluorescent signals to identify the edge of the inert outer layer. The development of visualization techniques to identify the thickness of inert outer layer and the patterns of charge elimination might lead to a better understanding on the mechanism of surface charge elimination via partial bromination reaction.

Future work

1. The patterns of charge distribution on support particles after modification are difficult to demonstrate based on the binding behaviours alone. The development of visualization techniques to identify the thickness of inert outer layer and the patterns of charge elimination/distribution might be one of the aspects worth further investigation.
2. An experiment should be conducted to examine the possibility of the reaction between sucrose and bromine at the exact condition applied for support preparation as described in chapter 2 in order to determine the cause of decrease support performance at the viscosity above 0.032 Pa.s.
3. The studies of integrated process by combining SEC/IEC column with other chromatographic column i.e. CIM™ DEAE (BIA Separation, Villach, Austria). SEC/IEC column may be applied first to capture impurities followed by CIM™ DEAE column for polishing and concentration or applied in polishing step to absorb small amount of impurities left after CIM™ DEAE column.
4. Identification of plasmid isoforms by analytical pDNA column available commercially i.e. MiniQ from GE Healthcare (Uppsala, Sweden) or CIMac™ pDNA Analytical Columns from BIA Separations (Villach, Austria) is also a good choice to help confirming the plasmid isoforms in addition to gel electrophoresis visualisation, restriction enzyme digestion and topoisomerase relaxation used in this thesis.

Appendix

A.1 Growth curve of *E. coli* containing pITT3 plasmid produced in Chapter 2

The fermentation was terminated after the cells reached late exponential phase at an OD_{600nm} of 2.13. The growth curve of *E. coli* was constructed using the software MicroFit v 1.0 (Institute of Food Research (IFR), Norwich, UK). The maximum specific growth rate (μ_{max}) and lag time calculated using this software were 1.32 h^{-1} and 5.43 h, respectively.

Fig. A.1 Growth curve of *E. coli* DH5α containing the pITT3 produced by fermentation in Chapter 2

A.2 Complete sequence of plasmid pORT3a-BAM2K

BASE COUNT 1017 a 981 c 1025 g 1086 t

ORIGIN

1 ctaggaaagc cacgttgtgt ctcaaaatct ctgatgttac attgcacaag ataaaaatat

61 atcatcatga acaataaaac tgtctgctta cataaacagt aatacaaggg gtgttatgag

121 ccatattcaa cgggaaacgt cttgctcgag gccgcgatta aattccaaca tggatgctga

181 tttatatggg tataaatggg ctcgcgataa tgtcgggcaa tcaggtgcga caatctatcg

241 attgtatggg aagcccgatg cgccagagtt gtttctgaaa catggcaaag gtagcgttgc

301 caatgatgtt acagatgaga tggtcagact aaactggctg acggaattta tgcctcttcc

361 gaccatcaag cattttatcc gtactcctga tgatgcatgg ttactcacca ctgcgatccc

421 cgggaaaaca gcattccagg tattagaaga atatcctgat tcaggtgaaa atattgttga

481 tgcgctggca gtgttcctgc gccggttgca ttcgattcct gtttgtaatt gtccttttaa

541 cagcgatcgc gtatttcgtc tcgctcaggc gcaatcacga atgaataacg gtttggttga

601 tgcgagtgat tttgatgacg agcgtaatgg ctggcctgtt gaacaagtct ggaaagaaat

661 gcataagctt ttgccattct caccggattc agtcgtcact catggtgatt tctcacttga

721 taaccttatt tttgacgagg ggaaattaat aggttgtatt gatgttggac gagtcggaat

781 cgcagaccga taccaggatc ttgccatcct atggaactgc ctcggtgagt tttctccttc

841 attacagaaa cggctttttc aaaaatatgg tattgataat cctgatatga ataaattgca

901 gtttcatttg atgctcgatg agtttttcta atcagaattg gttaattggt tgtaacactg

961 gcagagcatt acgctgactt gacgggacgg cggctttgtt gaataaatcg aacttttcct

1021 aggtaccccc tggcacgaca ggtttcccga ctggaaagcg ggcagtgagc gcaacgcaat

1081 taatgtgagt tagctcactc attaggcacc ccaggcttta cactttatgc ttccggctcg

1141 tatgttgtgt ggaattgtga gcggataaca atttcacaca ggaaacagct atgaccatga

1201 ttacgccaag cgcgcaatta accctcacta aagggaacaa aagctggagc gatgtacggg

1261 ccagatatac gcgttgacat tgattattga ctagttatta atagtaatca attacggggt

1321 cattagttca tagcccatat atggagttcc gcgttacata acttacggta aatggcccgc

1381 ctggctgacc gcccaacgac ccccgcccat tgacgtcaat aatgacgtat gttcccatag

1441 taacgccaat agggactttc cattgacgtc aatgggtgga gtatttacgg taaactgccc

1501 acttggcagt acatcaagtg tatcatatgc caagtacgcc ccctattgac gtcaatgacg

1561 gtaaatggcc cgcctggcat tatgcccagt acatgacctt atgggacttt cctacttggc

1621 agtacatcta cgtattagtc atcgctatta ccatggtgat gcggttttgg cagtacatca

1681 atgggcgtgg atagcggttt gactcacggg gatttccaag tctccacccc attgacgtca

1741 atgggagttt gttttggcac caaaatcaac gggactttcc aaaatgtcgt aacaactccg

1801 ccccattgac gcaaatgggc ggtaggcgtg tacggtggga ggtctatata agcagagctc

1861 tctggctaac tagagaaccc actgcttact ggcttatcga aattaatacg actcactata

1921 gggagaccca agctggctag caccatgctt gaaccgttcc aaattctctc tatctgctca

1981 tttatcttat ctgccctcca ttttatggcc tggaccagcc tgttaaccga agtcgaaacg

2041 ccaattcgta atgaatgggg ctgccgctgc aatgattcgt ccgatagctt actcacagaa

2101 gttgaaaccc ctacaaaaaa cgaatgggaa tgtcgctgca atgattcatc agattcctta

2161 ttaaccgaag tcgaaactct tactcgcaac ggctggggtt gtcgctgtag tgattctagt

2221 gatcctctcg tagtcgccgc atctatcatt ggtatcttac acttgatttt atggatttta

2281 gaccgcctct tttttaaatg tatctatcgc cgcttcaaat atggattaaa acgcggacct

2341 agcaccgaag gagtacctga aagtatgcgt gaagaatacc gccaagaaca acaaaacgct

2401 gtagacgttg atgatggtca ttttgtaaac attgaattag aataatggat ccactagtcc

2461 agtgtggtgg aattctgcag atatccagca cagtggcggc cgctcgagtc tagagggccc

2521 gtttaaaccc gctgatcagc ctcgactgtg ccttctagtt gccagccatc tgttgtttgc

2581 ccctcccccg tgccttcctt gaccctggaa ggtgccactc ccactgtcct ttcctaataa

2641 aatgaggaaa ttgcatcgca ttgtctgagt aggtgtcatt ctattctggg gggtggggtg

2701 gggcaggaca gcaaggggga ggattgggaa gacaatagca ggcatgctgg ggatgcggtg

2761 ggctctatgg cttctgaggc ggaaagaacc aggacctcga gggggggccc ggtacccaat

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2881 tcgttccact gagcgtcaga ccccgtagaa aagatcaaag gatcttcttg agatcctttt

2941 tttctgcgcg taatctgctg cttgcaaaca aaaaaaccac cgctaccagc ggtggtttgt

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3061 ataccaaata ctgtccttct agtgtagccg tagttaggcc accacttcaa gaactctgta

3121 gcaccgccta catacctcgc tctgctaatc ctgttaccag tggctgctgc cagtggcgat

3181 aagtcgtgtc ttaccgggtt ggactcaaga cgatagttac cggataaggc gcagcggtcg

3241 ggctgaacgg ggggttcgtg cacacagccc agcttggagc gaacgaccta caccgaactg

3301 agatacctac agcgtgagct atgagaaagc gccacgcttc ccgaagggag aaaggcggac

3361 aggtatccgg taagcggcag ggtcggaaca ggagagcgca cgagggagct tccaggggga

3421 aacgcctggt atctttatag tcctgtcggg tttcgccacc tctgacttga gcgtcgattt

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3541 cggttcctgg ccttttgctg gccttttgct cacatgttct ttcctgcgtt atcccctgat

3601 tctgtggata accgtattac cgcctttgag tgagctgata ccgctcgccg cagccgaacg

3661 accgagcgca gcgagtcagt gagcgaggaa gcggaagagc gcccaatacg caaaccgcct

3721 ctccccgcgc gttggccgat tcattaatgc agggccggcc tcatgaatgg tgttaagcgg

3781 gcggttttga gatgtaaact cgcccgttta acataatgga tcttgcgcgc accgcccgaa

3841 caccactcgc cacaaaaaac cgccggaacg tccaaaagta cgggttttgc tgcccgcaaa

3901 cgggctgttc tggtgttgct agtttgttat cagaatcgca gatccggctt caggtttgcc

3961 ggctgaaagc gctatttctt ccagaattgc catgattttt tccccacggg aggcgtcact

4021 ggctcccgtg ttgtcggcag ctttgattcg ataagcagca tcgcctgttt caggctgtct

4081agcttat cgatgataag cttcatgac //

A.3 Preliminary tests on monomodal microwave heating of water and water-support slurry

A.3.1 Method

A.3.1.1 Microwave heating profile of water at small volume

Distilled water (1 to 5 mL) in a 10 mL reaction vessel (CEM Corporation, Matthews, NC, USA) was heated under mixing in a CEM Discover S-Class microwave apparatus using a 'fixed power' method. A fixed amount of microwave power (10 to 40 W) was supplied for a fixed period of time (10 to 30 s). On-line reading of water temperature was taken immediately before and after heating using the CEM microwave's built-in infrared sensor (see Fig. A.2).

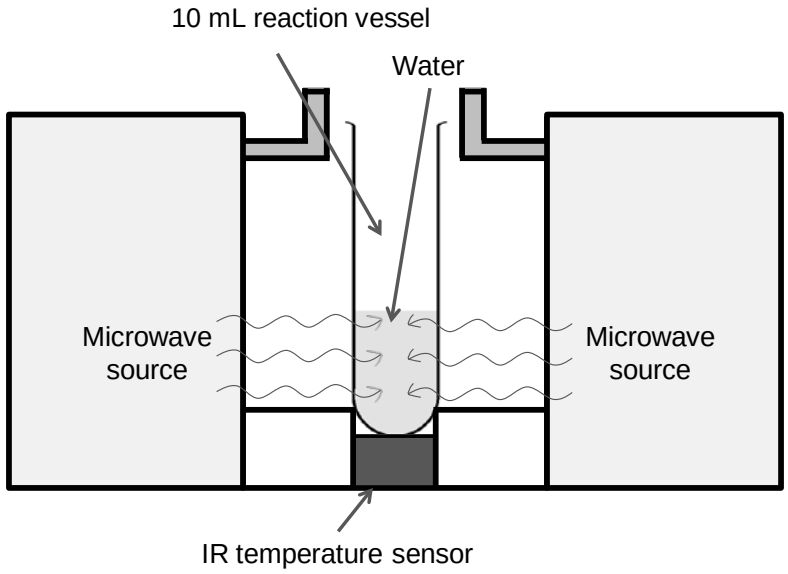

Fig. A.2 Setup for microwave heating of water in 10 mL reaction vessel

A.3.1.2 Microwave heating profile of water at large volume

Distilled water (10 to 50 mL) in a 100 mL round bottom flask was heated under mixing in a CEM Discover microwave apparatus using a 'fixed power' method. A fixed amount of microwave power (10 to 40 W) was supplied for a fixed period of time (10 to 30 s). On-line reading of water temperature was taken immediately before and after heating using the CEM microwave's built-in infrared sensor (see Fig. A.3).

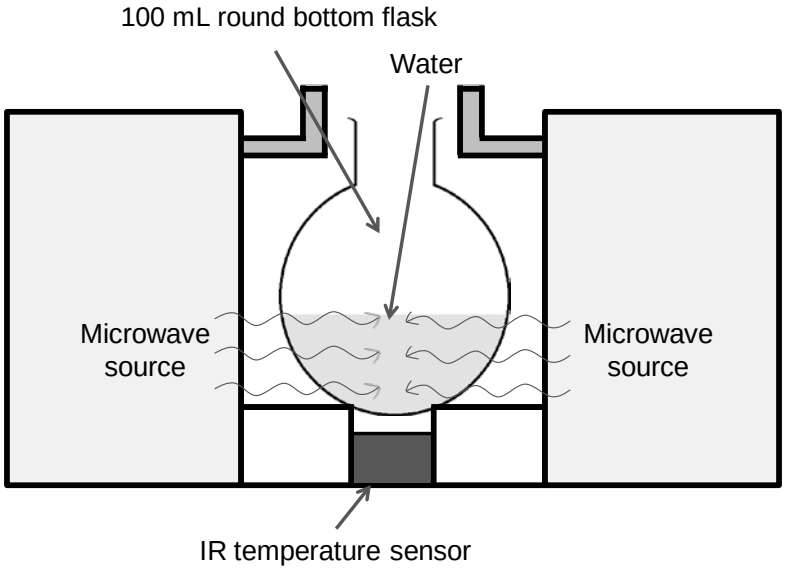

Fig. A.3 Setup for microwave heating of water in 100 mL round bottom flask.

A.3.1.3 Microwave heating profile of water-supports slurry

Two millilitres of Sepharose CL-6B in distilled water slurry (50-80% v/v supports) in a 10 mL reaction vessel was heated under mixing in a CEM Discover microwave apparatus using a 'fixed power' method. A fixed amount of microwave power (10 to 40 W) was supplied for a fixed period of time (10 to 30 s). On-line reading of water temperature was taken immediately before and after heating using the CEM microwave's built-in infrared sensor (see Fig. A.4).

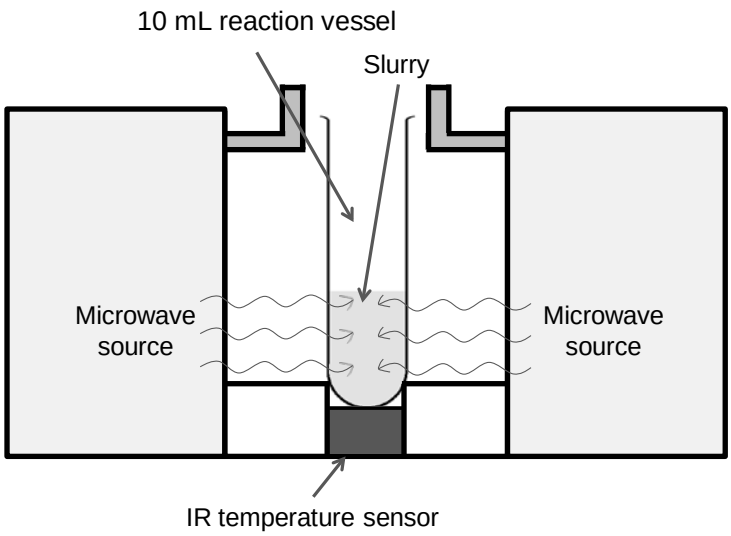

Fig. A.4 Setup for microwave heating of Sepharose CL-6B slurry in 10 mL reaction vessel.

A.3.2 Results and discussions

A.3.2.1 Microwave heating profiles in water

Temperature change (ΔT) profile against microwave power input (power x time/volume) in water is shown in Fig. A.5. Fixed power mode of microwave heating was applied to heat up 1-5 mL water in 10 mL reaction vessels. Data plots obtained by heating 3-5 mL water appeared to collapses on the same line, indicated that absorbance of microwave energy by water at these volumes remains consistent. However, when the volume of water decrease to 2 mL, data plots shifted slightly downward while those for 1 mL shifted down significantly further. The difference in heating profile at 1-2 mL water compared to at 3-5 mL may caused by an insufficient height of sample in the vessel to be fully irradiated by microwave radiation in reaction chamber. It was suspected that some microwave radiation in the CEM Discover chamber hits the reaction vessel at a height range above the maximum liquid height for the 1 mL and 2 mL samples (11 mm and 19 mm height respectively) and a loss of some microwave radiation was likely to occur (see Fig. A.6). As a result, the amount

of radiation absorbed by the 1-2 mL samples is less than that for 3-5 mL samples, which have liquid heights of 27-43 mm.

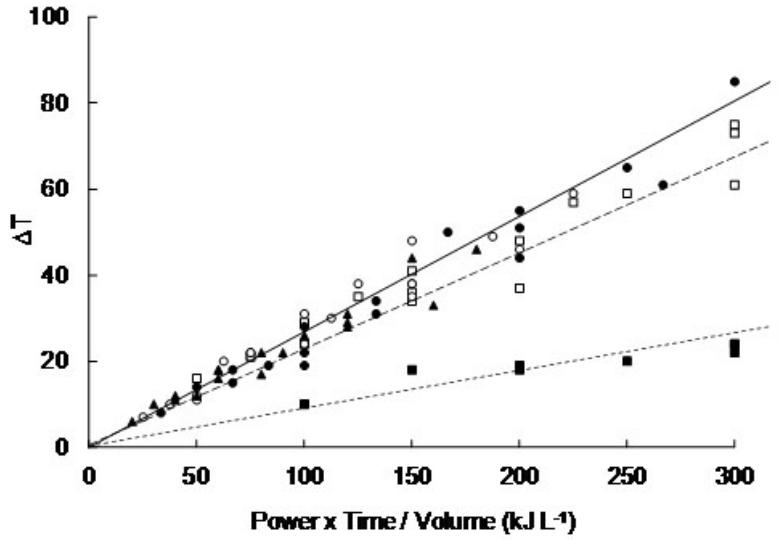

Fig. A.5 Effects of microwave energy on temperature change for microwave heating of 1-5 ml water in 10 mL reaction vessels using 'fixed power' mode. Symbols; ■ : 1 mL, □ : 2 mL, ● : 3 mL, ○ : 4 mL and ▲ : 5 mL.

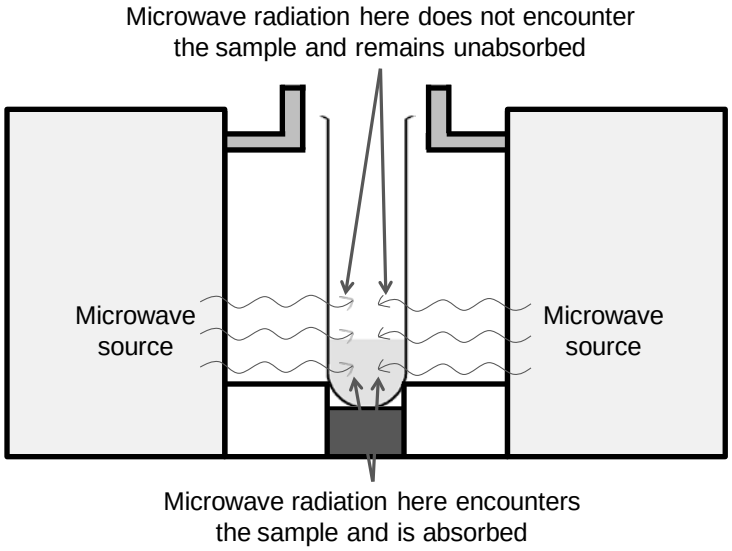

Fig. A.6 Schematic diagram showing microwave radiation on the small volume sample (1-5 mL in 10 mL reaction vessel) in irradiation chamber.

The similar heating profile was also observed when microwave heating was performed in a larger container (100 mL round bottom flasks) containing large volume of water (10-50 mL). Again, data plots obtained from heating of 30-50 mL water by microwave radiation overlapped each other to create a linear relationship. At lower volumes of 10 and 20 mL, heating profiles resulted in 2 distinguished data plots showing shallower slopes when less volume of water was applied (Fig A.7). This similar heating behaviour confirmed the previous findings when small amount of water was applied. The heights of water in this test were also measured. It was convincing that the lower heating performances for 10 and 20 mL water were caused by, again, the insufficient heights of the sample (13 and 19 mm, respectively) to be interacted with microwave radiation supplied in the chamber. The similar heating profiles obtained from 30-50 mL water indicated that microwave radiation fully interacted with liquid at this range of liquid height (24-31 mm). Effectiveness of microwave heating at small and large volume of water seemed to be similar, giving a slope of 0.25 and 0.2 for 3-5 mL water and 30-50 mL water, respectively.

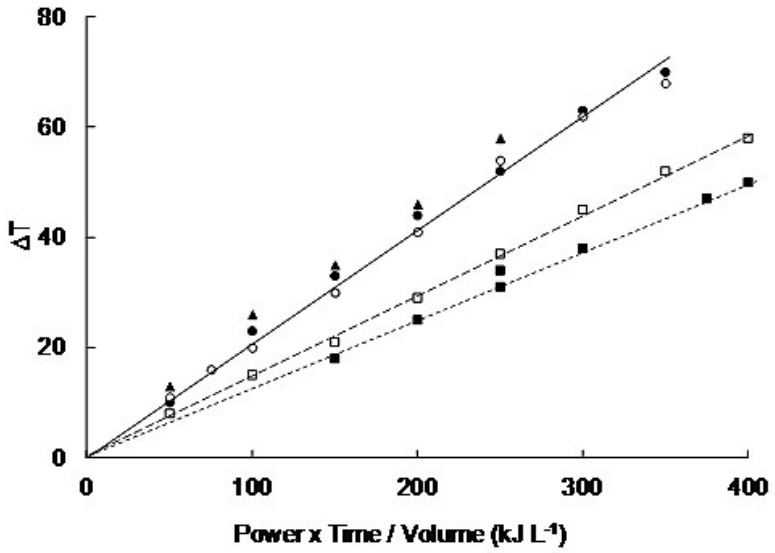

Fig. A.7 Effects of microwave energy on temperature change for microwave heating of 10-50 ml water in 100 mL round bottom flask using 'fixed power' mode. Symbols; ■ : 10 mL, □ : 20 mL, ● : 30 mL, ○ : 40 mL and ▲ : 50 mL.

A.3.2.2 Microwave heating profiles in water-support slurry

Increasing support content in the slurry led to increased microwave energy required to achieve a determined temperature rise (Fig. A.8). At 80% (v/v) Sepharose CL-6B content, approx. 50% increase in microwave energy was required to achieve the same temperature rise when only water was used. For example, 50^{o}C rise required 200 kJ /L for water and 300 kJ/L for an 80% (v/v) Sepharose CL-6B slurry. However, such extra energy requirement is considered to be relatively small due to the structure of Sepharose CL-6B which consists of approx. 94% water. The extra energy required is believed to be corresponded to the skeletal polymer of this support, cross- linked agarose.

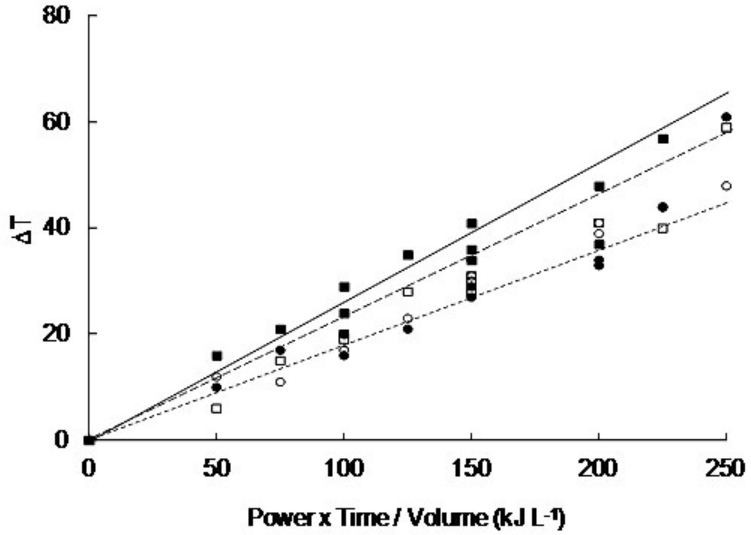

Fig. A.8 Effects of microwave energy on temperature change for microwave heating of 2 mL Sepharose CL-6B slurries in 10 mL reaction vessels with 'fixed power' mode. Symbols; ■ : 0% (v/v), □ : 50% (v/v), ● : 60% (v/v), and ○ : 80% (v/v).

A.4 Calibration curves

A.4.1 Bromine: for bromine assays

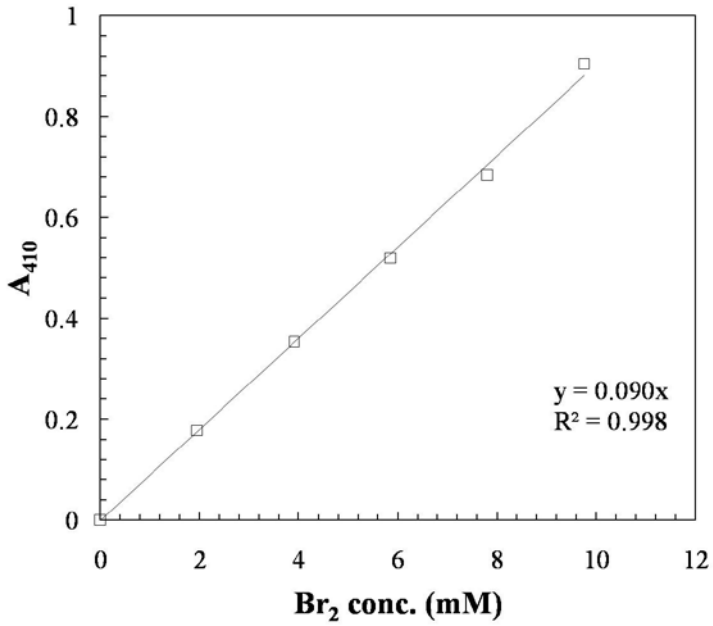

Fig. A.9 Bromine standard curve for bromine assays.

A.4.2 NaCl: for ionic capacity measurements

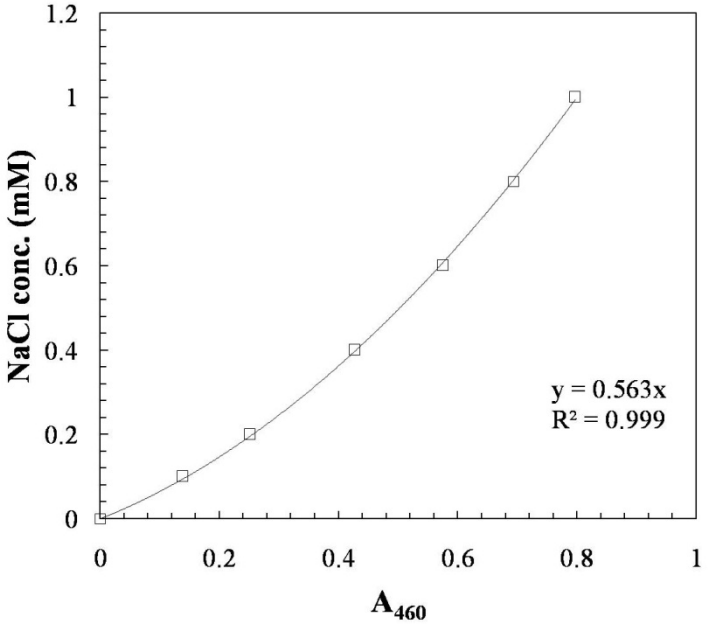

Fig. A.10 NaCl standard curve for ionic capacity measurements.

A.4.3 Calf thymus DNA: for DPA assays

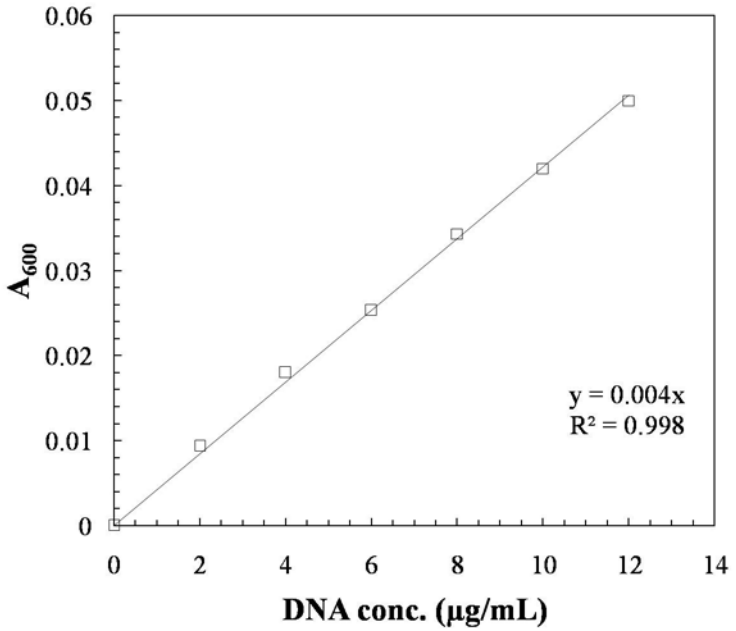

Fig. A.11 Calf thymus DNA standard curve for DPA assays.

A.4.4 BSA: for BCA assays in microcentrifuge tubes

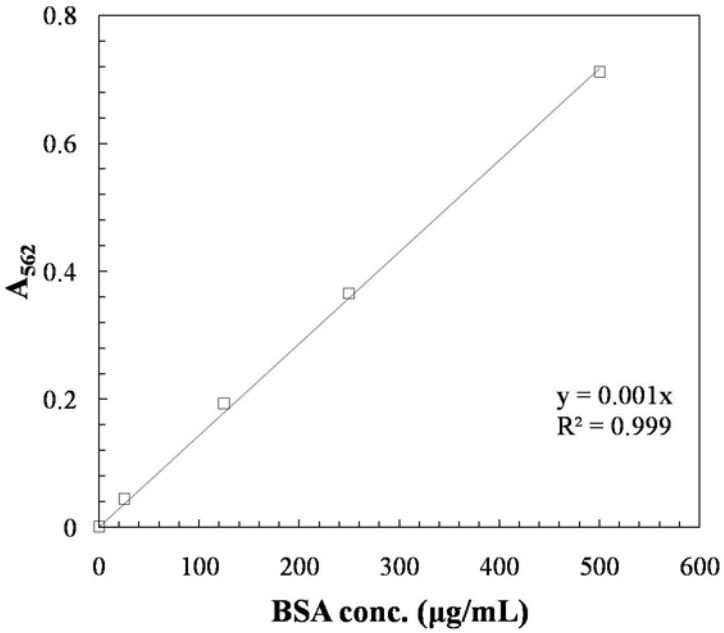

Fig. A.12 BSA standard curve for BCA assays in microcentrifuge tubes.

A.4.5 BSA: for BCA assays in microplates

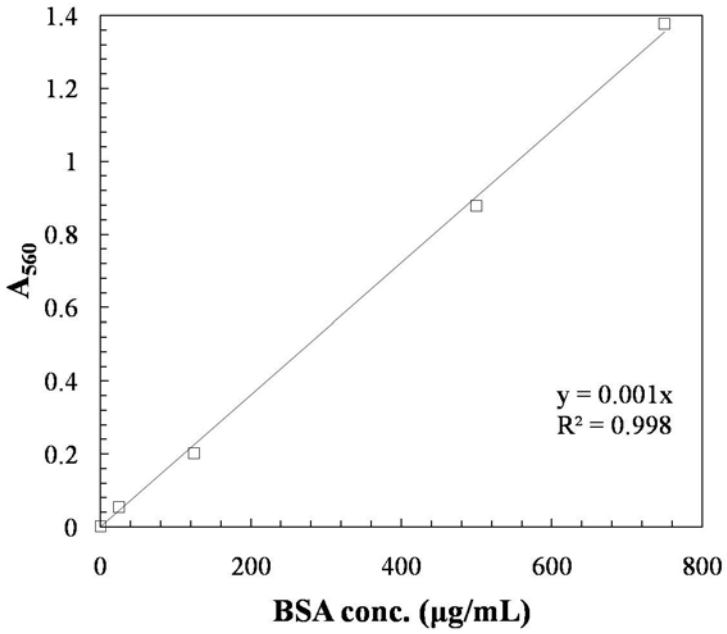

Fig. A.13 BSA standard curve for BCA assays in microplates.

A.4.6 RNA: for orcinol assays

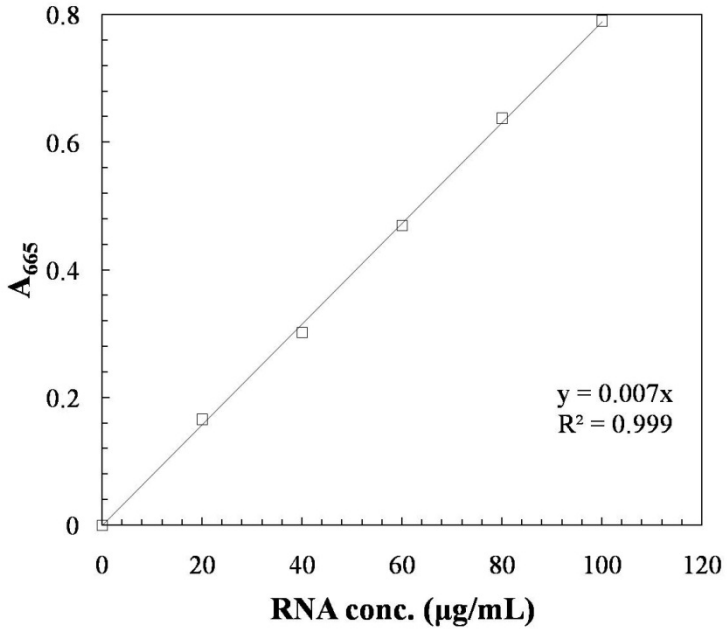

Fig. A.14 RNA standard curve for orcinol assays.

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