Biochemical Engineering Journal 93 (2015) 1–10

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

Preparation of liposome membrane adsorbers and testing for plasmid purification

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ARTICLE INFO

Article history:
Received 7 April 2014
Received in revised form 3 September 2014
Accepted 7 September 2014
Available online 16 September 2014

Keywords:
DNA vaccine
HIC
Liposomes
Membrane adsorbers
Membrane chromatography
Plasmid DNA purification

ABSTRACT

DNA therapeutics can prevent or overcome several diseases and use plasmid DNA (pDNA) to deliver specific genes to the hosts for the therapies. The demand for highly purified pDNA is increased. Hydrophobic interaction chromatography (HIC) using membrane adsorbers is a purification technique able to separate lysate contaminants from pDNA, with improved productivity when compared to the use of classic adsorbers but still relying on hydrophobic interactions between patches on the surface of biomolecules and those designed at the chromatographic matrix.

A final step in the downstream processing of pDNA was performed by HIC on membrane adsorbers functionalized with unilamellar liposomes of DOPE, DOPC, DLPA and DSPA. The chemical and morphological fibre structure characterizations of the derived membrane adsorbers are disclosed.

Confocal microscope images demonstrated the ability of these membranes to interact with pDNA under high kosmotropic salt concentration environment. The study of chromatographic performance of the adsorbers on pDNA purification showed that DLPA-membrane has acquired the lowest ligand density but a considerable membrane adsorbing capacity; conversely, the DOPC-membrane has highest binding capacity and high HIC performance parameters. Purification yields obtained are still lower than those found for other chromatographic membrane adsorbers, consistent with the promotion of unspecific interactions hindering pDNA purification. This work gives a new insight onto liposome-based membrane adsorbers application for the pDNA downstream purification process.

1. Introduction

Gene therapy and DNA vaccination have been broadly studied in the past two decades with the purpose of treating and preventing acquired diseases and genetic disorders such as infections, cancer and cystic fibrosis. The use of viral and nonviral vectors as a new generation of biotechnology products in the marketplace has reached later phases in clinical trials. These therapies depend on the introduction of specific nucleic acids in the cells of patients to restore, cancel, enhance or introduce a biochemical function [1]. The transport of therapeutic genes to the nucleus of target cells by nonviral vectors, such as naked or encapsulated plasmid DNA (pDNA), constitutes safer gene delivery alternative to viral vectors due to its lower toxicity and larger gene capacity [2]. For a DNA therapeutic to be successfully developed several scientific and technological challenges must be overcome at process scale for production and purification to be able to meet the product specifications and standards imposed by regulatory agencies like FDA [3,4]. Optimal host *Escherichia coli* (*E. coli*) strains allow the production of large amounts of therapeutical DNA at the industrial scale [5,6]. This operation encompasses fermentation, primary isolation and purification steps. At first, the host cells are cultivated in bioreactors and, within the process, the plasmid undergoes autonomous replication [7]. Then, a primary isolation is carried out by cell harvesting and a subsequent alkaline lysis [8,9], followed by precipitation and concentration of pDNA in the lysates as well as other differently concentrated host impurities like proteins, RNA, endotoxins and genomic DNA (gDNA) fragments which share, to some point, similar physicochemical characteristics with pDNA, i.e., negative charge (RNA, gDNA and endotoxins), molecular mass (gDNA and endotoxins) and hydrophobicity (endotoxins) [10,11]. To surpass the resemblances between pDNA and impurities, high resolution chromatographic supports with high

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http://dx.doi.org/10.1016/j.bej.2014.09.005
1369-703X/

dynamic binding capacity are required [12]. Due to their specific design for protein separations, classic chromatographic supports can only adsorb plasmids in the outer surface of beads, requiring larger columns which evidence low performance when separating these large polynucleotides. Convective flow devices, such as monolithic columns and membrane adsorbers, are therefore more attractive for plasmid purification from clarified lysates. The use of liposome carrying monolithic separation media enhances mass transfer and has great potential for chromatographic separation of nucleic acids [13]. Membrane chromatography has also been developed to purify large molecules as they enable better separation efficiency by increasing solute selectivity and productivity with minimum or negligible diffusive mass transfer limitations to the surface [14–16]. Furthermore, membrane adsorbers can be tailored to carry functional ligands which create a hydrophobic environment and promote hydrophobic interactions between pDNA (or its contaminants) and the matrix aiming at pDNA purification [9,16]. Recently, the use of liposomes immobilized in the surface of chromatographic matrices (ILC—immobilized liposome chromatography) has been under study to evaluate the interactions between lipid membranes and drugs. Since these structured phospholipid molecules have relatively small molecular mass and thus show little conformational change under stress conditions, are also able to self-aggregate, and, with a proper environment, can hold charged groups (i.e. phosphate or amine groups) and hydrogen-bond relating groups (i.e. carbonyl group) [17,18] able to interact with biological molecules.

Several phospholipids have particular features suitable to interact with nucleic acids. The unillamelar liposome 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) is a predominant lipid species in real biomembranes that preferentially assemble into lamellar phases. Other phospholipid, the 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) belongs instead to the category of lipids characterized by the propensity to form nonlamellar inverse phases. Real biological membranes are normally made of mixtures of lamellar and nonlamellar lipids and such mixed systems usually exist as lamellar phases (like DOPC). DOPE-based liposomes are frequently used for delivery of DNA into mammalian cells, the DOPE molecule itself being reported as enhancer of the release of the nucleic acid:liposome complex trapped within endosomes by a process of membrane destabilization within the vesicle.

DOPC is frequently arranged into phospholipid bilayers for efficient *in vivo* delivery of short interfering RNA (siRNA).

Phosphatidic acids (1,2-dilauroyl-*sn*-glycero-3-phosphate and 1,2-dioctadecanoyl-*sn*-glycero-3-phosphate—DLPA and DSPA, respectively) are also important constituents of cell membranes, playing important roles as precursors in the biosynthesis of other lipids, facilitating vesicle fission/fusion via its biophysical properties, and acting as signalling lipids.

Taking into consideration that structured forms of these phospholipids are known to interact with DNA and RNA, the interaction of these hydrophobic drugs with such membranes by liposome chromatography is a worth study to investigate how liposome immobilized membrane adsorbers would behave on the purification of plasmid DNA from its natural impurities by hydrophobic interaction chromatography.

In this work, we made use of a Sartorious Sartobind® aldehyde-activated membrane support that was derivatized with unilamellar liposomes to obtain lipidic ligands at the membrane surfaces and promote hydrophobic interactions between adsorber matrix and pDNA contaminants by a membrane chromatography technique for potential final pDNA purification step. Four liposome-forming molecules were chosen and for each newly developed membrane adsorber, a range of hydrophobic chromatography operating conditions was applied in the search for an optimized chromatographic method.

2. Materials and methods

2.1. Cell culture, alkaline lysis and primary purification

2.1.1. Cell culture and harvesting

Seed banks were prepared from agar plates with isolated colonies of *E. coli* DH5α transformed with the 6050 bp commercial plasmid pVAX1-LacZ (Invitrogen, Carlsbad, USA): a single colony was picked from a Petri dish pre-culture and inoculated in Luria–Bertani (LB) medium supplemented with 30 μg kanamycin/mL; cells were cultured to mid-exponential phase at 250 rpm, 37 °C, and frozen at −80 °C in sterile cryovials to which sterile glycerol was added to a final concentration of 15% (v/v). *E. coli* DH5α transformed cells from a cryovial were seeded in 100 mL Erlenmeyer shake flasks containing 30 mL of LB medium supplemented with 30 μg kanamycin/mL and incubated overnight at 37 °C and 250 rpm, up to an OD_{600} of 2 (pre-inocula). These were then used to inoculate 250 mL LB medium supplemented with 30 μg kanamycin/mL, also at 37 °C and 250 rpm, in 2000 mL Erlenmeyer flasks. The latter cultures were grown for 7–9 h until reaching the early stationary growth phase. Cells were then harvested by centrifugation in a refrigerated centrifuge at 3500 × *g* and 4 °C, for 15 min (Sorval, Osterode, Germany), and supernatants discharged [9].

Afterwards, bench-scale bioreactors of 1 L working capacity were used to inoculate the bacterial culture with an initial OD_{600} of approximately 0.1 in a complex medium [basal medium (Bacto peptone, 10 g/L; yeast extract, 10 g/L; $(NH_4)_2SO_4$, 3 g/L; K_2HPO_4, 3.5 g/L; KH_2PO_4, 3.5 g/L) 8.3 mL defrosted seed suplement solution (thiamine-HCl, 24 g/L; $MgSO_4{\cdot}7H_2O$, 240 g/L) per 1 L medium; 1.0 mL trace element solution [19] per 1 L medium] supplemented with 30 μg kanamycin/mL and glucose, 10 g/L, used as the primary carbon source. The bacterial culture progressed batch-wise under controlled temperature (set at 37 °C), pH value (set at 7.1) and combined stirring and aeration conditions (to maintain dissolved oxygen level at 30%). After 18 h of bacterial growth, cells were harvested in late exponential growth phase by centrifugation as described above [19].

2.1.2. Alkaline lysis and primary pDNA purification

The pellet resultant from cell harvesting was submitted to an alkaline lysis method [8]. Afterwards, cell debris, precipitated gDNA and the majority of proteins were removed by centrifugation. Isopropanol (0.7 volumes) was added to the resulting volume of pDNA-containing lysate supernatant and the mixture was left in static incubation for at least 2 h at 4 °C to precipitate all nucleic acids (pDNA, RNA and traces of gDNA). A new centrifugation step followed, at 10,000 × *g* and 4 °C, for 30 min (Sorval, Osterode, Germany), and the supernatant was discarded. The pellet was then washed with 70% (v/v) ethanol, centrifuged, redissolved in 10 mM Tris–HCl and later mixed with ammonium sulphate to the desired final concentration (Section 2.4.2). The mixture was again centrifuged for final clarification at 18,000 × *g* and 4 °C for 30 min (Eppendorf AG, Hamburg) and stored at −20 °C until further processing [9].

2.2. Membrane derivatization procedure

2.2.1. Membrane preparation and activation

Commercial Sartobind® Aldehyde membranes (nominal pore size of 0.45 μm) were carefully handled, cut into 25 mm diameter discs from a Din A4 sheet (Sartorius Stedim Biotech GmbH), washed three times with MilliQ water, at a water-to-membrane area ratio of 0.5 mL/cm^2, to get rid of protective and moistening chemicals and later dehydrated at 55 °C for 30 min (Memmert UFB500) until wrinkled. Then, aldehyde pre-activated membrane discs were incubated