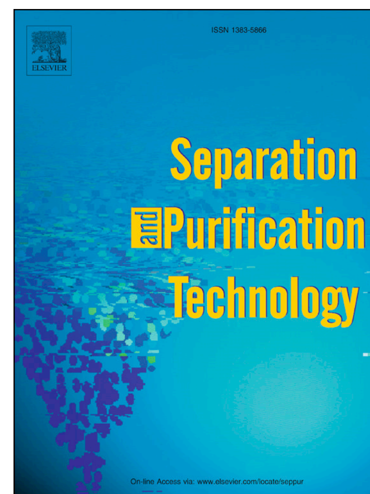
Accepted Manuscript



Influenza DNA **vaccine purification** using pHEMA cryogel support

Tiago Santos, Andreia Brito, Renato Boto, Pedro Sousa, Paulo Almeida, Carla Cruz, Cândida Tomaz

PII: S1383-5866(18)30964-X
DOI: https://doi.org/10.1016/j.seppur.2018.06.002
Reference: SEPPUR 14656

To appear in: *Separation and Purification Technology*

Received Date: 21 March 2018
Revised Date: 31 May 2018
Accepted Date: 1 June 2018

Please cite this article as: T. Santos, A. Brito, R. Boto, P. Sousa, P. Almeida, C. Cruz, C. Tomaz, **Influenza** DNA **vaccine purification** using pHEMA cryogel support, *Separation and Purification Technology* (2018), doi: https://doi.org/10.1016/j.seppur.2018.06.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Influenza DNA vaccine purification using pHEMA cryogel support

Tiago Santos[a], Andreia Brito[a], Renato Boto[a], Pedro Sousa[a], Paulo Almeida[a], Carla Cruz[a] and Cândida Tomaz[a]*

[a] CICS-UBI - Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal; Tel: +351 275 329 076; Fax: +351 275 329 099; *ctomaz@ubi.pt

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Highlights

- Poly(2-hydroxyethyl methacrylate) cryogel was synthesized by cryogelation
- Purification of Influenza DNA vaccine was achieved using poly(2-hydroxyethyl methacrylate) cryogel
- Economic and efficient method to purify Influenza DNA vaccine

Abstract

Influenza virus is a huge financial and social burden for health care systems over the world. Currently, traditional approaches are not effective in the fight of the epidemy and new alternatives like DNA vaccines have been developed. However, the downstream process of DNA vaccines is a constant challenge in the biotechnology industry. Cryogels has several advantages over traditional supports and have been tested as stationary phase in chromatographic separations. In this work, a method based on poly(2-hydroxyethyl methacrylate) cryogel was used to purify the plasmid NTC7482-41H-VA2 HA, which express the Influenza hemagglutinin gene. For this purpose, the cryogel was synthesized by cryo-polymerization of 2-hydroxyethyl methacrylate and characterized by scanning electron microscopy. The purification of supercoiled isoform of the plasmid NTC7482-41H-VA2 HA from a clarified lysate sample was achieved in a two-step experiment using NaCl and the dynamic binding capacity of pHEMA cryogel was determined. The assessment of DNA vaccine allowed to conclude that the level of contaminants such as proteins, genomic DNA, RNA and endotoxins are in accordance with FDA agency.

Keywords;

Influenza; DNA vaccine; pHEMA cryogel; supercoiled purification; DNA vaccine assessment

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

1. Introduction

Currently, respiratory disease caused by influenza virus is a considerable financial and social burden for public health care systems [1-4]. Influenza vaccines are usually efficient and safe to protect against infection; however, they need to be updated annually, because the genes of influenza viruses change constantly over time as the virus replicates. Every year, traditional vaccines are prepared from embryonated chicken eggs and inoculated individually with each virus type, which is time consuming and highly limiting in the case of influenza pandemic [5]. Additionally, concerns about zoonotic risk and limited immunological protection have been increasing the development of new methodologies for effective immunization, in case of a new influenza outbreak [5]. Taking this into account, researchers focus their efforts in producing new alternatives to traditional vaccines, such as DNA vaccines, [6] which present technical and economic advantages over traditional ones [7]. For example, they can mimic the effects of attenuated vaccines by focus on raising a humoral response against hemagglutinin (HA) the more abundant, immunodominant glycoprotein on the surface of the influenza virus [7]. The induction of HA-specific antibodies after a natural infection reduce the severity of the disease by inhibiting the entry of the virus in the host cells in a future contact [8]. Therefore, the HA gene is usually inserted into plasmid DNA (pDNA) of influenza DNA vaccines to promote protection against the virus. However, regulatory agencies have rigid requirements concerning the final pDNA sample purity, which should have more than 80% of the supercoiled (sc) isoform [9]. To obtain a highly pure sc pDNA sample, the purification process needs to be optimized, from upstream to downstream, being the major concerns related with sc pDNA purification.

Affinity chromatography is the most powerful technology for the purification of sc pDNA both in the analytical and large scale. Moreover, affinity chromatography has several advantages related to the nature of stationary phases and composition of mobile phases in comparison with other types of chromatography [10, 11]. The process is also more effective regarding the time and number of steps necessary to purify sc pDNA with success.

Recently, several chromatography supports based on ethylenediamine and agmatine monoliths were used to purify a HA influenza deoxyribonucleic acid-based vaccine [12, 13]. These monoliths have several advantages over conventional stationary phases,

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

since they present excellent mass transfer properties and flow independence resolution [14]. However, cryogels have emerged as alternative matrices not only to conventional supports but also to monoliths due to their larger pore size and economical and ease production [14]. In fact, supermacroporous cryogels are found to have interesting applications in the purification of biomolecules [15, 16]. The unique features (e.g. high porosity, mass transfer and mechanical strength) of cryogels make them attractive matrices for the purification of particles with a larger size, such pDNA [15, 16]. Poly(2-hydroxyethyl methacrylate) (pHEMA) is one of the most widely used hydrophilic polymers in biomedical applications [15]. Recently, the poly(hydroxyethyl methacrylate-*N*-methacryloyl-L-histidine (pHEMAH) cryogel was used to purify both pDNA and RNA molecules from lysate *E. coli* complex samples [17, 18]. The *N*-methacryloyl-L-histidine methyl ester (MAH) ligands were tested in two different stationary phase forms: a pHEMAH affinity cryogel and pHEMAH magnetic nanoparticles. The preliminary pDNA adsorption studies showed that both matrices retain sc and open circular (oc) isoforms of native pDNA.

Considering these evidences, the pHEMA cryogel was synthetized by polymerization of 2-hydroxyethyl methacrylate (HEMA), to be applied as stationary phase in affinity chromatography for purification of the sc isoform of NTC7482-41H-VA2 HA plasmid expressing influenza virus HA gene.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

2. Materials and Methods

2.1. Materials

Tris(hydroxymethyl)aminomethane (Tris) was obtained from Merck (Darmstadt, Germany) and sodium chloride and sodium acetate from Fisher Scientific (Fair Lawn, NJ, USA). HEMA and ammonium persulfate (APS), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Econo-Pac Chromatography Columns, Econo-Pac Flow adaptor, *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED) and *N*,*N*'-methylenebisacrylamide (MBAAm) were bought from BioRAD (Hercules, CA, USA). All buffers used for the chromatographic experiments were freshly prepared with deionized ultra-pure grade water, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The GreenSafe Premium and the NZYMaxiprep commercial kit used in the pDNA pre-purification were obtained from NZYTech (Lisbon, Portugal). Plasmid pVAX1-*LacZ* (6050 bp) was acquired from Invitrogene (Carlsband, CA, EUA) and plasmid NTC7482-41H-VA2 HA (6471 bp) was kindly provided by Nature Technology Corporation (Lincoln, USA).

2.2. Methods

2.2.1. Synthesis of pHEMA cryogel

Cryogel was synthesized within an Econo-Pac column (1.5 by 12 cm; Bio-Rad). HEMA was polymerized in free radical polymerization by using APS and TEMED as initiator. Water and MBAAm were included in the polymerization recipe as the pore-former and crosslinker, respectively. HEMA (0.675 mL) was dissolved in 2.5 mL of deionized water and added to a second solution prepared with MBAAm (0.142 g) dissolved in 5 mL deionized water. Then, the cryogel was produced by free radical polymerization initiated by addition of TEMED (0.0125 mL) and APS (0.010 g). After adding APS and TEMED the solution was stirred for 1 min. Thereafter, the polymerization mixture was frozen at −16 °C for 24 h in an acetone bath and then thawed at room temperature. After washing with 300 mL of deionized water, the cryogel was stored in buffer containing 2% sodium azide at 4 °C until use.

ACCEPTED MANUSCRIPT

2.2.2. SEM micrographs for microstructural determination of pHEMA cryogel

The micrographs of pHEMA were obtained using Scanning Electron Microscope (SEM. The samples were mounted on an aluminum board using double-sided adhesive tape and then made electrically conductive by coating with gold using an Emitech K550 sputter coater (London, UK). Then, the samples were analyzed on a Hitachi S-2700 (Tokyo, Japan) operating at an accelerating voltage of 20 kV at 400× magnifications.

2.2.3. Swelling measurement

The swelling degree was estimated as amount of water in swollen cryogel per gram of dried polymer. The water-holding capacity of the cryogels was determined by the swelling degree Q in equation 1:

$$Q = (m_{eq} - m_{dry})/m_{dry} \quad (1)$$

where m_{eq} is the equilibrium weight of the gel immersed in water, and m_{dry} is the weight of the corresponding freeze-dried sample.

2.2.4. Plasmid production and isolation

The plasmids pVAX1-*LacZ* and NTC7482-41H-VA2 HA were amplified by a cell culture of *E. coli* DH5α. Growth was carried out overnight in shake flasks (250 rpm) at 37 °C using a terrific broth medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) supplemented with 30 μg/mL of kanamycin sulfate. After production, cells were recovered by centrifugation at 3900 RCF for 50 min at 4 ºC and pellets were stored at -20 ºC. Thereafter, pDNA was purified using NZYTech Plasmid Maxiprep kit according to the supplier's protocol to obtain the native pDNA (sc and oc isoforms). The protocol is based on alkaline lysis procedure followed by the binding of pDNA to the NZYTech anion-exchange resin under appropriate low salt and pH conditions. After that, the impurities were removed by a medium salt wash. Finally, plasmid was eluted with high salt conditions and then is concentrated and desalted by isopropanol precipitation. The resulting pDNA preparations were dissolved in 10 mM sodium acetate pH 5.0 buffer (10 mM sodium acetate

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

and acetic acid). These samples were used for initial evaluation of chromatographic retention behavior.

Crude extract of pDNA with all of contaminants (genomic DNA (gDNA), RNA, proteins, endotoxins) was obtained through a modified alkaline lysis method described by Diogo and collaborators [19]. The pDNA samples concentration was measured with a NANOPhotometer (Implen). All the samples were stored at -20 °C.

2.2.5. Chromatographic experiments with native pDNA and lysate samples using pHEMA cryogel support

The Econo-Pac Flow adaptor was assembled into an Econo-Pac column containing pHEMA cryogel to perform the chromatographic experiments in an ÄKTA purifier system controlled by UNICORN 5.1 software (GE Healthcare Biosciences, Uppsala, Sweden). The solutions used in chromatographic runs were filtered through a 0.20 µm pore size membrane (Whatman, Dassel, Germany) and degassed before use. Experiments were performed at room temperature and pH 5.0. The absorbance of each run was continuously measured at 260 nm. Fractions were pooled according to the chromatograms and were concentrated and desalted with Vivaspin concentrators (Vivascience) for further analysis by gel agarose electrophoresis. The fractions recovered in each chromatographic experiment were analyzed by horizontal electrophoresis using 15-cm-long 0.8% agarose gels (Hoefer, San Francisco, CA, USA), stained with GreenSafe Premium (1 µg/mL). Electrophoresis was performed at 110 V, for 40 min, with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and revealed under UV radiation in a transilluminator system (ILC Lda, Lisbon, Portugal).

2.2.5.1. Separation of RNA from pVAX1-LacZ lysate sample

The column was first equilibrated with 55 mM NaCl in 10 mM sodium acetate pH 5.0. The pVAX1-*LacZ* solution (200 µg pDNA/mL) was loaded onto column using a 200 µL loop at flow rate of 1 mL/min. After elution of unbound species, the ionic strength of mobile phase was increased to 600 mM of NaCl in the same buffer.

2.2.5.2. Purification of sc NTC7482-41H-VA2 HA plasmid from lysate sample.

The column was first equilibrated with 60 mM NaCl in 10 mM sodium acetate pH 5.0. The NTC7482-41H-VA2 HA plasmid solution (100 µg pDNA/mL) was loaded onto column using a 200 µL loop at flow rate 1 mL/min. After elution of unbound species, the ionic strength of mobile phase was increased to 600 mM of NaCl in the same buffer.

2.2.6. Dynamic binding capacity

Dynamic binding capacity (DBC) is defined as the amount of target molecules that bind to the matrix under standard flow conditions and should be determined under specific flow and load characteristics [11]. DBC experiments were performed with 0.01, 0.05 and 0.1 mg/mL of NTC7482-41H-VA2 HA plasmid solution, at flow-rate of 1 mL/min. For this purpose, cryogel was synthesized onto Econo-Pac column with 1 cm of diameter and 0.8 cm of height. The column was equilibrated with 10 mM sodium acetate and thereafter, it was overloaded with the pDNA solution under the same conditions. DBC was determined by recording breakthrough curves and calculating the amount of bound pDNA per mL of support at 10% and 50% of breakthrough curve at 10 and 50% of maximal absorbance value. Afterwards, the elution of the bound pDNA was achieved by increasing the NaCl concentration from 0 M to 1.5 M in 10 mM sodium acetate. Finally, cryogel was washed with several column volumes of ultra-pure grade deionized water.

2.2.7. Analytical chromatography

The content of sc NTC7482-41H-VA2 HA present in clarified *E. coli* DH5α lysate and in purified fractions was determined with CIMac™ pDNA analytical column by a modified analytical method [20]. CIMac™ pDNA analytical column consists of a disk-shaped poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous polymer matrix, with 0.32 mL of volume, 15.0 mm length and 5.2 mm diameter. The NTC7482-41H-VA2 HA plasmid

ACCEPTED MANUSCRIPT

concentration in each sample was calculated by using a calibration curve constructed with NTC7482-41H-VA2 HA plasmid standards of 2.5–75 μg/mL, purified with a commercial NZYTech kit. All samples were prepared by diluting the highest concentration with 200 mM Tris–HCl (pH 8.0). The analytical column was equilibrated with 600 mM NaCl in 200 mM Tris–HCl buffer (pH 8.0) and after the injection of 20 μL of sample, a linear gradient to 700 mM NaCl in 200 mM Tris–HCl buffer (pH 8.0) was established at 1 mL/min. After the chromatographic runs, the CIMac™ pDNA analytical column was washed with several column volumes of ultra-pure grade deionized water.

2.2.8. Impurities assessment

2.2.8.1. Proteins

Protein quantification in NTC7482-41H-VA2 HA samples was performed using the micro-BCA (bicinchoninic acid) protein assay kit from Pierce, in accordance with the specifications of the manufacturer. A calibration curve was prepared with the standards of protein bovine serum albumin (BSA) (0.01–0.1 mg/mL) diluted in 10 mM Tris–HCl (pH 8.0). A fraction of each sample (10 μL) was added to 200 μL of BCA in a microplate and incubated for 30 min at 37 °C and then cooled to room temperature. The absorbance was measured at 570 nm in microplate reader.

2.2.8.2. Genomic DNA

Real-time polymerase chain reaction (PCR) was used to evaluate the existence of gDNA in the purified samples. The analyses were performed in an iQ5 Multicolor real-time PCR detection system (Bio-Rad), Specific primers (sense – 5′-ACACGGTCCAGAACTCCTACG-3′ and antisense – 5′-CCGGTGCTTCTTCTGCGGGTAACGTCA-3′) were used to amplify a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following changes in fluorescence of the DNA binding dye SYBR Green/Fluorescein. The calibration curve to achieve the gDNA concentration was constructed by serial dilutions of the *E. coli* DH5α gDNA sample, purified with the Wizard gDNA purification kit (Promega), in the range of 0.005–50 ng/μL.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

2.2.8.3. Endotoxins

Endotoxin contamination was assessed by using the ToxiSensorTM Chromogenic Limulus Amebocyte Lysate (LAL) Endotoxin Assay Kit (GenScript, USA, Inc.), according to the supplier's protocol. The calibration curve (0.005 to 0.1 EU/mL) was constructed using a provided stock solution of 10 EU/mL. To avoid external endotoxin interference, all the samples were diluted or dissolved in non-pyrogenic water, which was also used as blank. All the tubes and tips used to perform this quantification were endotoxin-free and the entire procedure was performed inside of a laminar flow cabinet.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

3. Results and Discussion

3.1. Characterization of pHEMA cryogel

Supermacroporous pHEMA cryogel was synthetized by free-radical cryo-polymerization of HEMA with MBAAm as a crosslinker in the presence of APS/TEMED as an initiator/activator pair. The pHEMA cryogel support was used as affinity chromatography support for the purification of sc NTC7482-41H-VA2 HA plasmid. The chemical structure of pHEMA cryogel support disregarding crosslinking substructure was presented in Figure 1.

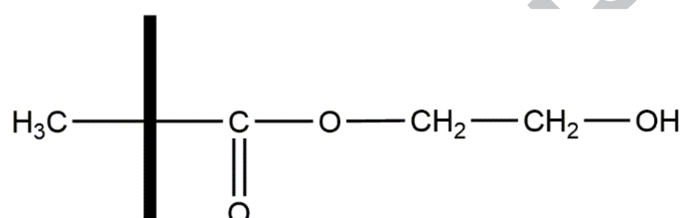


Figure 1 – Chemical structure of pHEMA cryogel support

The polymerization in a frozen state was the critical step of process, because contribute to the formation of macroporous structure. In order to full characterize the structure of pHEMA cryogel, SEM was performed, and micrograph with 400× magnification is shown in Figure 2. The results reveal a large continuous interconnected pore structure, with pore size estimated between 10-100 µm. This structure allowed the flow-through of mobile phase with a negligible mass transfer resistance.

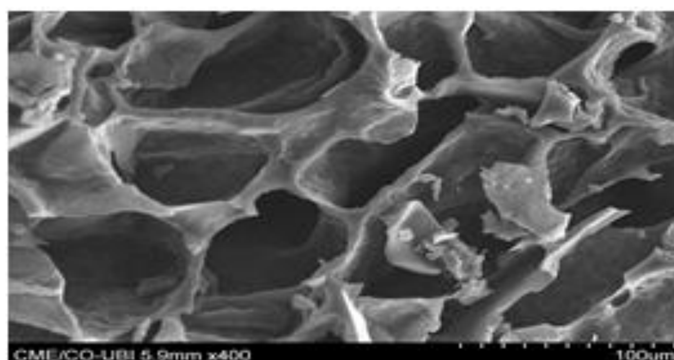


Figure 2 – SEM micrograph of pHEMA cryogel support with 400× magnification.

ACCEPTED MANUSCRIPT

The cryogel synthetized had a highly elastic sponge structure. The swelling behavior of cryogels is an important feature to evaluate, since increases the pore size and the surface area/volume ratio, facilitating the flow of pDNA through the pores. When the cryogel was compressed, the water accumulated inside the pores was released. The reverse behavior was also observed, when the cryogel was submerged in water, rapidly recover its initial size and shape. The equilibrium swelling degree of the pHEMA cryogel support was 6.80 g of H_2O/g of dry cryogel and is in accordance with the recent reports [21, 22].

3.2. Purification of sc NTC7482-41H-VA2 HA plasmid *from a crude extract using pHEMA cryogel support*

Supermacroporous cryogels have been studied as potential tools in bioseparation [16]. Thus, the goal of this work was to investigate the efficacy of pHEMA cryogel support, to selectively purify the sc isoform of NTC7482-41H-VA2 HA plasmid. This plasmid contains the sequence HA that is the more abundant, immunodominant glycoprotein on the surface of the influenza virus. A preliminary screening with clarified lysate containing the model plasmid pVAX1-*LacZ* was performed to evaluate the experimental conditions of pHEMA cryogel using different salt gradients. The use of an increased stepwise gradient of NaCl in purification method has shown tremendous advantages over $(NH_4)_2SO_4$, such as cost reduction and less environmental impact [23]. Moreover, the use of NaCl in mobile phase was successful applied in the purification of sc pDNA with agmatine monolith [13], naphthalene tripodal support [23] and amino acid supports [24-26]. Regarding that, the evaluation of retention/elution profile of pVAX1-*LacZ* on pHEMA cryogel was performed by manipulating the ionic strength. After injecting the clarified lysate containing pVAX1-*LacZ*, all nucleic acids were retained in the cryogel using 10 mM sodium acetate buffer pH 5.0 without salt. The chromatographic profile and gel agarose electrophoresis are presented in Figure 3. Thereafter, the increase of salt in mobile phase to 120 mM NaCl allowed the elution of most species, except a small portion of sc pVAX1-*LacZ* that was retained and eluted in the next step using 600 mM NaCl. In last step, the salt concentration was increased to 1.5 M NaCl, and no species were eluted. By analysis of gel agarose electrophoresis of the Figure 3, it is

ACCEPTED MANUSCRIPT

possible to verify that pHEMA cryogel support shows higher affinity for pDNA than for RNA which is an advantage in the purification process.

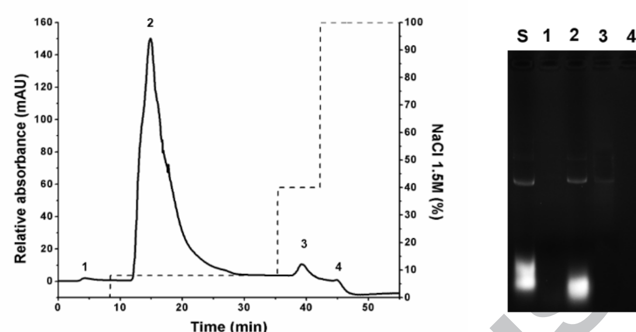


Figure 3 - Chromatographic profile and gel agarose electrophoresis of pVAX1-*LacZ* on pHEMA cryogel support. Elution was performed at 1 mL/min by stepwise increasing gradient of NaCl concentration in the eluent, as represented by the dashed line. Lane S – Clarified lysate sample; Lane 1 – Peak 1; Lane 2 – Peak 2; Lane 3 – Peak 3 and Lane 4 – Peak 4.

The separation of pVAX1-*LacZ* isoforms (oc+sc) from RNA was achieved in a two-step increased gradient from 55 mM to 600 mM NaCl. The results are presented in Figure 4. The first step promoted the binding of both isoforms to the cryogel, unlike the RNA that was eluted in the flow-through due to its lower charge density. The second step allowed the elution of pVAX1-*LacZ* isoforms, by increasing the salt concentration in eluent buffer, which weakened the binding of sc isoform onto pHEMA cryogel promoting its elution. This behaviour can be explained by the reduction of affinity interactions with the increase of the ionic strength in the eluent. Indeed, the interaction of sc pDNA with pHEMA cryogel was weakened by competition, being the binding sites of sc isoform masked by the salt molecules**.**

The separation performance of pHEMA cryogel support is similar to the L-arginine modified monolith previously reported [28], in which RNA eluted at an early stage under low salt conditions. Therefore, the obtained result allowed to confirm the separation of pVAX1-*LacZ* isoforms from RNA.

ACCEPTED MANUSCRIPT

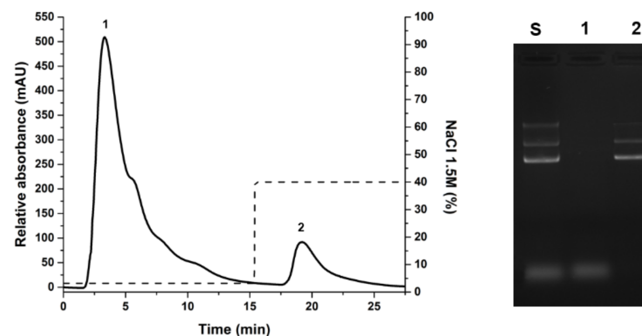


Figure 4 - Chromatographic profile and gel agarose electrophoresis showing the separation of pVAX1-*LacZ* isoforms from RNA on pHEMA cryogel support. Elution was performed at 1 mL/min by stepwise gradient increasing of NaCl concentration as eluent and is represented by the dashed line. Lane S – Clarified lysate sample; Lane 1 – RNA recovered from peak 1; Lane 2 – pVAX1-*LacZ* isoforms recovered from peak 2.

After the preliminary experiments with pVAX1-*LacZ* isoforms, the purification of sc NTC 7482-41H-VA2 HA isoform was achieved from clarified lysate and involved two-step increased gradient of 60 mM to 600 mM NaCl in sodium acetate pH 5.0. The sc NTC 7482-41H-VA2 HA retention was observed when salt concentrations were kept below 60 mM of NaCl, while RNA, gDNA and oc DNA elute. The elution of sc NTC 7482-41H-VA2 HA was then performed with a second step gradient by increasing the concentration of NaCl to 600 mM in sodium acetate pH 5.0. The chromatographic profile and the fractions analysed by gel electrophoresis are presented in Figure 5.

The results showed that pHEMA cryogel had an unexpected ability to purify sc NTC 7482-41H-VA2 HA isoform even without any ligand immobilized in the matrix, and only by manipulation of the ionic strength. As already reported, the deformation induced by torsional strain of the sc isoform promotes a higher exposition of pDNA bases and the involvement of different interactions, thus improving the selectivity for separation of this isoform [25].

Previous studies used MAH ligand in two different stationary phase forms: pHEMAH cryogel [18] and pHEMAH magnetic nanoparticles [17]. The pHEMAH cryogel showed ability to retain native pDNA (oc and sc isoforms) but was not able to completely separate the RNA from the crude lysate [18]. On the other hand, the

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

magnetic nanoparticles of pHEMAH purified pDNA from RNA, however the separation between sc and oc isoforms was not achieved [17, 18].

Therefore, pHEMA cryogel emerges as an alternative chromatographic support to purify sc NTC 7482-41H-VA2 HA isoform with reduced costs using a stationary phase without specific ligands and under low concentrations of salt.

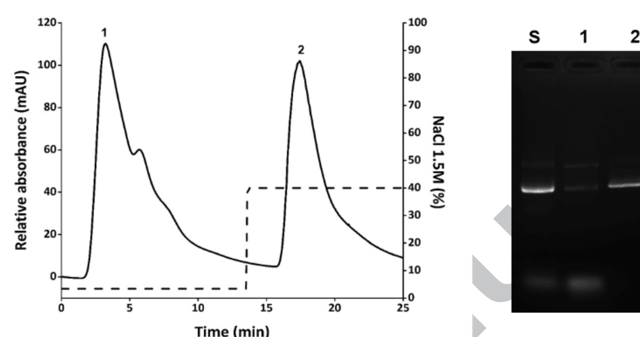


Figure 5 - Chromatographic profile and gel agarose electrophoresis showing the separation of sc NTC 7482-41H-VA2 HA from RNA and oc isoform using the pHEMA cryogel support. Elution was performed at 1 mL/min by stepwise gradient increasing the NaCl concentration in the eluent, as represented by the dashed line. Lane S – Clarified lysate sample; Lane 1 – RNA, oc pDNA and partial sc pDNA recovered from peak 1; Lane 2 – pDNA recovered from peak 2.

3.3. Quantitative analysis of sc NTC7482-41H-VA2 HA plasmid

The recovery yield, concentration and quality of sc NTC7482-41H-VA2 HA plasmid were assessed by a CIMac™ pDNA analytical column, according to the modified analytical method [20]. This method allows the elution of RNA species in the flowthrough at 600 mM NaCl in 200 mM Tris–HCl (pH 8.0), while oc and sc isoforms of pDNA are separated during a 10 min linear gradient from 600 to 700 mM NaCl in 200 mM Tris–HCl (pH 8.0). After the construction of the calibration curves by injection of native NTC7482-41H-VA2 HA plasmid standards under these elution conditions, each component present in clarified lysate samples was quantified. The results of concentration, recovery yield and purity of each peak by analysis with CIMac™ pDNA analytical column, are presented in Table 1.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1 - Quantitative analysis of NTC7482-41H-VA2 HA plasmid recovery yield from clarified lysate sample using pHEMA cryogel support.

Sample	Total pDNA (µg)	pDNA oc fraction (µg)	pDNA sc fraction (µg)	pDNA recovery (%)	Purity of sc pDNA (%)
Clarified lysate	36.49	0.87	38.20	-	-
Peak 1	5.06	3.31	2.22	13.85	43.90
Peak 2	25.25	0.36	24.89	69.19	98.60

As shown in Table 1, with this purification strategy a 69.19% of yield and 98.60% of purity was obtained for sc NTC7482-41H-VA2 HA. These values are in accordance with FDA requirements, which refer that homogeneity of sc pDNA should be higher than 97%. The results indicate that the pHEMA cryogel is selective for sc pDNA and allows its recovery with a high purity and yields [24,30]. The purity level of sc NTC 7482-41H-VA2 HA plasmid described for agmatine monolith with an increased stepwise of NaCl was 98.3%, while the recovery yield was 45.3% after two chromatographic runs [13]. Similar results were described for ethylenediamine monolith also using an increased gradient of NaCl, with a purity level and recovery yield of 97.1% and 47%, respectively [12]. Therefore, our strategy results in similar levels of purity and improved recovery yield of sc NTC 7482-41H-VA2 HA plasmid, compared to other approaches developed for the purification of NTC 7482-41H-VA2 HA vaccine using affinity supports [12].

3.3.1- Impurities assessment

The presence of proteins, gDNA, endotoxins and RNA in DNA vaccine preparation is an important aspect concerning safety [30]. These impurities could be molecules of various innate immune receptors, leading to undesirable biological effects and reduction of transfection efficiency [31]. The structural and chemical similarities of these impurities with pDNA can be a bottleneck in downstream process [27]. Thus, in order to evaluate the presence of impurities in purified sc NTC 7482-41H-VA2 HA plasmid

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

fraction, proteins, gDNA, endotoxins and RNA were determined by BCA protein assay, real-time PCR, Chromogenic Limulus Amebocyte Lysate (LAL) endotoxin assay and gel agarose electrophoresis, respectively. The results are presented in Table 2.

Table 2 – Proteins, gDNA, endotoxins and RNA assessment of plasmid NTC7482-41H-VA2 HA clarified lysate sample and sc NTC7482-41H-VA2 HA plasmid sample purified by pHEMA cryogel support.

Sample	Proteins (µg/mL)	gDNA (ng gDNA/µg pDNA)	Endotoxins (EU/mg sc pDNA)	RNA (µg)
Clarified lysate	68.89	5.43	0.99	428.89
Peak 2 (sc NTC 7482-41H-VA2 HA plasmid)	Non-detectable	0.60	0.94	Non-detectable
FDA requirements	Non-detectable	<2	<40	Non-detectable

Results from the BCA protein assay showed that purified sc NTC 7482-41H-VA2 HA plasmid fraction of peak 2 had undetectable levels of proteins. Despite the great difficulty in removing gDNA due to its structural similarities to pDNA, [31] the real-time PCR analysis showed a great reduction of gDNA content after the chromatographic step using pHEMA cryogel (0.60 ng gDNA/µg pDNA) compared to clarified lysate (5.43 ng gDNA/µg pDNA). The presence of endotoxins in DNA vaccines can lead to an abrupt, systemic response very similar to anaphylaxis [32]. Taking this into account, the levels of endotoxins were analysed and reveal low levels in plasmid fraction of peak 2 (0.94 EU/mg sc pDNA). Finally, the results obtained from agarose gel electrophoresis presented in Figure 5 showed that RNA was undetectable in sc NTC 7482-41H-VA2 HA fraction of peak 2.
Overall, the content of impurities complies the requirements of regulatory agencies for DNA vaccines preparation.

3.3.2- Dynamic binding capacity

Dynamic binding capacity (DBC) of a stationary phase is an important parameter in pDNA purification, as showed in previous studies [12-14]. The method consists in

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

measure the amount of pDNA that will bind to the column under certain experimental conditions, before occurrence of a break in breakthrough curve [33]. Once the stationary phase is saturated, pDNA concentration in the output reaches to its inlet concentration [33]. The DBC information allows the assessment of loading conditions and column lifetime, avoiding extra process scale-up, processing time, costs and pDNA loss [33, 34]. In previous reports, it was showed that pDNA feedstock concentration directly affect DBC [12-14]. Therefore, DBC was determined for pHEMA cryogel using native NTC7482-41H-VA2 HA plasmid feedstock concentrations (0.010, 0.05 and 0.100 mg/mL) and 10 mM acetate buffer pH 5.0 (10 mM sodium acetate and acetic acid) as mobile phase at 1 mL/min. The DBC was determined at 10 and 50% breakthroughs for native NTC7482-41H-VA2 HA plasmid and the obtained breakthrough curves showed a sigmoidal profile (Figure 6). The results (Table 3) showed an improvement in capacity with the increase of pDNA concentrations.

Table 3 - Dynamic binding capacity obtained for pHEMA cryogel support. The breakthrough experiments were performed with 0.01, 0.05 and 0.10 mg/mL of native NTC7482-41H-VA2 HA plasmid solution at room temperature using 1 mL/min flow rate.

% Breakthrough at 1 mL/min	**[pDNA]**		
	0.01 mg/mL	**0.05 mg/mL**	**0.1 mg/mL**
10	0.010	0.067	0.142
50	0.070	0.224	0.385

The DBC at 10% of breakthrough for a NTC7482-41H-VA2 HA plasmid feedstock concentration of 0.01 mg/mL was 0.01 mg/mL, whilst for 0.05 and 0.1 mg/mL, the DBC values were 0.067 and 0.142 mg/mL, respectively. The results for 50% of breakthrough showed an increase in DBC, for plasmid feedstock concentrations of 0.01, 0.05 and 0.1 mg/mL, with DBC values of 0.07, 0.224 and 0.385 mg/mL, respectively. The results are in accordance with previous reports, where the DBC also increased with pDNA feedstock concentration [12-14].

ACCEPTED MANUSCRIPT

By comparing our results with others cryogels, pHEMA had less DBC than pHEMAH [18], pHEMA-Cibacron Blue F3GA cryogel [35] and P(HEMA-MAPA)-FD [36], perhaps due to the lack of ligands coupled to the support. Nevertheless, the DBC values are similar to those obtained with Sepharose matrixes with an immobilized ligand, like DAPP [10] or L-histidine [37]. In monoliths, the DBC values are significantly higher, however they also use a ligand immobilized in the matrix [13, 20], whereas in this work no ligands were required for sc pDNA purification. Moreover, the yield obtained in the purification of NTC7482-41H-VA2 HA vaccine using the pHEMA cryogel was superior to those obtained with monoliths of higher DBC [12,13].

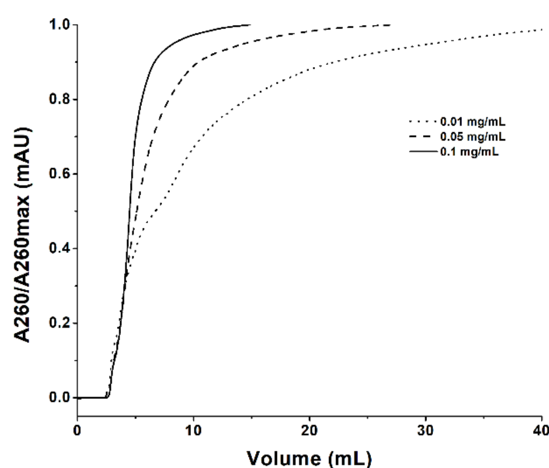


Figure 6 - Breakthrough curves of native NTC7482-41H-VA2 HA plasmid solution (0.01, 0.05 and 0.1 mg/mL) obtained with pHEMA cryogel support at room temperature using 1 mL/min flow rate.

ACCEPTED MANUSCRIPT

4. Conclusions

The purification of DNA vaccines remains a challenge for the biotechnology industry. In this work, we approached an innovative strategy using pHEMA cryogel as stationary phase for the purification of the influenza DNA vaccine NTC7482-41H-VA2 HA. The pHEMA cryogel was prepared by cryogelation and the characterization by SEM revealed a large continuous interconnected pore structure. The chromatographic studies allowed the separation of supercoiled isoform of NTC7482-41H-VA2 HA from a clarified lysate sample using 60 and 600 mM NaCl in the first and second steps, respectively. The complete characterization of pHEMA cryogel was achieved by determining the dynamic binding capacity, which value at 50% of breakthrough was 0.368 mg/mL for a feedstock concentration of 0.1 mg/mL pDNA. Finally, the assessment of proteins, endotoxins and gDNA presence in the Influenza DNA vaccine revealed values in accordance with FDA agency. The overall process also showed 98.1% of purity and 69.19% of recovery yield. The main novelty of this method is that it is not necessary to derivatize the support with ligands, since the matrix itself interacts specifically with the sc form of the DNA vaccine. On the other hand, the use of low amounts of salt provide an economic, environmental and efficient method to purify the Influenza DNA vaccine NTC7482-41H-VA2 HA.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Acknowledgments

Tiago Santos acknowledges the research fellowship BI1 IF/00959/2015 from FCT project "Projeto de Investigação Exploratória" ref. IF/00959/2015 entitled "Nucleolin targeting by G-quadruplex aptamers for cervical cancer therapy" financed by Fundo Social Europeu e Programa Operacional Potencial Humano and FLAD-Healthcare 2020 project ref. 45/2018 entitled "Development of drug delivery nanocarrier for HPV infection". This work was supported by FCT project "Projeto de Investigação Exploratória" ref. IF/00959/2015 entitled "Nucleolin targeting by G-quadruplex aptamers for cervical cancer therapy" financed by Fundo Social Europeu e Programa Operacional Potencial Humano and FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491), by National Funds by FCT (Project UID/Multi /00709/2013).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

[1] N. Principi, S. Esposito, P. Marchisio, R. Gasparini, P. Crovari, Socioeconomic impact of influenza on healthy children and their families, Pediatr Infect Dis J, 22 (2003) S207-210.

[2] M. Keech, P. Beardsworth, The impact of influenza on working days lost: a review of the literature, Pharmacoeconomics, 26 (2008) 911-924.

[3] R. Gasparini, D. Amicizia, P.L. Lai, D. Panatto, Clinical and socioeconomic impact of seasonal and pandemic influenza in adults and the elderly, Hum Vaccin Immunother, 8 (2012) 21-28.

[4] M.E. Klepser, Socioeconomic Impact of Seasonal (Epidemic) Influenza and the Role of Over-the-Counter Medicines, Drugs, 74 (2014) 1467-1479.

[5] P.C. Soema, R. Kompier, J.-P. Amorij, G.F.A. Kersten, Current and next generation influenza vaccines: Formulation and production strategies, European Journal of Pharmaceutics and Biopharmaceutics, 94 (2015) 251-263.

[6] D. Bicho, J.A. Queiroz, C.T. Tomaz, Influenza Plasmid DNA Vaccines: Progress and Prospects, Curr Gene Ther, 15 (2015) 541-549.

[7] M.A. Kutzler, D.B. Weiner, DNA vaccines: ready for prime time?, Nature reviews. Genetics, 9 (2008) 776-788.

[8] S.J. Gamblin, J.J. Skehel, Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins, The Journal of Biological Chemistry, 285 (2010) 28403-28409.

[9] P.A. Shamlou, Scaleable processes for the manufacture of therapeutic quantities of plasmid DNA, Biotechnol Appl Biochem, 37 (2003) 207-218.

[10] C. Caramelo-Nunes, D. Bicho, P. Almeida, J.C. Marcos, C.T. Tomaz, Dynamic binding capacity and specificity of 3,8-diamino-6-phenylphenanthridine-Sepharose support for purification of supercoiled plasmid deoxyribonucleic acid, J Chromatogr A, 1307 (2013) 91-98.

[11] C. Caramelo-Nunes, M.F. Gabriel, P. Almeida, J.C. Marcos, C.T. Tomaz, Purification of plasmid DNA from clarified and non-clarified Escherichia coli lysates by berenil pseudo-affinity chromatography, J Chromatogr B Analyt Technol Biomed Life Sci, 904 (2012) 81-87.

[12] D. Bicho, B.F. Santos, C. Caramelo-Nunes, A. Sousa, F. Sousa, J.A. Queiroz, C.T. Tomaz, Application of ethylenediamine monolith to purify a hemagglutinin influenza deoxyribonucleic acid-based vaccine, Separation and Purification Technology, 154 (2015) 320-327.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[13] D. Bicho, C. Caramelo-Nunes, A. Sousa, F. Sousa, J.A. Queiroz, C.T. Tomaz, Purification of influenza deoxyribonucleic acid-based vaccine using agmatine monolith, Journal of Chromatography B, 1012-1013 (2016) 153-161.

[14] D. Bicho, A. Sousa, F. Sousa, J. Queiroz, C. Tomaz, Effect of chromatographic conditions and plasmid DNA size on the dynamic binding capacity of a monolithic support, J Sep Sci, 37 (2014) 2284-2292.

[15] V.I. Lozinsky, I.Y. Galaev, F.M. Plieva, I.N. Savina, H. Jungvid, B. Mattiasson, Polymeric cryogels as promising materials of biotechnological interest, Trends in Biotechnology, 21 (2003) 445-451.

[16] V.I. Lozinsky, F.M. Plieva, I.Y. Galaev, B. Mattiasson, The potential of polymeric cryogels in bioseparation, Bioseparation, 10 (2001) 163-188.

[17] I. Perçin, V. Karakoç, S. Akgöl, E. Aksöz, A. Denizli, Poly(hydroxyethyl methacrylate) based magnetic nanoparticles for plasmid DNA purification from Escherichia coli lysate, Materials Science and Engineering: C, 32 (2012) 1133-1140.

[18] I. Percin, E. Saglar, H. Yavuz, E. Aksoz, A. Denizli, Poly(hydroxyethyl methacrylate) based affinity cryogel for plasmid DNA purification, Int J Biol Macromol, 48 (2011) 577-582.

[19] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, S.A. Martins, G.N. Ferreira, D.M. Prazeres, Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography, Biotechnol Bioeng, 68 (2000) 576-583.

[20] A. Sousa, A.M. Almeida, U. Cernigoj, F. Sousa, J.A. Queiroz, Histamine monolith versatility to purify supercoiled plasmid deoxyribonucleic acid from Escherichia coli lysate, J Chromatogr A, 1355 (2014) 125-133.

[21] E. Özgür, N. Bereli, D. Türkmen, S. Ünal, A. Denizli, PHEMA cryogel for in-vitro removal of anti-dsDNA antibodies from SLE plasma, Materials Science and Engineering: C, 31 (2011) 915-920.

[22] M. Uygun, B. Akduman, S. Akgöl, A. Denizli, A New Metal-Chelated Cryogel for Reversible Immobilization of Urease, Applied Biochemistry and Biotechnology, 170 (2013) 1815-1826.

[23] T. Santos, Z. Proença, J.A. Queiroz, C. Tomaz, C. Cruz, Plasmid purification by using a new naphthalene tripodal support, Separation and Purification Technology, 188 (2017) 81-89.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

[24] T. Santos, P. Pereira, F. Sousa, J.A. Queiroz, C. Cruz, Purification of supercoiled G-quadruplex pDNA for in vitro transcription, Separation and Purification Technology, 163 (2016) 59-71.

[25] F. Sousa, T. Matos, D.M.F. Prazeres, J.A. Queiroz, Specific recognition of supercoiled plasmid DNA in arginine affinity chromatography, Analytical Biochemistry, 374 (2008) 432-434.

[26] A. Sousa, F. Sousa, J.A. Queiroz, Biorecognition of supercoiled plasmid DNA isoform in lysine-affinity chromatography, Journal of Chromatography B, 877 (2009) 3257-3260.

[27] F. Sousa, D.M. Prazeres, J.A. Queiroz, Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA, Trends in biotechnology, 26 (2008) 518-525.

[28] A. Soares, J.A. Queiroz, F. Sousa, A. Sousa, Purification of human papillomavirus 16 E6/E7 plasmid deoxyribonucleic acid-based vaccine using an arginine modified monolithic support, Journal of Chromatography A, 1320 (2013) 72-79.

[29] C.M. Ongkudon, M.K. Danquah, Process optimisation for anion exchange monolithic chromatography of 4.2kbp plasmid vaccine (pcDNA3F), Journal of Chromatography B, 878 (2010) 2719-2725.

[30] G.N.M. Ferreira, G.A. Monteiro, D.M.F. Prazeres, J.M.S. Cabral, Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications, Trends in Biotechnology, 18 (2000) 380-388.

[31] J.A. Williams, Vector Design for Improved DNA Vaccine Efficacy, Safety and Production, Vaccines, 1 (2013) 225-249.

[32] Z. Wei, W. Huang, J. Li, G. Hou, J. Fang, Z. Yuan, Studies on endotoxin removal mechanism of adsorbents with amino acid ligands, Journal of Chromatography B, 852 (2007) 288-292.

[33] M. Bencina, A. Podgornik, A. Strancar, Characterization of methacrylate monoliths for purification of DNA molecules, J Sep Sci, 27 (2004) 801-810.

[34] F. Smrekar, A. Podgornik, M. Ciringer, S. Kontrec, P. Raspor, A. Štrancar, M. Peterka, Preparation of pharmaceutical-grade plasmid DNA using methacrylate monolithic columns, Vaccine, 28 (2010) 2039-2045.

[35] D. Cimen, F. Yilmaz, I. Percin, D. Turkmen, A. Denizli, Dye affinity cryogels for plasmid DNA purification, Mater Sci Eng C Mater Biol Appl, 56 (2015) 318-324.

ACCEPTED MANUSCRIPT

[36] R. Uzek, L. Uzun, S. Senel, A. Denizli, Nanospines incorporation into the structure of the hydrophobic cryogels via novel cryogelation method: an alternative sorbent for plasmid DNA purification, Colloids Surf B Biointerfaces, 102 (2013) 243-250.

[37] F. Sousa, D.M. Prazeres, J.A. Queiroz, Dynamic binding capacity of plasmid DNA in histidine-agarose chromatography, Biomed Chromatogr, 21 (2007) 993-998.

ACCEPTED MANUSCRIPT