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Rapid Production of a Plasmid DNA Encoding a Malaria Vaccine Candidate via Amino-Functionalized Poly(GMA-*co*-EDMA) Monolith

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Malaria is a global health problem; an effective vaccine is urgently needed. Due to the relative poverty and lack of infrastructure in malaria endemic areas, DNA-based vaccines that are stable at ambient temperatures and easy to formulate have great potential. While attention has been focused mainly on antigen selection, vector design and efficacy assessment, the development of a rapid and commercially viable process to manufacture DNA is generally overlooked. We report here a continuous purification technique employing an optimized stationary adsorbent to allow high-vaccine recovery, low-processing time, and, hence, high-productivity. A 40.0 mL monolithic stationary phase was synthesized and functionalized with amino groups from 2-Chloro-N,N-diethylethylamine hydrochloride for anion-exchange isolation of a plasmid DNA (pDNA) that encodes a malaria vaccine candidate, VR1020-PyMSP4/5. Physical characterization of the monolithic polymer showed a macroporous material with a modal pore diameter of 750 nm. The final vaccine product isolated after 3 min elution was homogeneous supercoiled plasmid with gDNA, RNA and protein levels in keeping with clinical regulatory standards. Toxicological studies of the pVR1020-PyMSP4/5 showed a minimum endotoxin level of 0.28 EU/mg pDNA. This cost-effective technique is cGMP compatible and highly scalable for the production of DNA-based vaccines in commercial quantities, when such vaccines prove to be effective against malaria. © 2008 American Institute of Chemical Engineers *AIChE J,* 54: 2990–2998, 2008
Keywords: malaria vaccine, plasmid DNA, methacrylate monolith, liquid chromatography

Introduction

Malaria, an infectious disease caused by parasites of the *Plasmodium* genus, is a major world health problem. Each year, up to 2.7 million people die as a result of the parasite infection.[1] The socioeconomic prospects of people who live in malaria endemic areas are adversely affected by the burden of this disease. The World Health Organization has rated malaria as the single biggest component of the total disease burden in Africa.[2] Therefore, an affordable, safe, and effective vaccine is urgently needed to enhance currently available malaria control measures. Protection in humans is believed

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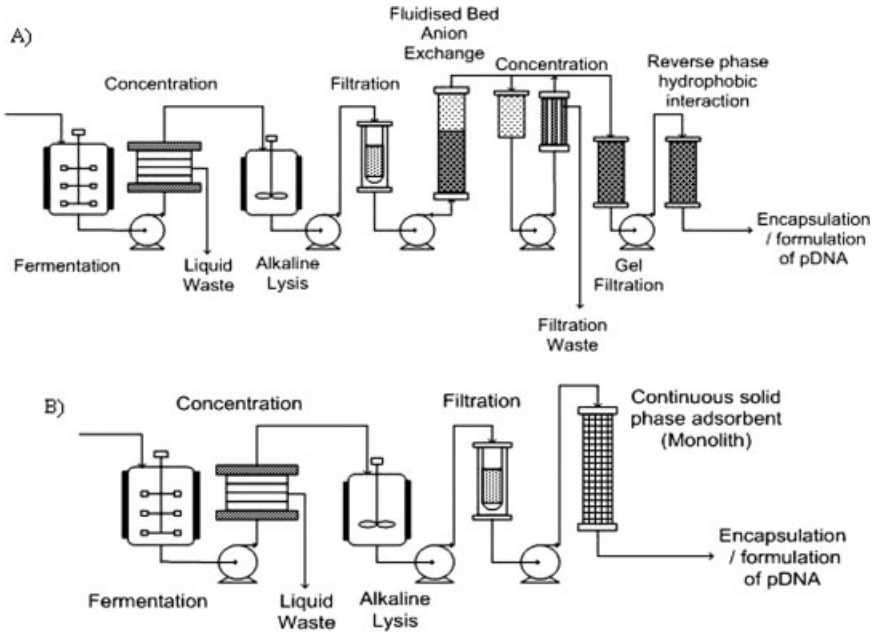

Figure 1. (A) A schematic flow diagram showing the current state of pDNA vaccine production. Several unit operations are incorporated into downstream processes, and (B) A process flow diagram showing improvements on current pDNA vaccine production technique by incorporating a monolith.

to include antibodies targeting the asexual blood stage parasites, such as those that block merozoite entry and inhibit parasite multiplication within erythrocytes.[3] A number of malaria proteins have been demonstrated to be potential vaccine candidates, and are in various stages of preclinical and clinical evaluation. Due to the relative poverty and lack of infrastructure in malaria endemic areas, a successful immunisation strategy will depend critically on cheap and scalable methods of vaccine production, distribution and delivery. Affordable and effective vaccine formulations which are stable at ambient temperatures and cheaper to produce would significantly enhance widespread vaccine deployment. One promising technology is the production and delivery of malaria vaccines using pDNA.

pDNA vaccines have the advantages of being cheaper to produce, stable at ambient temperature and easy to formulate. Several DNA constructs expressing malaria vaccine candidates, mainly of pre-erythrocytic stages, have been evaluated and shown promising results; especially in heterologous prime-boost approach.[4] A multiepitope DNA vaccine derived from a genomic *Plasmodium chabaudi* DNA expression library of 30,000 plasmids has been shown to induce strain-transcending immunity in mice against parasite challenge.[5] If a DNA vaccine for malaria proves to be effective, the ultimate scale of production is potentially huge—over 2.6 billion doses for the 40 % of the world's population living in malaria risk regions.[6] There are some general indications suggesting that pDNA dosage amounts will range from the microgram to milligram scale.[7] In addition, the size and nature of plasmids pose new processing and formulation challenges to biochemical engineers as pure supercoiled pDNA is the desired topological form for vaccine delivery.[8] Hence, the establishment of a rapid and commercially-viable manufacturing process for supercoiled pDNA is essential for vaccine delivery.

Although various methods are available for pDNA purification on a laboratory scale applying commercially-available kits, chromatography is widely accepted as the industrial method for producing pDNA with purities acceptable for therapeutic use. Electrostatic isolation of pDNA via anion-exchange liquid chromatography is the most frequently used technique for plasmid purification owing to its simplicity, widespread application, high-resolving power and high capacity.[9–13] However, the performance of anion-exchange

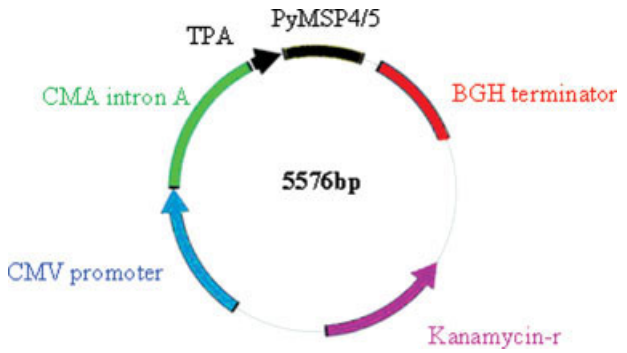

Figure 2. Schematic representation of pVR1020-PyMSP4/5.

The predicted mature PyMSP4/5 coding sequence (black bar) is fused at its 5′ end to a TPA secretion signal (black arrow). The recombinant plasmid contains a human cytomegalovirus (CMV) early promoter (blue), a CMV untranslated region including intron A (green), bovine growth hormone (BGH) terminator (red), and a kanamycin resistance gene for drug selection during bacterial cell growth (purple). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

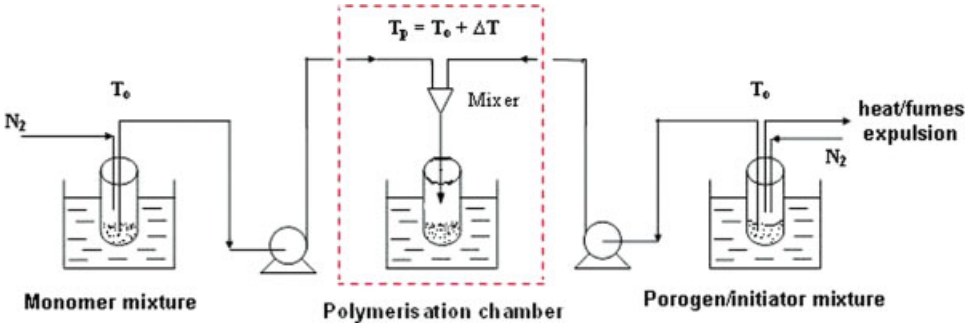

Figure 3. A schematic representation of the large-volume methacrylate polymer synthesis methodology via heat expulsion.

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

liquid chromatography for large-scale purification process hinges on the pore structure of the stationary phase.[13,14]

Macroporous methacrylate monoliths display distinct advantages compared to particulate stationary phases for biomolecule purification. The most essential feature of monolithic supports is that all of the mobile phase is forced to flow through the large pores of the monolith, thereby allowing the penetration of large pDNA molecules to the internal surface area, but at reduced pressure drops, even at high-flow rates.[15–18] This convective mass transfer enhances plasmid retention, binding capacity, purification yield or productivity and the economics of the purification process. Here we report a cost-effective, nontoxic and scalable technique for the rapid isolation of a pDNA encoding PyMSP4/5, a homologue of *P. falciparum* merozoite surface protein 4 (MSP4) and 5 (MSP5) in rodent malaria species *P. yoelii*. MSP4 and MSP5 are two glycosylphosphatidylinositol (GPI)-anchored integral membrane proteins that are potential components of a subunit vaccine against malaria.[19,2] Their single homologues (MSP4/5) in rodent malaria species have structural features similar to both MSP4 and MSP5,[21] and have shown to be highly effective at protecting mice against lethal challenge following immunisation with recombinant protein expressed in *E. coli*.[22] Immunisation with DNA vaccines encoding MSP4/5 provided protection against *P. chabaudi* blood stage infection,[23] but not in a more stringent challenge model of *P. yoelii*.[24] Both MSP4 and MSP5 are selected by Malaria Vaccine Initiatives as potential vaccine candidates for preclinical development and manufacture (http://www.malariavaccine.org/ab-current_projects.htm). Their potential as two components of a multistage malaria vaccine based on DNA immunization is also being investigated.[3] We examined the isolation of the plasmid pVR1020-PyMSP4/5 using a methacrylate monolithic adsorbent from *E. coli* DH5α-pVR1020-PyMSP4/5 lysate. The synthesis and characterization of the polymeric resin are presented. The possibility of amino-functionalized methacrylate monolith for a single-stage anion-exchange purification of the plasmid vaccine is investigated with the view of enabling reduced number of unit operations in the downstream process, thus, improving vaccine recovery

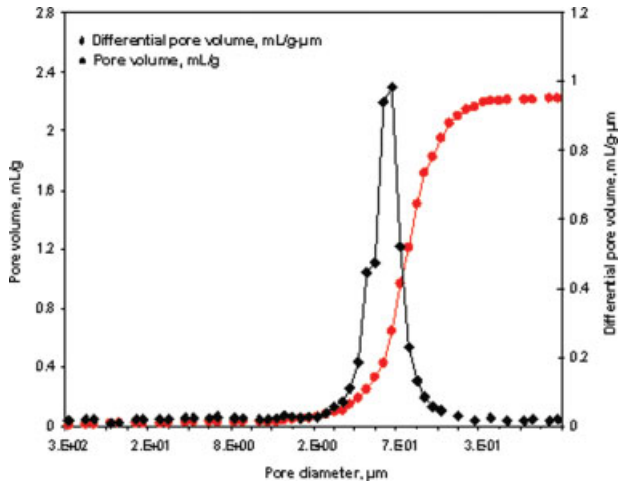

Figure 4. Average cumulative pore volume and differential pore volume against pore diameter of the methacrylate monolithic polymer using Hg intrusion porosimeter.

The plot shows a modal pore diameter of 750 nm existing in the matrix and a total pore volume of 2.20 mL/g. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

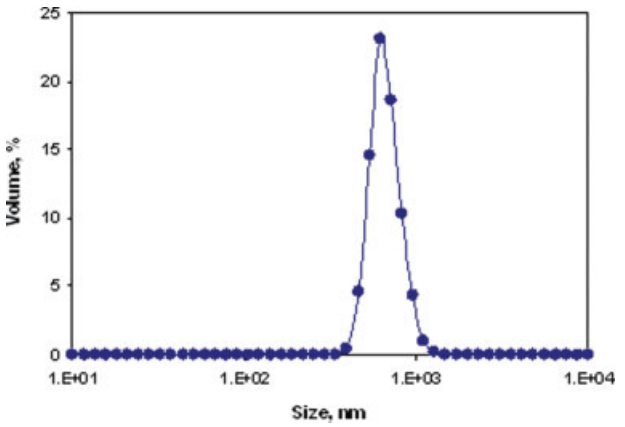

Figure 5. pVR1020-PyMSP4/5 molecular size analysis in TE buffer (25 mM Tris-HCl, pH = 8) using a zetasizer (Malvern zetasizer, ZEN 3600, U.K.).

A hydrodynamic size of ~600 nm was obtained. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1. Summary of the Pore Characteristics of the Methacrylate Polymer

Radial Positions	Total Intrusion Volume, ml/g	Modal Pore Diameter, nm	Porosity, %	BET Surface Area, m^2/g
Centre	2.21 ± 0.04	753.8 ± 2.5	79.6 ± 2.2	7.21 ± 0.02
6 mm	2.19 ± 0.08	751.5 ± 2.3	77.1 ± 2.3	7.12 ± 0.01
12 mm	2.18 ± 0.02	749.2 ± 2.2	76.3 ± 2.1	7.07 ± 0.04

The polymer feedstock compositions: EDMA/GMA mixture (40/60 % v/v) combined with cyclohexanol/AIBN mixture in the proportion 25/75 % v/v. Polymerization was performed at 60 °C. Data reported represent the mean and standard deviation of three replicates $\overline{X} \pm SD$, N = 3.

and productivity, while maintaining plasmid integrity (Figure 1). Comparison between the nature and characteristics of the final purified malaria vaccine and that of regulatory standards is also presented.

Experimental

Materials

Ethylene glycol dimethacrylate (EDMA) (MW 198.22, 98 %), Glycidyl methacrylate (GMA) (MW 142.15, 97 %), Cyclohexanol (MW 100.16, 99 %), Azobisisobutyronitryl (AIBN) (MW 164.21, 98 %), Methanol (HPLC grade, MW 32.04, 99.93 %), 2-Chloro-*N*,*N*-diethylethylamine hydrochloride (DEAE-Cl) (MW 172.10, 97 %) and Bradford reagent were purchased from Sigma-Aldrich. Agarose (Promega), Sodium dodecyl sulfate (Amresco, MW 288.38, 99.0 %), Sodium carbonate (SPECTRUM, MW 105.99, 99.5 %), Tris (Amresco, MW 121.14, 99.8 %), EDTA (SERVA, MW 292.3, Analytical grade), Ethidium bromide (Sigma, MW 394.31, 10 mg/mL), 1 kbp DNA ladder (BioLabs, New England).

Chromatographic unit

The chromatographic unit (Biologic DuoFlow system, BIORAD) is designed with two F10 pumps for a maximum flow rate of 10 mL/min at 24 MPa, QuadTec UV/Vis detector, SV5-4 select valve, BioLogic maximizer mixer, AVR9-8 switching valves, BioLogic Maximizer buffer blending system, pH monitor, F40 workstation, BioFrac fraction collector, AVR 7-3 sample inject valve, BioLogic rack, BioLogic DuoFlow software, USB bitbus communicator and Dell controller.

Chromatographic column

The chromatographic column employed is a BIORAD glass column 10 cm × 2.5 cm (Econo-column chromatography columns, Cat # 737-2512) connected with a movable flow adaptor (Flow Adaptor, 2.5 cm column ID, 1–14 cm functional length, Cat # 738-0017).

Plasmid sample

Construction of the plasmid pVR1020-PyMSP4/5 (Figure 2) has been described previously.[24] pVR1020-PyMSP4/5 was isolated from *E. coli* DH5α-pVR1020-PyMSP4/5 bacteria after alkaline lysis using Wizard plus SV Maxipreps according to the manufacturer's instructions (Promega). The hydrodynamic size of the pVR1020-PyMSP4/5 vaccine molecule was measured to be ~600 nm in 25 mM Tris-HCl, pH = 8 using a zetasizer (Malvern Zetasizer, ZEN 3600, U.K.).

Experimental methods

Preparation of Clarified Lysate from Concentrated Cell Paste. Concentrated frozen cells of *E. coli* DH5α-pVR1020-PyMSP4/5 were thawn and resuspended by adding 150 mL of 0.05 M Tris-HCl, 0.01 M EDTA, pH 8 buffer to 15 g of bacterial cell paste and vortexing until a uniform suspension was obtained. The resuspended cells were contacted and homogeneously mixed with the same volume of lysis solution (0.2 M NaOH, 1 % SDS) for 5 min to release intercellular products. Neutralization was performed by the addition of an equal volume of 3 M CH_3COOK at pH = 5.5 to the lysed cell suspension to renature pDNA under the set pH condition. After gently mixing for 5 min, the mixture of pDNA-containing lysate, and the precipitated impurities, mainly gDNA was separated at 4,600 × g for 1 h to obtain a clarified lysate.

Synthesis of methacrylate monolith

As shown in Figure 3, a novel heat expulsion technique was adopted to minimize the development of a significant radial temperature gradient which causes a nonuniform pore-size distributions along the length of large-volume monoliths synthesized via bulk polymerization. Cyclohexanol/AIBN

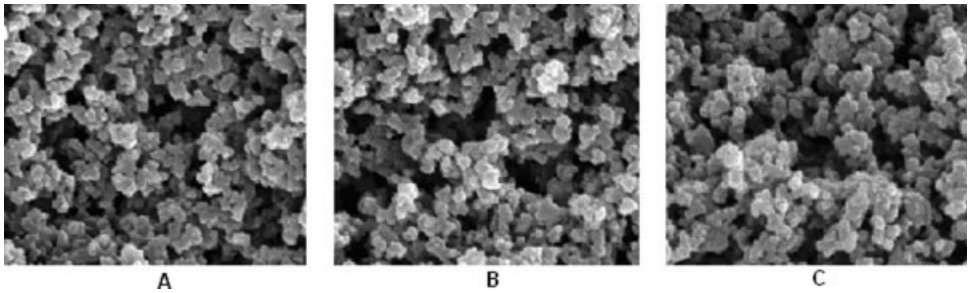

Figure 6. SEM pictures of the monolithic polymer. Pictures A, B and C show the micrographs of samples sliced from the different radial positions; center, 6 mm and 12 mm, respectively.

The samples show identical morphology.

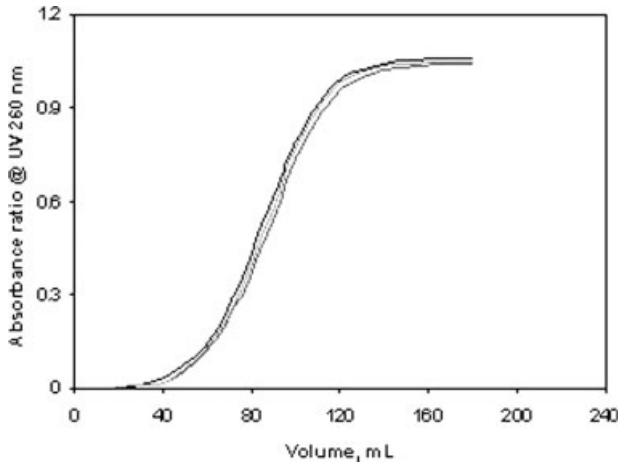

Figure 7. Dependency of the flow rate on the dynamic binding capacity.

Conditions: flow rate, 6 mL/min, 8 mL/min and 10 mL/min; sample, 9.54 μg/mL pVR1020-PyMSP4/5 in a 25 mM Tris-HCl, 2 mM EDTA pH 8; detection, UV at 260 nm.

mixture and the monomer mixture were preheated to a temperature T_o = 50 °C and isothermally pumped simultaneously into the polymerization mold (10 cm × 2.5 cm BIORAD, Cat # 737–2512), which is immersed in a water bath at T_p = 60 °C. The heat/fumes resulting from the AIBN decomposition was expelled prior to the isothermal pumping. The choice of monomer mixture and AIBN/cyclohexanol flow rates is very critical and dependent on the volume of polymer to be prepared and the polymerization time. The polymerization feedstock compositions were as follows; EDMA/GMA mixture (40/60 % v/v) was combined with cyclohexanol/AIBN mixture in the proportion 25/75 % v/v making a solution with total volume 40 mL. AIBN (1 % weight with respect to monomer) was used to initiate the polymerization process. Both the monomer mixture and cyclohexanol/AIBN mixture were sonicated for 10 min, and sparged with N_2 gas to expel dissolved O_2 prior to preheating. The polymerization was allowed to proceed for 15 h. Another monolith was synthesized under identical conditions in an identical mold for characterization. The polymer resin was washed to remove all porogens and other soluble materials with methanol in a Soxhlet extractor for 20 hours and dried at 75 °C.

Table 2. Data on pVR1020-PyMSP4/5 Binding Capacity Analysis of Poly(GMA-*co*-EDMA) Monolithic Polymer (modal pore size 750 nm) at Different Flow Rates; 6 mL/min, 8 mL/min and 10 mL/min

Flow Rates, mL/min	ΔP, MPa	Capacity at 10 % Breakthrough, mg/mL	Total Binding Capacity, mg/mL
6.0	0.10	14.40 ± 0.21	17.81 ± 0.21
8.0	0.12	14.22 ± 0.34	17.64 ± 0.23
10.0	0.15	14.15 ± 0.32	17.32 ± 0.19

Data reported represent the mean and standard deviation of three replicates $\overline{X}$ ± SD, N = 3.

Amino functionalization of epoxy groups

The methacrylate polymer was washed with 0.5 M Na_2CO_3, 1.0 M NaCl, pH = 11.5 followed by 60 g/L solution of DEAE-Cl, and the reaction was allowed to proceed for 15 h at 60 °C. The resulting polymer was washed with DI water for 1 hour and dried at 75 °C. The ligand density was found to be 1.49 mmol DEAE-Cl/g by weight difference measurement.

Physical characterization of the methacrylate polymer

The porous properties in the dry state were studied by mercury intrusion porosimeter (Autopore III, USA). The specific surface areas were obtained from nitrogen adsorption/desorption isotherms at 77 K (Micromeritics ASAP 2010, USA). Microscopic analysis of the polymer was carried out using JEOL JSM-6300F high-resolution field emission scanning electron microscope, Japan at 15 kV.

Dynamic binding capacity determination

Plasmid vaccine sample purified with Wizard plus SV Maxipreps was dissolved in 25 mM Tris-HCl, 2 mM EDTA pH 8 to a concentration of 9.54 μg/mL. The solution was pumped through the monolithic column at different flow rates (6 mL/min, 8 mL/min and 10 mL/min), and the outlet concentration was measured at 260 nm UV absorbency. The dynamic binding capacity of the monolithic column was calculated as 10 % of the final absorbency value of the breakthrough curve. The monolithic column was regenerated with 400 mL of 25 mM Tris-HCl, 2 mM EDTA, 2 M NaCl, pH = 8.

Table 3. Binding Capacity of the Amino-Functionalized Polymer with Modal Pore Size 750 nm and Ligand Density 1.49 mmol/g Measured at 10 mL/min in Repeated Loadings with Column Regeneration

Loading	1	2	3	4	5	6	7	8	9	10
Capacity at 10 % breakthrough, % of max.	100	97.5	95.5	89.5	81.3	75.4	70.1	65.2	60.8	55.3
Total binding capacity, % of max.	100	100	100	96.6	90.5	86.8	82.2	74.5	70.8	63.9
Column Regenerated after the 10th Loading										
Loading	11	12	13	14	15	16	17	18	19	20
Capacity at 10 % breakthrough, % of max.	100	95.4	88.3	82.4	76.1	70.5	65.3	60.8	55.9	51.7
Total binding capacity, % of max.	100	100	97.5	93.5	87.1	84.2	76.8	70.9	66.3	59.4
Column Regenerated after the 20th Loading										
Loading	21	22	23	24	25	26	27	28	29	30
Capacity at 10 % breakthrough, % of max.	100	100	92.7	89.5	83.6	78.3	72.4	66.5	59.8	53.7
Total binding capacity, % of max.	100	100	100	96.5	91.5	86.2	82.3	75.8	69.9	62.3

Anion-exchange purification of pDNA malaria vaccine

BIORAD glass column 10 cm × 2.5 cm containing 40 mL of amino-functionalized monolithic polymer was connected with a movable adaptor (Flow Adaptor, 2.5 cm column ID, 1–14 cm functional length), and configured to the BIORAD chromatographic unit. Chromatographic purification of pDNA vaccine was performed using 25 mM Tris-HCl, 2 mM EDTA, χ M NaCl, pH = 8 as buffer A and 25 mM Tris-HCl, 2 mM EDTA, 2.0 M NaCl, pH = 8 as buffer B. Prior to the purification experiment, the polymer was equilibrated with 5 column volumes of buffer A. 30 mL of the feedstock lysate was applied to the column at different flow rates. After washing the unbound and weakly retained molecules, the ionic strength of the buffer system was increased to elute bound species.

Endotoxin level estimation of the pDNA vaccine

Endotoxin analysis of the plasmid vaccine samples were performed using limulus amoebocyte lysate (LAL) technique according to the manufacturer's instruction (Sigma).

Column sanitation and regeneration procedure

Column sanitation was performed by washing with 500 column volumes of a solution containing 0.5 M sodium hydroxide, 2 M NaCl and 10 % ethanol followed by another 500 column volumes of 10 % ethanol solution. Column regeneration was performed with 500 column volumes of 25 mM Tris-HCl, 2.0 M NaCl, pH = 8 and equilibration was done with the appropriate starting buffer until a constant UV baseline was obtained.

Qualitative and quantitative analysis of the pDNA vaccine

The purity and concentration of pDNA malaria vaccine samples were estimated from UV absorbance at 260 nm and 280 nm. Nucleic acid analysis and characterization were performed using EtBr agarose gel electrophoresis technique with a 1 kbp DNA ladder. Gel was made up in x 50 dilution of TAE buffer (242 g of Tris-base, 57.1 mL CH_3COOH, 9.305 g of EDTA), stained with 3 μg/mL EtBr and run at 66 V for 2 hours. The resulting fractionated nucleic acid gel was visualised, photographed (BIORAD, Universal Hood II, Italy), and analyzed with quantity one software for band intensities. Protein level estimation was performed using Bradford assay according to the manufacturer's instructions (Sigma-Aldrich).

Results and Discussion

Pore characteristics of the monolithic polymer

The results obtained from the pore analysis show a common unimodal pore-size distribution for different samples sliced from different radial positions (center, 6 mm and 12 mm), with an identical maximum occurring pore diameter of 750 nm according to Figure 4. This value shows a suitable pore diameter of the monolith as a stationary phase for the plasmid vaccine molecule penetration and retention considering the plasmid (pVR1020-PyMSP4/5) molecular hydrodynamic size of ~600 nm (Figure 5). The total pore volume of the polymer is 2.20 mL/g, and the BET surface area obtained from N_2 adsorption/desorption isotherm at 77 K is 7.1 m^2/g. About 75 % of the pores within the matrix have diameters greater than 650 nm. Table 1 shows a summary of the pore characteristics of the polymer. Scanning electron micrographs in Figure 6 reveals the homogeneous porous network structure of the polymer matrix.

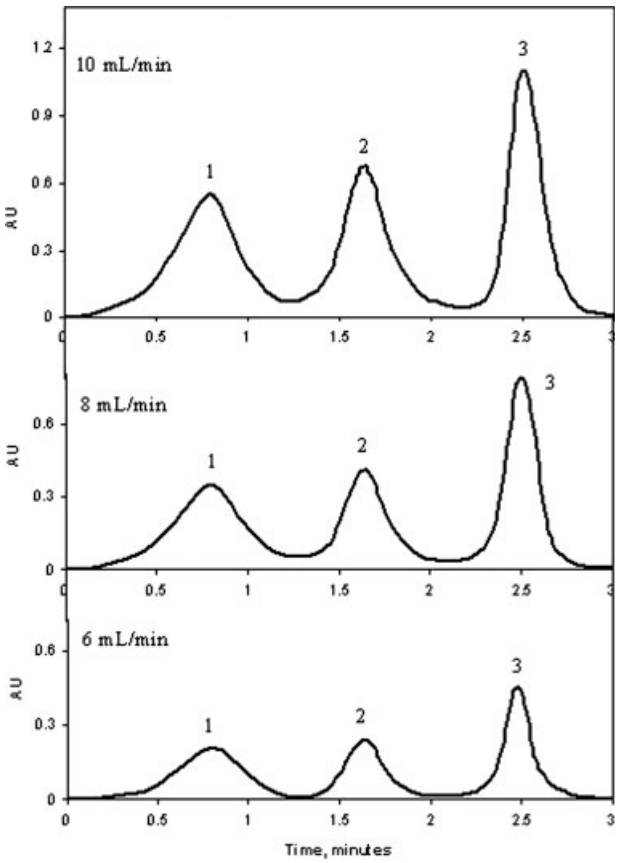

Figure 8. Effect of the flow rate on resolution for the isolation of pVR1020-PyMSP4/5 from *E. coli* DH5α-pVR1020-PyMSP4/5 clarified lysate at three different flow rates (6 mL/min, 8 mL/min and 10 mL/min).

Mobile phase: 25 mM Tris-HCl, 2 mM EDTA, 0.2 M NaCl, pH 8 (buffer A), and 25 mM Tris-HCl, 2 mM EDTA, 2.0 M NaCl, pH 8 (buffer B). Gradient elution: 0–0.325 M for 102 s and Step elution, 0.325–0.75 M for 78 s. Peaks 1, 2 and 3 represent RNA, proteins and pVR1020-PyMSP4/5 vaccine fractions, respectively.

Dynamic binding capacity of the monolithic polymer

Rapid preparative-scale purification of plasmid vaccines hinges predominantly on the use of stationary phases with high retention capacity maintained at high-flow rates with low-pressure drops. Hence, it is imperative to study the dynamic binding capacity at different flow rates. Analysis was performed by loading the plasmid vaccine sample on the monolithic column at three different flow rates; 6 mL/min, 8 mL/min and 10 mL/min. After each loading, elution was performed with 1 M NaCl in the binding buffer. The results are as shown in Figure 7 and Table 2. Since the normalized

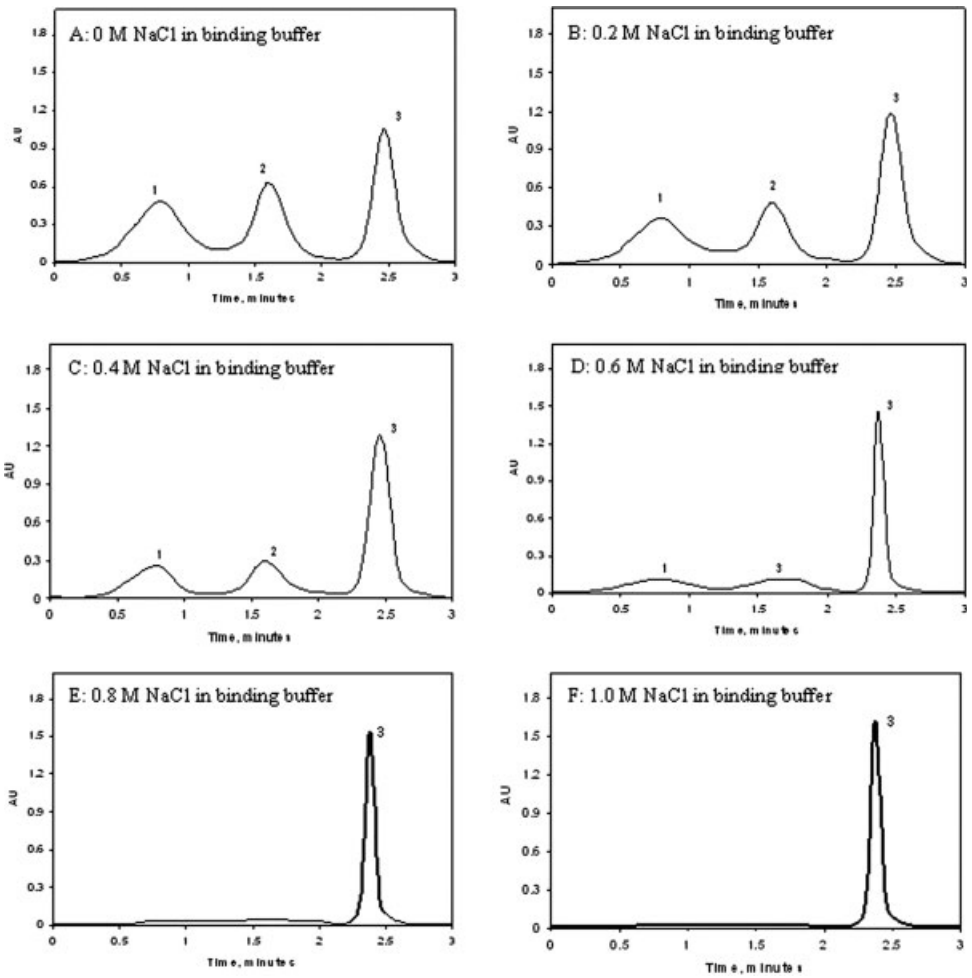

Figure 9. Effect of ionic strength of binding buffer on retention and elution of pVR1020-PyMSP4/5 from *E. coli* DH5α-pVR1020-PyMSP4/5 clarified lysate.

Chromatograms show reduction in the copurification of RNA and protein contaminants with increasing salt concentration. Stationary phase: amino-functionalized methacrylate monolith with active group density 1.49 mmol/g polymer and modal pore size 750 nm. Mobile phase: 25 mM Tris-HCl, 2 mM EDTA, *x* NaCl, pH = 8. Sample: 30 mL of clarified lysate. Flow rate; 10 mL/min. Gradient elution: 0–0.325 M for 102 s and Step elution, 0.325–0.75 M for 78 s. Peaks 1, 2 and 3 represent RNA, proteins and pVR1020-PyMSP4/5 vaccine fractions, respectively.

breakthrough curves overlap each other at the different flow rates, it can be concluded that the binding capacity is not affected by increasing flow rates. The capacity of the polymer is 0.59 g of pVR1020-PyMSP5/5, which gives a binding capacity of 14.2 mg/mL of support. As shown in Table 3, the binding capacity persisted after several applications of the polymer.

Isolation of pDNA malaria vaccine from clarified bacteria lysate

Figure 8 shows the resulting chromatograms for the isolation of the pDNA malaria vaccine from clarified lysate at the different flow rates; 6 mL/min, 8 mL/min and 10 mL/min. The chromatogram shows co-purification of protein and RNA resulting from the electrostatic interaction between the positively charged matrix and negatively charged RNA and protein molecules accompanying the target plasmid vaccine molecules in the clarified lysate. Bound RNA, proteins and the pDNA vaccine molecules were eluted, respectively as first, second and third peaks on the chromatogram. Peak elution of the molecules is in order of increasing anionic charge density. Increasing the ionic strength of the binding buffer was adopted to minimize the adsorption of low-charge density contaminants; RNA and protein (Figure 9). Under this condition, impurities gradually flow through, and the entire capacity of the polymer is fully utilized for the pDNA vaccine molecules adsorption. The final pDNA vaccine product obtained was a homogeneous supercoiled pDNA free from gDNA and RNA contaminations as shown by the EtBr agarose gel electrophoresis in Figure 10.

Endotoxin level estimation of pDNA malaria vaccine sample

Endotoxin levels of the different pDNA malaria vaccine samples obtained from binding buffers with different ionic strengths were determined to study the effect of salt concentration on the endotoxin concentration accompanying the plasmid vaccine. The vaccine samples were serially diluted

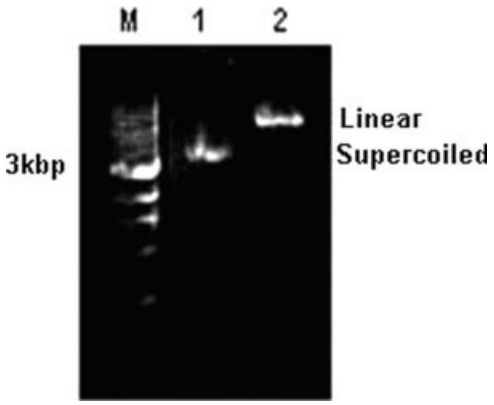

Figure 10. Results from EtBr agarose gel electrophoresis of pVR1020-PyMSP4/5 fraction from the final chromatographic purification with binding buffer 25 mM Tris-HCl, 2 mM EDTA, 1.0 M NaCl, pH = 8.

Analysis was performed using 1 % agarose in TAE x 1 buffer, 3 μg/ml EtBr at 66 V for 2 h. Lane M is 1 kbp DNA ladder; lane 1 represents supercoiled pDNA fraction, and lane 2 shows band for linear form obtained from BamHI cleavage at the sequence -G-G-A-T-C-C- of the final plasmid vaccine. Gel picture reveals no band for RNA or gDNA contaminants.

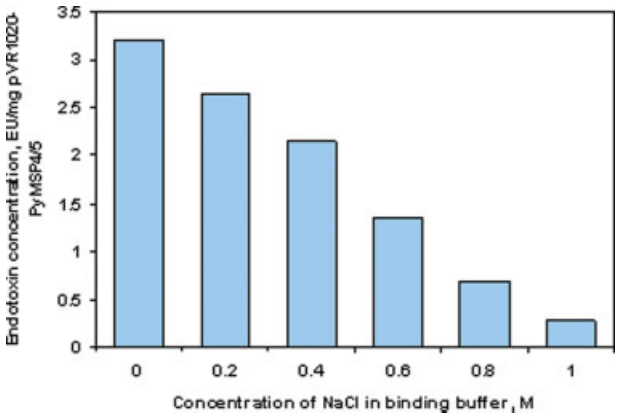

Figure 11. Effect of NaCl concentration on pVR1020-PyMSP4/5 vaccine endotoxin level.

The analysis shows a gradual decrease in endotoxin level from 3.21 EU/mg pDNA to 0.28 EU/mg pVR1020-PyMSP4/5 for 0 M and 1.0 M NaCl, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with endotoxin-free water in combination with E-TOXATE (Sigma, Catalogue No. 9154), and compared to a serially diluted endotoxin standard (*E. coli* 0.55:B5 lipopolysaccharide) with 10,000–20,000 endotoxin units (EU) per vial. The analysis shows a gradual decrease in endotoxin level from 3.21–0.28 EU/mg pDNA vaccine with increasing salt concentration in the binding buffer from 0–1.0 M, respectively (Figure 11). Endotoxins present in *E. coli* are primarily lipopolysaccharide complex units enclosed in its outer envelope. The presence of high-salt concentrations in the binding buffer causes an osmotic shrinkage via the primary hydrophobic sites for larger molecular size endotoxin units, thereby decreasing molecular size of the lipopolysaccharide complexes. This makes them to easily flow through the monolithic polymer with minimal interaction; thus, causing a decrease in endotoxin level accompanying the pDNA vaccine fraction. Also, the exposed hydrophobic groups on the lipopolysaccharide complexes cause weaker or no interaction with the polymer even at lower ionic strengths of the binding buffer, hence, can easily be washed off.

Quality and purity analysis of the plasmid vaccine product

The purified pVR1020-PyMSP4/5 malaria vaccine product was sterile filtered to meet release or administration specifications. The purified malaria vaccine product specifications was adjudged as in Table 4 to be in conformity with defined values of regulatory agencies for key contaminants such as proteins, RNA, gDNA, endotoxins and nonsupercoiled pDNA (open circular or linear). The most commonly used analytical technique for examining nucleic acid purity and quality is EtBr agarose gel electrophoresis. This technique is based on the different migration rates (from negative terminal to the positive terminal) of the nucleic acid components in the vaccine sample. The different components can be visualised, photographed, identified and quantified. Other methods like qPCR, HPLC ribose assay, BCA assay (or Bradford assay and SDS page), and LAL assay can be used to determine gDNA, RNA, proteins and endotoxin levels, respectively.

Table 4. Properties of the Purified pVR1020-PyMSP4/5 Malaria Vaccine Product

Components	Regulatory Standards for pDNA Vaccine Delivery [23]	Properties of Purified Plasmid Malaria Vaccine Sample	Remarks
% of supercoiled pDNA	>90%	92.5 ± 1.3 (band densitometric analysis)	Conforms to regulatory standards
% of *E. coli* gDNA	<1%	Undetected by EtBr agarose gel electrophoresis with sensitivity < 0.01%	Conforms to regulatory standards. Quantitative techniques could be employed to estimate exact concentrations.
% RNA	<0.1 %	Undetected by EtBr agarose gel electrophoresis with sensitivity < 0.01%	Conforms to regulatory standards. Quantitative techniques could be employed to estimate exact concentrations.
Endotoxin	<0.5 EU/mg pDNA	0.28 ± 0.11 EU/mg pDNA vaccine (by LAL assay)	Conforms to regulatory standards
% Proteins	<1 %	0.26 ± 0.08 % by Bradford assay	Conforms to regulatory standards

Data reported represent the mean and standard deviation of three replicates $\overline{X}$ ± SD, N = 3.

Conclusion

The need for commercially viable processes to manufacture a plasmid-based malaria vaccine is easy to overlook when attention is focused principally on vector design and establishment of early clinical results. pDNA is a large molecule and has properties that are similar to those of its contaminants. This, coupled with the low-initial concentration of plasmid in the host cell, create unique challenges that require detailed process engineering design to establish reproducible manufacturing methodologies that comply with cGMP. The body of work expounded in this article uses a commercially-viable technique for the rapid isolation of a pDNA malaria vaccine using a 40.0 mL methacrylate monolithic stationary phase. Characterization of the methacrylate polymer showed suitable pore properties for high-retention of the pDNA vaccine molecules. The final vaccine product obtained after 3 min elution was a supercoiled pDNA vaccine molecule with gDNA, RNA, protein and endotoxin levels that met regulatory standards for vaccine delivery. The polymer displays the potential to reduce the number of unit operations in post-clarification plasmid downstream processing from greater than three to a single-stage purification. This technique presents a great breakthrough in plasmid-based malaria vaccine production as downstream processes can now be carried out effectively and efficiently to ultimately enhance the productivity of large-scale pDNA vaccine manufacture. We are currently examining the safety profile of this pDNA preparation, examining its immunogenicity and protective efficacy by different formulations and delivery regimes.

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Literature Cited

1. Dunachie SA. Prime-boost strategies for malaria vaccine development. *J Exp Biol*. 2003;206:3771–3779.
2. Gallup JL, Sachs JD. The economic burden of malaria. *Am J Trop Med Hyg*. 2001;64:85–96.
3. Kumar S, Epstein JE, Richie TL, Nkrumah FK, Soisson L, Carucci DJ, Hoffman SL. A multilateral effort to develop DNA vaccines against falciparum malaria. *Trends Parasitol*. 2002;18:129–135
4. Moore AC, Hill AV. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. *Immunol Rev*. 2004; 199:126–143.
5. Scorza T, Grubb K, Smooker P, Rainczuk A, Proll D, Spithill TW. Induction of strain-transcending immunity against Plasmodium chabaudi adami malaria with a multiepitope DNA vaccine. *Infect Immun*. 2005;73(5):2974–2985.
6. World Health Organisation, Malaria Fact sheet N° 94, May 2007, viewed 5th Oct 2007,www.who.int/mediacentre/factsheets/fs094/en/.
7. Nabel GJ, Nabel EG, Yang Z, Fox BA, Plautz GE, Gao X, Huang L, Shu S, Gordon D, Chang AE. Direct Gene Transfer with DNA-Liposome Complexes in Melanoma: Expression, Biologic Activity, and Lack of Toxicity in Humans. *Proceedings of the National Academy of Sciences*. 1993;90:11307–11311.
8. Levy MS, O'Kennedy RD, Ayazi-Shamlou P, Dunnill P. Biochemical engineering approaches to the challenges of producing pure plasmid DNA. *Trends in Biotechnol*. 2000;18:296–305.
9. Danquah MK, Forde GM. Towards the design of a scalable and commercially viable technique for plasmid purification using a methacrylate monolithic stationary phase. *J Chem Technol Biotechnol*. 2007;82:752–757.
10. Durland R, Eastman E. Manufacturing and quality control of plasmid-based gene expression systems. *Adv Drug Deliver*. 1998;30:33–48.
11. Diogo MM, Queiroz JA, Prazeres DMF. Chromatography of plasmid DNA. *J Chromatogr A*. 2005;1069:3–22.
12. Prazeres DMF, Schluep T, Cooney C. Preparative purification of supercoiled plasmid DNA using anion exchange chromatography. *J Chromatogr A*. 1998;806:31–45.
13. Danquah MK, Forde GM. The suitability of DEAE-Cl active groups on customized poly(GMA-co-EDMA) continuous stationary phase for fast enzyme-free isolation of plasmid DNA. *J Chromatogr B*. 2007;853(1–2):38–46.
14. Svec F. Recent developments in LC column technology. *LC-GC Europe*. 2003;16(6a):24–28.
15. Jungbauer A, Hahn R. Monolith for fast bioseparation, bioconversion and their applications in biotechnology. *J Sep Sci*. 2004;27:767–778.
16. Zochling A, Hahn R, Ahrer K, Urthaler J, Jungbauer A. Mass transfer characteristics of plasmids in monoliths. *J Sep Sci*. 2004;27:819–827.
17. Podgornik A, Jancar J, Merhar M, Kozamernik S, Glover D, Cucek K, Barut M, Strancar A. Large-scale monolithic columns: design and properties. *J Biochem Biophys Methods*. 2004;60:179–189.
18. Wen J, Guillo C, Ferrance JP. DNA extraction using a tetramethyl orthosilicate-grafted photopolymerized monolithic solid phase. *J Anal Chem*. 2006;78(5):1673–1681.
19. Marshall VM, Silva A, Foley M, Cranmer S, Wang L, McColl DJ, Kemp DJ, Coppel RL. A second merozoite surface protein (MSP-4) of Plasmodium falciparum that contains an epidermal growth factor-like domain. *Infect Immun*. 1997;65(11):4460–4467.
20. Marshall VM, Tieqiao W, Coppel RL. Close linkage of three merozoite surface protein genes on chromosome 2 of Plasmodium falciparum. *Mol Biochem Parasitol*. 1998;94(1):13–25.
21. Kedzierski L, Black CG, Coppel RL. Characterisation of the merozoite surface protein 4/5 gene of Plasmodium berghei and Plasmodium yoelii. *Mol Biochem Parasitol*. 2000;105:137–147.
22. Kedzierski L, Black CG, Coppel RL. Immunization with recombinant Plasmodium yoelii merozoite surface protein 4/5 protects mice against lethal challenge. *Infect Immun*. 2000;68(10):6034–6037.
23. Rainczuk A, Smooker PM, Kedzierski L, Black CG, Coppel RL, Spithill TW. The protective efficacy of MSP4/5 against lethal Plasmodium chabaudi adami challenge is dependent on the type of DNA vaccine vector and vaccination protocol. *Vaccine*. 2003;21(21–22):3030–3042.
24. Wang L, Kedzierski L, Schofield L, Coppel RL. Influence of glycosylphosphatidylinositol anchorage on the efficacy of DNA vaccines encoding *Plasmodium yoelii* merozoite surface protein 4/5. *Vaccine*. 2005;23:4120–4127.

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