Separation and Purification Technology 93 (2012) 75–82

Contents lists available at SciVerse ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Grafting hydrophobic and affinity interaction ligands on membrane adsorbers: A close-up "view" by X-ray photoelectron spectroscopy

Luís Raiado-Pereira [a,b], Ana P. Carapeto [c], Ana M. Botelho do Rego [c], Marília Mateus [a,b,*]

[a] Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais 1, 1049-001 Lisboa, Portugal
[b] Department of Bioengineering (DBE), Instituto Superior Técnico, Technical University of Lisbon, Portugal
[c] Centro de Química-Física Molecular, Institute of Nanoscience and Nanotechnology, DEQ, IST, Technical University of Lisbon, Av. Rovisco Pais 1, 1049-001 Lisboa, Portugal

ARTICLE INFO

Article history:
Received 25 January 2012
Received in revised form 21 March 2012
Accepted 22 March 2012
Available online 29 March 2012

Keywords:
HIC
Membrane chromatography
pDNA chromatography
Phenylboronate affinity chromatography
XPS

ABSTRACT

The surface chemistry of chromatographic media plays a crucial role in their selectivity and binding capacities, enabling, or not, their use for the purification of therapeutic biomolecules. This work focuses on the XPS analysis of two different chromatographic membrane adsorbers to be integrated in the downstream processing of pDNA: (i) by hydrophobic interaction – alkyl and phenyl ligands, and (ii) by phenylboronate affinity interaction.

The analysis of the Sartobind® epoxy precursor membrane evidenced both the major C 1s components expected for a cellulose membrane, at 286.7 and 288.1 eV as well as its major O 1s components at 532.9 and 533.5 eV. Also present in the C 1s region a peak at 289.0 ± 0.2 eV attributed to an ester group used as reinforcement of the cellulose and, likely derived from a carbonyldiimidazole (CDI) pre-activation. Traces of nitrogen (N/C ~0.0035) helped corroborating the latter premise. The subsequent grafting to obtain a hydrophobic alkyl based membrane was followed by XPS. Global quantitative results attested the success of these reactions. Calculated atomic ratios for N/C and N/O bestowed information on the arrangement of the ligands during grafting and elucidaded the chromatographic behavior.

The phenyl membrane adsorber presented the typical aromatic peak at 284.7 eV. On par with the previous Sartobind® epoxy membrane, the peak at ~289.0 eV assigned to an ester group was present.

Functionalization with 3-aminophenylboronic acid used two strategies one in which it was directly linked to the surface and an analogous strategy using sorbitol as shielding agent to prevent reaction of the boronate acid of the ligand with the epoxy. Quantitative XPS analysis demonstrated the fragility of this ligand, suffering from boronate hydrolysis, and the inefficacy of the sorbitol as shielding agent; concerning chromatography, the sorbitol methodology demonstrated a slight improvement.

1. Introduction

Developments in gene therapy and nucleic acid vaccination have put plasmid DNA in the front line amongst the next generation of biopharmaceutical products, promising a therapeutic field for prevention and cure of diseases like HIV, cancer, malaria and influenza virus. Unlike other applications, for which there is the possibility of using crude cell extracts of varying degrees of purity, plasmid DNA (pDNA) used for therapeutic gene transfer must be highly purified, free of contaminating bacterial components, in agreement with specifications from regulatory agencies [1]. To achieve such standards chromatography has been the dominant process for the purification of pDNA. However, classic chromatographic media has been reported unsatisfactory to purify large pDNA molecules. In these cases, whichever the basis of the interaction, pDNA binding is relegated to the surface [2], due to the small pore sizes more suitable for protein purification [3,4]. Therefore, membrane adsorbers and monolith-technology have become valuable alternatives as chromatographic media. Their large pores and the absence of transport limitations improve the binding capacity of pDNA molecules and allow a significant throughput increase [5–8]. Plasmid capacities of 6 and 15 mg/mL have been reported for monoliths and membranes, respectively [3].

Hydrophobic interaction and phenylboronate affinity (PBA) chromatographic membranes were the main focus of this study. Hydrophobic interaction chromatography (HIC) has been widely used both as analytical and preparative technique for pDNA isolation [9]. The separation of pDNA from impurities in *Escherichia coli* alkaline lysates by HIC relies in the different hydrophobicities of pDNA isoforms, single-stranded nucleic acids (e.g. RNA and fragmented gDNA), and endotoxin impurities [10–15]. In solutions bearing high concentrations of a kosmotropic salt (e.g. ammonium

* Corresponding author at: Institute for Biotechnology and Bioengineering (IBB), Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais 1, 1049-001 Lisboa, Portugal. Tel.: +351 218419136; fax: +351 218419062.
E-mail address: marilia.mateus@ist.utl.pt (M. Mateus).

1383-5866/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.seppur.2012.03.028

sulfate), pDNA molecules have the hydrophobic bases packed and shielded inside the double helix, thus, hydrophobic interactions with the HIC media are minimal. Conversely, single stranded nucleic acids impurities have a higher exposure of the hydrophobic bases and therefore interact strongly with the hydrophobic ligands [14]. Highly hydrophobic endotoxins are further retained in an HIC column when compared with pDNA [9,14]. Inherently, bead based HIC has lower capacity than ion exchange chromatography and requires larger columns and quantities of buffers, as well as being inconvenient for handling and disposal. Membrane adsorbers easily overcome these problems as they can be stacked to achieve the desired capacity, posing little problem with pressure drops and dispersion of solutes.

Affinity ligands bind specifically and reversibly to target molecules by intermolecular forces (e.g. hydrogen bonds, van der Waals forces). Phenylboronate ligands (PBL) undergo affinity interactions with cis-diol groups, such as the ones present in oligos and polyribonucleotides (e.g. ribonucleosides, ribonucleotides and ribonucleic acids), through a pH dependent reversible esterification mechanism [16–18]. In ribonucleic acids the cis-diol group is located at the 2′ and 3′ position of the ribose, and form tetrahedral complexes in aqueous environment with the boronate acid moieties [17]. Interaction will not occur if the polynucleotides or nucleotides are either 2′ or 3′ phosphorylated due to blockage of one of the hydroxyl groups. Typically, the optimal pH for esterification lies above the pK_a of the boronate acid. Binding of most diols such as carbohydrates are best performed at pH 9.0. However, it is not always certain that the boronate esterification will take place in these conditions for it also depends on the diol species involved. Phenylboronate ligands are also able to establish hydrophobic and aromatic π–π interactions, secondary ionic interactions (between boronates and diols, through coulombic attraction or repulsion effects), hydrogen bonding via the hydroxyl groups and coordination interactions. All these interactions are important as they modulate the strength of bonding [16,19]. Recently, 3-aminophenylboronate (APBA) in controlled pore glass (CPG) beads have been reported to adsorb RNA impurity species that bear the cis-diol groups directly from plasmid-containing lysates at pH 5.2 [19], far below the pK_a of the APBA.

The specific interactions established at the surface of chromatographic media within its close microenvironment depend fundamentally on the physical–chemical characteristics of the surface. Functionalization by heterogeneous grafting of ligands can modulate the interactions of solutes with the surface of the media. However, the way these ligands arrange themselves upon grafting is not often totally understood. X-ray photoelectron spectroscopy (XPS) is a technique of relevant importance for the study of surfaces, as it allows the chemical characterization of its first atomic layers [20]. The XPS study described herein aimed at characterizing the surface chemistry of HIC and PBA chromatographic membranes, and at establishing a comparison with their chromatographic performance. While providing knowledge on the heterogeneous functionalization methodologies, XPS also conveyed insight on the mechanisms that govern the chromatography.

2. Materials and methods

2.1. Membrane modification

The membrane modification was carried on a precursor membrane, the epoxy activated Sartobind®Epoxy 75 affinity membranes (nominal pore size of 0.45 μm) [21]. The functional ligands were grafted onto the precursor epoxy membrane in two different ways, (i) to obtain a hydrophobic alkyl ligand, and (ii) to obtain a phenyl-boronate affinity membrane. To achieve both modifications the membranes were cut into 25 mm diameter discs from a Din A4 sheet from Sartorius Stedim Biotech GmbH. Membrane discs were handled carefully with the contact limited to the edges of the discs to avoid damaging the glossy surface of the membrane.

A pre-commercial prototype of a phenyl membrane adsorber kindly provided by Sartorius Stedim Biotech GbmH, was also included in the set of studied membranes.

2.1.1. Hydrophobic alkyl membranes

The alkyl membrane modification was carried out in a three step process: amination, re-epoxidation and blocking. The starting epoxy membrane (Sample 0 in Fig. 1) was reacted with 1,6-diaminehexane resulting in an aminated membrane (Sample 1 in Fig. 1) [22,23]. Free amines were then reacted with 1,4-butanediol diglycidyl ether (BUDGE) (Sample 2 in Fig. 1). The remaining free epoxy groups were finally blocked by further reacting the membrane with ethanolamine (Sample 3 in Fig. 1) [22,23]. The membranes were washed with water between each reaction step and analyzed immediately after drying.

2.1.2. Phenylboronate affinity membranes

The phenylboronate membrane modification was carried out via amino chemistry using 3-aminophenylboronic acid (APBA) in two different approaches (Fig. 2): The first approach (Fig. 2a) used the method described in [16]. The second approach (Fig. 2b) was based on the methodology described in the first method, but using 100 mM sorbitol that bound to the boronate moiety of the molecule preventing it from bind through this site.

2.2. XPS analysis

XPS analysis was performed with a XSAM800 (KRATOS) spectrophotometer operating in the fixed analyser transmission (FAT) mode, with a pass energy of 20 eV and a non-monochromatised Al Kα X-radiation (1486.7 eV). Except when referred otherwise the take off angle of analysis was 90°. The freeware program XPS peak version 4.1 was used for peak fitting. Data acquisition and treatment details were described elsewhere [24]. The charge shift (no flood gun was used) was corrected using the binding energy of sp^3 carbon bound to carbon and/or hydrogen at 285 eV as reference. X-ray source satellites were subtracted. For quantification purposes, sensitivity factors were 0.66 for O 1s, 0.25 for C 1s, 0.42 for N 1s, and 0.13 for B 1s [24].

2.3. Bacterial culture

Pre-innocula of *E. coli* DH5α harboring the 6050 bp commercial plasmid pVAX1-LacZ (Invitrogen, Carlsbad, CA) were grown overnight in 100 mL shake flasks containing 30 mL of LB medium with 30 μg/mL kanamycin at 37 °C and 250 rpm up to an optical density at 600 nm (OD_{600nm}) of 3.5. Larger culture volumes (250 mL) were inoculated with the appropriate amount of overnight culture to an OD_{600nm} of 0.2 and incubated under the same conditions for 8 h to harvest the cells in late exponential growth phase. *E. coli* DH5α without plasmid was also grown under the same conditions, but with no kanamycin present. Cells were harvested in a refrigerated centrifuge at 3500*g* for 15 min (Sorval Inc., Osterode, Germany).

Samples containing plasmid DNA with different degrees of purity were prepared by carrying out the following purification process: an alkaline lysis method [25] was used to recover intracellular components; cellular debris were centrifuged and discarded, then the plasmid (along with other impurities) in the supernatant was precipitated by adding 0.7 volumes of isopropanol and left in a static incubation for 30 min at 4 °C; a centrifugation step at 10,000*g* then follows for 30 min to separate the plasmid; the