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Adsorption behavior of large plasmids on the anion-exchange methacrylate monolithic columns

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ABSTRACT

The objective of this study was to investigate the behavior of large plasmids on the monolithic columns under binding and nonbinding conditions. The pressure drop measurements under nonbinding conditions demonstrated that the flow velocities under which plasmid passing monolith became hindered by the monolithic pore structure depended on the plasmid size as well as on the average monolith pore size; however, they were all very high exceeding the values encountered when applying CIM monolithic columns at their maximal flow rate. The impact of the ligand density and the salt concentration in loading buffer on binding capacity of the monolith for different sized plasmids was examined. For all plasmids the increase of dynamic binding capacity with the increase of salt concentration in the loading solution was observed reaching maximum of 7.1 mg/mL at 0.4 M NaCl for 21 kbp, 12.0 mg/mL at 0.4 M NaCl for 39.4 kbp and 8.4 mg/mL at 0.5 M NaCl for 62.1 kbp. Analysis of the pressure drop data measured on the monolithic column during plasmid loading revealed different patterns of plasmid binding to the surface, showing "car-parking problem" phenomena under certain conditions. In addition, layer thickness of adsorbed plasmid was estimated and at maximal dynamic binding capacity it matched calculated plasmid radius of gyration. Finally, it was found that the adsorbed plasmid layer acts similarly as the grafted layer responding to changes in solution's ionic strength as well as mobile phase flow rate and that the density of plasmid layer depends on the plasmid size and also loading conditions.

1. Introduction

The use of plasmid DNA (pDNA) as a vector for gene therapy and particularly for DNA vaccination has been extensively investigated during the last decade. Despite poor immunogenicity that it is often stated as a main disadvantage, pDNA vectors have inherent advantages over viral vectors, including safety and production simplicity [1]. Therefore, the percentage of naked/plasmid DNA vectors among all the vectors used in gene therapy clinical trials grew from 11% in year 2002 to 17.7% in 2009 [2]. In 2007 the U.S. Department of Agriculture issued a conditional license for a plasmid DNA vaccine to treat canine melanoma [3,4]. In the same year first phase 3 clinical trial of pDNA based product for human patients with angiogenesis was successfully finished and a new drug application for treatment of critical limb ischemia is now under review by Japanese Ministry of Health, Labor and Welfare [5]. Additionally, investigations of DNA vaccines generated very encouraging results in treatments against malaria and AIDS [6–8] and because of relatively fast production process in comparison to conventional virus-based vaccines, DNA vaccines are also being explored for their utility in influenza vaccination [9,10].

At present, most clinical trials involve plasmids from 5 to 20 kbp in size. However, future requirements for multigene vectors including extensive control regions may require the production of larger plasmids or even mega-sized artificial chromosomes [11]. The target form, most stable for the therapeutic use, is supercoiled (*sc*) pDNA, which is, however, still very sensitive and can be degraded during the purification process [12]. The degree of degradation due to shear forces increases severely with the plasmid size [11,13].

Despite these problems, the rapid evolution of gene therapy and DNA vaccines results in an increasing interest in producing large quantities of pharmaceutical grade pDNA [14–16]. Downstream processing of plasmid DNA usually comprises of several chromatographic steps using different types of interactions [17]. Most of the chromatographic resins used for biotechnological processes are optimized for purification of much smaller proteins, consequently their capacity for bigger molecules such as plasmid DNA is rather low [17,18]. However, chromatographic matrixes suitable for purification of large shear-sensitive particles should not only exhibit high capacity for large molecules but also be highly permeable not to damage the molecules. In addition, their active surface

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should be available by convective transport in order to avoid slow diffusion of large molecules resulting in long process times that can also lead to degradation of target molecules. Because of that only few chromatographic matrixes are currently effective for purification of large biomolecules, such as superporous supports [19,20], monoliths [21–24] and adsorptive membranes [25]. Convective interaction media (CIM) anion-exchange methacrylate monolithic columns have already been successfully used in the industrial scale purification process of pharmaceutical grade small plasmid DNA [12]. Additionally, developing new hydrophobic interaction CIM methacrylate monoliths enabled the purification of pDNA solely on monolithic columns [24]. Last but not least, it has also been shown recently that monolithic structure is suitable for purification of plasmids with the size up to 62 kbp [23].

The objective of this study was to investigate the chromatographic behavior of large plasmids on the monolithic columns. The impact of chromatographic system and methacrylate monolithic column on the degradation of plasmid molecules was studied and the capacity measurements varying salt concentration in loading buffer were performed for different sized plasmids. By analyzing pressure drop data some physical features of the plasmids in the flow solution as well as of the plasmids being adsorbed on the surface were characterized. Implementing this results some parameters of downstream process could be optimized.

2. Materials and methods

2.1. Materials and instrumentation

All CIM diethylaminoethyl (DEAE) weak anion exchange monolithic columns, 8 mL tube, 76 μL analytical column prototype and custom made 12 μL microanalytical columns packed into CIM 8 mL tube housing or specially designed stainless steel housing for analytical columns, were obtained from BIA Separations (Ljubljana, Slovenia). Plasmids R388, R6K and TP114 (hosts: *Escherichia coli*) were purchased from DSMZ (Braunschweig, Germany) and plasmid pEGFP-N1 (host: *Escherichia coli* DH5α) was purchased from Clontech Laboratories (Mountain View, CA 94043). pDNA conformations and RNA were analyzed by agarose gel electrophoresis (AGE, Bio-Rad, Richmond, VI, USA). Plasmid lengths were determined using 1 kbp Plus DNA Ladder (Invitrogen, Eugene, OR, USA). Agarose gel was made of SeaKem LE Agarose (Lonza Group, Basel, Switzerland) and TBE buffer (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.0). Luria–Bertani broth, potassium acetate, tris(hydroxymethyl)aminomethane (Tris), boric acid, sodium chloride, sodium hydroxide and hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany). Antibiotics ampicillin, kanamycin and sulfanilamide, sodium dodecyl sulfate (SDS) and DNase-free ribonuclease A (RNase A) from bovine pancreas were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was obtained from Kemika (Zagreb, Croatia). All solutions were prepared using water purified by a Watek IWA-80 roi (Ledeč nad Sázavou, Czeck Republic) water purification system and analytical grade reagents. Buffer solutions were prepared by dissolving a known mass of buffering species into ca. 80% of the desired final volume of deionized water, titrating with HCl and adding the deionized water to yield the final solution volume. Finally, buffers were filtered through a 0.45 μm pore size filter composed of Sartolon polyamide (Sartorius, Goettingen, Germany).

Cell density, pDNA purity and concentration were measured by spectrophotometer Smart Spec 3000 (BioRad, Richmond, VI, USA), sample centrifugations and sample analysis were performed using Sorvall RC5C Plus centrifuge (Kendro, Newtown, CT, USA) and agarose gel electrophoresis (AGE, BioRad, Richmond, VI, USA), respectively. All chromatographic experiments were performed using a gradient Knauer HPLC system (Berlin, Germany) consisting of two Knauer Type 64 analytical pumps, an injection valve with 100, 500 or 1000 μL sample loops, a Knauer UV–vis absorbance detector model K-2500 with a 10 mm optical path cell and a conductivity monitor Amersham Biosciences (GE Healthcare, Uppsala, Sweden), all connected via a Knauer interface box to a personal computer for real time data acquisition by Eurochrom 2000 software. For loading the sample into an 8 mL tube the peristaltic pump (Ismatec, Glattbrugg, Switzerland) was used. During pulse response and capacity measurements pressure drop on the monolithic columns was measured by differential manometer (MidWest Instruments, Sterling Heights, MI, USA).

2.2. Methods

2.2.1. Cell lysis protocol

Bacteria were lysed using modified alkaline lysis procedure according to Birnboim and Doly [26]. The liquid cultures of the bacterium *Escherichia coli* with different plasmids were cultivated overnight at 37 °C on Luria–Bertani broth containing proper antibiotic. After harvest, cells were resuspended in TE buffer (50 mM Tris with 10 mM EDTA), pH 8.0, containing 100 μg/mL DNase-free RNase and treated with cell lysis buffer containing 0.2 M NaOH and 1% SDS followed by neutralizing solution (3 M potassium acetate, adjusted to pH 5.5 with glacial acetic acid, chilled to 4 °C). Precipitated material, including cell debris, most chromosomal DNA, some RNA and proteins, was removed by centrifugation at 9000 × *g* for 10 min followed by clarification through a 0.45/0.2 μm Sartobran P filter (Sartorius, Goettingen, Germany).

2.2.2. Purification of plasmids

Plasmid purification was performed by AEX chromatography as described in literature [23]. For this purpose 8 mL CIM DEAE tube monolithic column with ligand density 0.4 mmol/g and average pore diameter 1400 nm was used. Conductivity of the plasmid sample solution, obtained after alkaline lysis, was adjusted to proper value (*i.e.*, the value where RNA does not bind to the column) by adding NaCl. The column was first equilibrated with TE buffer (50 mM Tris with 10 mM EDTA) with NaCl, pH 7.2, with the same conductivity as sample solution. After that plasmid sample was loaded and column was washed with loading buffer. The elution step was performed by linear gradient to TE buffer with 1.5 M NaCl, pH 7.2. Collected plasmid fractions were analyzed for RNA content by anion exchange chromatography (AEC) and AGE. Finally, pure plasmid was precipitated by 2-propanol and dissolved in appropriate buffer.

2.2.3. Electrophoresis and sample purity and quantity

Agarose electrophoresis was run in a horizontal gel electrophoresis. The running buffer was TBE (40 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8.0). Method was performed for 60 min at 80 V. For staining ethidium bromide was used. Additionally, DNA concentration and purity were measured by spectrophotometer. One unit of OD 260 nm in a 10 mm cuvette corresponded to 50 μg/mL dsDNA. Absorption measurements were taken at wavelengths of 260 nm and 280 nm and 260/280 absorption ratios were calculated from the absorption readings. The absorption ratios of clarified cell lysates were between 1.9 and 2.2 and of pure plasmid samples between 1.8 and 2.0. Protein concentrations were between 100 and 200 μg/mL for clarified cell lysates and under 20 μg/mL for pure plasmid samples, estimated according to Bradford method [27]. The absence of RNA was additionally confirmed by AEC on prototype CIM DEAE analytical monolithic column (column volume (CV) 76 μL) with 0.4 mmol/g ligand density and average pore diameter 1400 nm. The column was first equilibrated with TE buffer, pH 7.2,