Journal of Chromatography A, 1218 (2011) 5273–5278

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Monolith-based immobilized metal affinity chromatography increases production efficiency for plasmid DNA purification

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ARTICLE INFO

Article history:
Received 18 February 2011
Received in revised form 3 June 2011
Accepted 8 June 2011
Available online 17 June 2011

Keywords:
Immobilized metal affinity chromatography (IMAC)
Monolith column
Particle-based column
Plasmid DNA purification
RNA dynamic binding capacity

ABSTRACT

Immobilized metal affinity monolith column as a new class of chromatographic support is shown to be superior to conventional particle-based column as plasmid DNA (pDNA) purification platform. By harnessing the affinity of endotoxin to copper ions in the solution, a majority of endotoxin (90%) was removed from the alkaline cell lysate using $CuCl_2$-induced precipitation. RNA and remaining endotoxin were subsequently removed to below detection limit with minimal loss of pDNA using either monolith or particle-based column. Monolith column has the additional advantage of feed concentration and flowrate-independent dynamic binding capacity for RNA molecules, enabling purification process to be conducted at high feed RNA concentration and flowrate. The use of monolith column gives three fold increased productivity of pDNA as compared to particle-based column, providing a more rapid and economical platform for pDNA purification.

1. Introduction

Plasmid DNA (pDNA) vaccine consists of circular and double stranded extra chromosomal DNA encoding specific gene of one or more protein antigens [1]. In the host cells, the production of protein antigens induces humoral (antibody) and cellular (T cell) immune responses [2]. Plasmid DNA vaccines, compared to the conventional vaccines, have potential advantages such as eliminating the risk of viral infections via vaccination process and life-long immunity against several diseases in a single dose [3]. The quality of pDNA for therapeutics has to meet regulatory specifications in terms of purity, homogeneity and potency (>95% supercoiled (sc) pDNA, undetectable RNA on agarose gel electrophoresis and <0.1 EU/μg plasmid by LAL assay for endotoxin) [4].

pDNA purification using affinity precipitation (with compacting agents such as spermine [5] and spermidine [5,6] or high salt concentration such as 2.5 M ammonium sulfate [7]) and various chromatography procedures (e.g. size, charge, hydrophobicity, affinity-related chromatography) [8] have been reported. The scaling up to an industrial process scale for conventional particle-based column chromatography, however, has some disadvantages including high pressure drop, low flowrate and channeling flow [9]. Besides for macromolecules such as pDNA and RNA, the access to intraparticle pores of particle-based column is restricted by the large size of macromolecules, hence resulting in low binding capacity and throughput as the interaction between the target molecules and immobilized metal ions occurs mainly on the external particle surface of the chromatographic support [10].

Recently, monolith-based chromatography suitable for separation and purification of macromolecules is developed [11–13]. In a monolith column, due to high porosity, the pressure drop is low and stationary phase structure is intact even at a high flowrate [14]. Moreover, the separation and purification process based on convective flow can result in flow-independent resolution and dynamic binding capacity even for macromolecules [15,16]. Using $CaCl_2$ precipitation and a combination of monolith-based anion exchange and hydrophobic interaction chromatographic procedures, separation of scpDNA from impurities such as open circular (oc) pDNA, genomic DNA (gDNA), RNA and endotoxin was achieved [12]. The purification of pDNA harboring *lacO* nucleotide sequences from pDNA lacking *lacO* was also enabled in a single unit operation using LacI based peptide-monolith construct [13].

In our previous study, $CuCl_2$-induced precipitation followed by Cu^{2+}-iminodiacetic acid (IDA) based immobilized metal affinity chromatography (IMAC) were conducted to purify pDNA from its contaminants in the batch binding mode [17]. In brief, pDNA, RNA and endotoxin were precipitated by free Cu^{2+} ions from alkaline cell lysate while the majority of other impurities remain in the supernatant. After pDNA and RNA were transferred into bulk solution by adding EDTA to the precipitates, RNA could be selectively

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doi:10.1016/j.chroma.2011.06.040

captured by Cu^{2+} ions immobilized on the IDA particles, enriching pDNA in the unbound fraction. It was shown that RNA and endotoxin were completely removed from alkaline cell lysate without significant loss of pDNA. The interaction of nucleic acids with immobilized Cu^{2+} ions is based on affinity between exposed aromatic nitrogen bases in the nucleic acids and the metal ions [18]. Hence, pDNA where aromatic nitrogen bases were hidden within the double stranded helical structure did not show affinity to the immobilized metal ions while single stranded RNA with exposed aromatic nitrogen bases exhibited interaction with the metal ions.

However, the purification of pDNA in the chromatographic mode would be indispensable for efficient large scale operation. Since RNA is the major contaminant in the alkaline cell lysate following the $CuCl_2$-induced clearance of the majority of endotoxin, this study first investigated the dynamic binding capacity of RNA molecules to metal affinity monolith or particle-based column using feedstock solutions with or without the co-presence of pDNA at different feed concentrations and flowrates and assessed the RNA removal efficiencies and pDNA productivities in pDNA purification process at various chromatographic separation conditions.

2. Experimental

2.1. Materials

The particle-based and monolith IMAC columns used were HiTrap Chelating HP column (GE Healthcare, 17-0408-01) and CIM IDA disk monolithic column (BIA separation, 217.3010), respectively. Reagents used were tris (Applichem, A2264.1000), sodium chloride (Duksan, SEE0-32201), agarose (Promega, DV3125), ethidium bromide solution (Fluka, 46067), 1 kb plus 100 bpDNA ladder (Mbiotech, 10103), low MW DNA ladder (New England Biolabs, N3233S) and gel loading dye blue 6× (Biolabs, B7021). Baker's yeast RNA (R6750), endotoxin removal solution (E4274), cupric chloride dihydrate (C3279), imidazole (I2399) and EDTA disodium dihydrate (E5134) were from Sigma. SnakeSkin Dialysis Tubing with a nominal MWCO of 3.5 kDa (68035) was supplied by Perbio Science, and Endofree DNA Maxi purification kit (12362) by Qiagen.

2.2. Fermentation and alkaline cell lysis

DH5-α mutant harboring plasmid pcDNA3.1D, a 7.3 kb high copy plasmid (100–200/cell) which contains 1.8 kb dengue fever antigenic gene (NS3), was kindly provided by Bioprocessing Technology Institute (Singapore). Cells were grown on 12 g/l yeast extract (Conda Pronadisa, 1702.00), 6 g/l tryptone (Conda Pronadisa, 1612.00), 5 g/l glucose (Sigma, G8270), 6 g/l K_2HPO_4 (Aldrich, 221317) and 0.48 g/l $MgSO_4$ (Sigma–Aldrich, M7506) using 250 ml shake flasks in a shaking incubator (50 ml culture volume, 16 h, 37 °C, 250 rpm). Cell lysate was obtained using alkaline cell lysis procedures as described previously [19].

2.3. $CuCl_2$-induced precipitation prior Cu^{2+}-IDA based IMAC

To 30 ml of alkaline cell lysate, powdered $CuCl_2$ was added to a final concentration of 0.75 M, incubated on the mixer at 30 rpm for 15 min and centrifuged (19,000 × *g* for 1 min). After washing the precipitate with 30 ml (2 × 15 ml) of Tris buffer (20 mM Tris, pH 7), 30 ml of 50 mM EDTA in Tris buffer was added to the precipitate. 20 ml of the resulting supernatant was dialyzed against Tris–NaCl buffer (20 mM Tris, 0.75 M NaCl, pH 7) using SnakeSkin Dialysis Tubing (12–16 h) and concentrated using Amicon Ultra-15 Centrifugal Filter Units with a nominal MWCO of 3 kDa (Millipore, UFC900324). The dialyzed solution was filtered with 0.2 μm Minisart NML from Sartorius (16534-K) prior to CIM IDA disk monolith or HiTrap Chelating HP column loading.

2.4. Dynamic binding capacities for RNA molecules in various feedstock solutions using monolith and particle-based columns

Both CIM IDA disk monolithic column (comprising 3 disks of 0.34 ml volume per disk) and HiTrap Chelating HP column (1 ml) were washed with water (2 × 5 column volume (CV)), charged with 100 mM $CuCl_2$ (5 CV), washed with water (2 × 5 CV), and equilibrated with Tris–NaCl buffer (2 × 10 CV). Baker's yeast RNA was stripped of endotoxin using endotoxin removal solution. The purified RNA devoid of endotoxin (<0.05 EU/ml) was dissolved in Tris–NaCl buffer to give RNA concentrations in the range of 0.05, 0.1, 0.5, 1.0 and 2.0 mg/ml. RNA solutions were processed at flowrate of 0.5, 1, 2, 3, 6 and 9 ml/min for CIM IDA disk monolith column, and 0.5, 1, 2 and 3 ml/min for HiTrap Chelating HP column using a FPLC system (Bio-Rad, 760-2266) equipped with UV detector at 260 nm for continuous monitoring of RNA content in the flow-through.

The above procedures were repeated for cocktail solution (containing pDNA from Endofree DNA purification kit (<0.05 EU/ml) and purified RNA) and partially purified alkaline cell lysate with 222 EU/mL endotoxin (using $CuCl_2$-induced precipitation followed by EDTA resolubilization and dialysis against Tris–NaCl buffer) at 1 ml/min feed flowrate. In both cases, pDNA at concentrations of 0.016 and 0.002 mg/ml co-existed with RNA at concentrations of 1.0 and 0.1 mg/ml, respectively.

RNA molecules bound to the columns for various feedstock solutions were eluted using 500 mM imidazole. The amount of eluted RNA was quantified by agarose gel densitometry method as described in Section 2.5.1, and termed as dynamic binding capacity of RNA.

All experiments were performed in duplicates and the results averaged.

2.5. Analytical methods

2.5.1. Plasmid DNA (pDNA) and RNA analysis

Standard pure pDNA or RNA was quantified at 260 nm by UV/Vis spectrophotometer (WPA Biowave-2, Biochrom, 80-3003-75). 1 or 2% agarose gel electrophoresis was conducted in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8) using Mupid 2-plus (Advance, AD110) at 100 V for 25 min. Denatured loading samples were prepared prior to mixing with 6× loading dye as follows: 20 μl analyte, 150 μl formamide (Junsei, 377F0440, 98.5, v/v%)and 30 μl water were mixed, heated for 5 min at 95 °C, and immediately chilled on ice for 5 min in order to eliminate the effects of secondary RNA structure and base pairing on RNA electrophoretic mobility. The gel (containing 0.01% ethidium bromide) was then imaged by GelDoc-It Imaging System (UVP, 97-0139-04) and pDNA or RNA quantified with purified pDNA or Baker's yeast RNA as standard, respectively, using VisionWorksLS software equipped with the imaging system.

2.5.2. Endotoxin analysis

Endotoxin detection reagents used were Pyrochrome Chromogenic Endotoxin Testing (C1500), Control Standard Endotoxin (EC010) and LAL Reagent Water (WP1001) from Cape Cod Incorporated. The samples for endotoxin analysis were prepared in sterile polystyrene culture tubes (BD Falcon, 352052). Each sample was mixed with pyrochrome reagent and incubated at 37 °C for 30 min prior to UV absorbance reading at 405 nm using a microplate reader (Molecular Devices, EMax). Endotoxin concentration was calculated from endotoxin standard curve using SoftMax Program 4.6 according to manufacturer's instructions.