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Nanotechnology in Process Biotechnology: Recovery and Purification of Nanoparticulate Bioproducts Using Expanded Bed Adsorption

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ABSTRACT

In recent years developments in production of pharmaceutical and biotechnological products such as plasmid DNA (pDNA) as putative gene therapy vectors and protein nanoparticles as drug delivery vehicles have increased. In this study, a rapid and efficient scaleable purification protocol allowing obtaining concentrated, pure NBPs was developed. However, expanded bed adsorption (EBA) of NBPs was carried out and the dynamic binding capacity was calculated. The overall process yield of recovery of the NBPs was more than 80%, which was a superior result in expanded bed chromatography. The generic application of expanded bed adsorption for the recovery and adsorption of nanoparticulate bioproducts is strongly indicated.

Keywords: BSA nanoparticle, integrated process, nanobioproduct, plasmid DNA
Abbreviations: NBP, nanobioproduct

INTRODUCTION

Production of a nanobioproduct (NBP) by genetically engineered microorganisms, yeasts and animal cells became a very important technique for the preparation of pharmaceuticals (Anspach *et al*. 1999). Such products are characterized by critical size ranges (20-300 nm in diameter) which distinguish them from protein macromolecules (Jahanshahi *et al*. 2005). The size of NBPs strongly influences all the other properties such as diffusion coefficient, viscosity and shear force sensitivity (Urthaler *et al*. 2005). They are also specified by a complexity of surface chemistry and internal organization which poses new challenges in separation science and engineering (Jahanshahi *et al*. 2004). NBPs such as protein nanoparticles (NPs) act as suitable carrier for drug delivery, since they are biodegradable, non-toxic and non antigenic (Jahanshahi *et al*. 2008), Plasmid DNA (pDNA)-based gene therapy belongs to the category of non-viral systems (Urthaleret *et al*. 2005), thus, generally, their production is considered more scalable and it is necessary to eventually obtain a pure, defined substance of guaranteed purity and potency. However, the feedstocks from which NBPs are prepared are generally complex, containing solid and dissolved biomass of various sizes and molecular masses, respectively. This cannot be achieved with a single purification step but is usually achieved with by a combination of different unit operations that account for the different separation necessities (**Fig. 1**). Chromatography in expanded mode is considered as the method with highest resolution, therefore being essential for producing of such products suited for therapeutic and pharmaceutical applications. The most commonly used techniques are anion exchange (AIEC) (Eon-Duval and Burke 2004). Using this methodology, nanobioproducts could be purified in a single step from unclarified feedstock using the anion exchanger streamline DEAE. Expanded bed adsorption is based on controlled stable fluidization, thus combining the hydrodynamic properties of a fluidized bed with the chromatographic properties of a packed bed. The fluidization allows particulate matter to pass through the bed unhindered. The expanded bed principle, i.e. the formation of stable fluidization with a minimum of back-mixing, channeling and turbulence in the bed, allows the formation of several mass transfer units or several theoretical plates in the expanded bed, mimicking the performance of a traditional packed chromatography column (Pharmacia Biotech 1997). In this method since the liquid moves through the pores of these adsorbents by convective flow, mass transport is not diffusion-limited and therefore enhanced. In addition, the majority of binding sites are accessible to NBPs, resulting in an increased capacity (Tennikova and Svec 1993; Svec and Frechet 1995). This paper shows how expanded bed

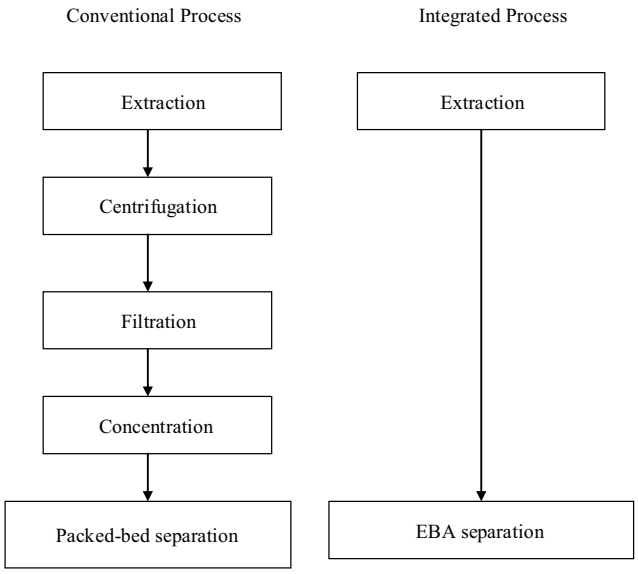

Fig. 1 Schematic presentation of nanobioproduct purification.

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adsorption has been used successfully to capture target NBPs from crude unclarified feed material. The applications cover different types of feed material; describe adsorption from bacterial culture (plasmid DNA NPs) and protein NP (BSA NPs) solution.

MATERIALS AND METHODS

Materials

BSA (fraction V, purity 98%), Tween-20, ethanolamine and glutaraldehyde (25% solution), were commercially supplied by Sigma-Aldrich (St. Louis, USA). Streamline DEAE adsorbent was provided by Amersham Bioscience (Uppsala, Sweden). The *Pseudomonas aeruginosa* bacteria containing immune plasmids to the the antibiotics penicillin and ampicillin were developed in-house. Luria–Bertani (LB) medium was from the Institute of Immunology in Iran. Kanamycin was from Roche Molecular Biochemicals (Mannheim, Germany). Other chemicals were bought from Merk (Darmstadt, Germany) and were of analytical grade.

Preparation of NBPs

The bovine serum albumin NPs (BSANPs) were prepared by a co-acervation method (Zhang *et al*. 2004). For preparation of plasmid DNA 250 mL of LB medium containing 30 µg mL^{-1} of kanamycin (Sigma-Aldrich) were inoculated with 1000 ml of an overnight shake flask culture (250 rpm, 37°C). Cells were harvested by centrifugation at 5000 rpm for 10 min (4°C), resuspended in 40 mL suspension buffer (10 mM EDTA, 50 mM Tris–HCl, pH = 8.0) and lysed by lysis buffer (0.1 M NaOH, 1% SDS). 40 ml cold (4°C) neutralization buffer (3 M potassium acetate, 10 mM EDTA, pH = 8.0) was added to the lysate for 30 min. Precipitated materials, including cell debris, chromosomal DNA, and proteins were removed by centrifugation at 6700 rpm for 20 min (Pi *et al*. 2007). High-molecular-mass RNA was eluting using 20 µg mL^{-1} RNase for 30 min at 37°C.

Equipment

A customized NBG column (Shahavi *et al*. 2008) was used in these experiments. The purification experiments were carried out at 22°C with a settle bed height of 6.0 ± 0.2 cm; the columns have a flow adapter that is positioned 0.5 ± 0.2 cm above the bed surface to suit the specific step of NBPs purification and a series of pumps and valves, connected through the adapter and bottom of the columns, to control the flow rate and direction of the buffer and sample loading. Thus, it is feasible to perform preliminary EBA trials with a little ingenuity and standard chromatographic equipment. A sintered glass was fitted onto the lower end of the columns to act as a flow distributor and a support for the bed. The liquid from the outlet of the columns was transferred through a UV detector. The outlet signal was monitored with a flow UV detector at 280 and 260 nm. The UV detector was placed as near as possible to the column outlet to reduce the dead volume in the experimental system (Tong *et al.* 2002). Proper column vertical alignment was confirmed in all experiments.

The mechanism of EBA in anion-exchange purification

When the adsorbent is packed in the column, it lies close together and leaves little room for large aggregates and clumps to manoeuver (**Fig. 2A**). As equilibration buffer is injected for at least 30 min from below, the adsorbent becomes fluidized and the adsorbent forms a stable concentration gradient when their sedimentation velocity equals the upward liquid flow velocity (**Fig. 2B**). As the sample feedstock is injected, the particulates and cell debris move freely around the adsorbent beads and eventually leave through the top of the column. As with any chromatographic step, the adsorbent then undergoes strenuous washing to limit non-specific interactions between the particulates and adsorbents. Meanwhile, the compounds of interest interact with the beads and are retained on the column (**Fig. 2C**). The column is then allowed to pack, the flow is reversed, and the compound is eluted from the adsorbents as in traditional methods (**Fig. 2E**) (Amersham). However, this last step can be perform both packed-mode and fluidized-bed mode (**Fig. 2D**). By contrast, EBA column allows improved elution peak resolution. In the case of plasmid DNA recovery, after washing with buffer (0.5 M NaCl, 10 mM EDTA, 50 mM Tris-HCl, pH = 8.0) to remove unbound material, an upward elution flow was then started with 0.5-1 M salt gradient (NaCl) in TE buffer (10 mM EDTA, 50 mM Tris-HCl, pH = 8.0) used to displace the different nucleic acids, which in principle should elute in order of increasing overall net charge (Prazeres *et al.* 1998).

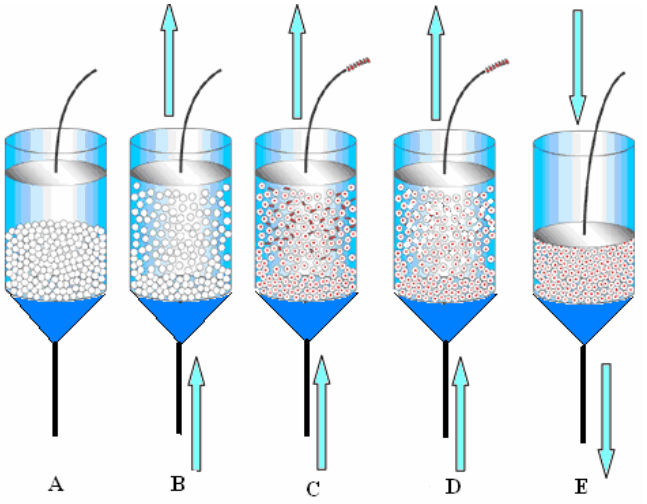

Fig. 2 Operating procedure of expanded bed adsorption. Details of A-E in text.

Dynamic adsorption of nanobioproducts by expanded bed

The breakthrough behavior of nanobioproducts in the expanded beds of the streamline DEAE was investigated by frontal adsorption experiments (Bruce and Chase 2001). The breakthrough curves measured within the expanded bed were used to calculate the total amount of NBPs bound in the column at various extents of adsorbate breakthrough. The experimental parameters are summarized in **Table 1**. A series of batch experiments had previously been performed to yield the adsorption isotherm and, from that, the maximum capacity (q_{max}) and dissociation constant (K_d), shown in **Table 1**. For each nanobioproduct all experiments were carried out at 20°C with a settled bed height of 6.0 ± 0.2 cm. BSA NP solution and unclarified plasmid DNA in equilibration buffer were used. In each run before applying feed material, the bed was allowed to expand stably at least 30 min with the equilibration

Table 1 Summary of BTC experimental parameters.

	BSA nanoparticle	**Plasmid DNA**
Adsorbent	STREAMLINE DEAE	STREAMLINE DEAE
Settled bed height (cm)	6.0 ± 0.2	6.0 ± 0.2
Expanded bed height (cm)	7.8, 9.6, 12, 13.8	7.8, 9.6, 12, 13.8
Equilibration/wash buffer	0.01 M Tris-HCl	0.5 M NaCl ,10 mM EDTA, 50 mM Tris-HCl
Buffer pH	7.5	8
Concentration of feed stock	2.5 (mg mL^{-1})	60 (µg mL^{-1})
q_{max}	21.23 (mg mL^{-1})	44.2 (µg mL^{-1})
k_d	0.95 (mg mL^{-1})	9.98 (µg mL^{-1})

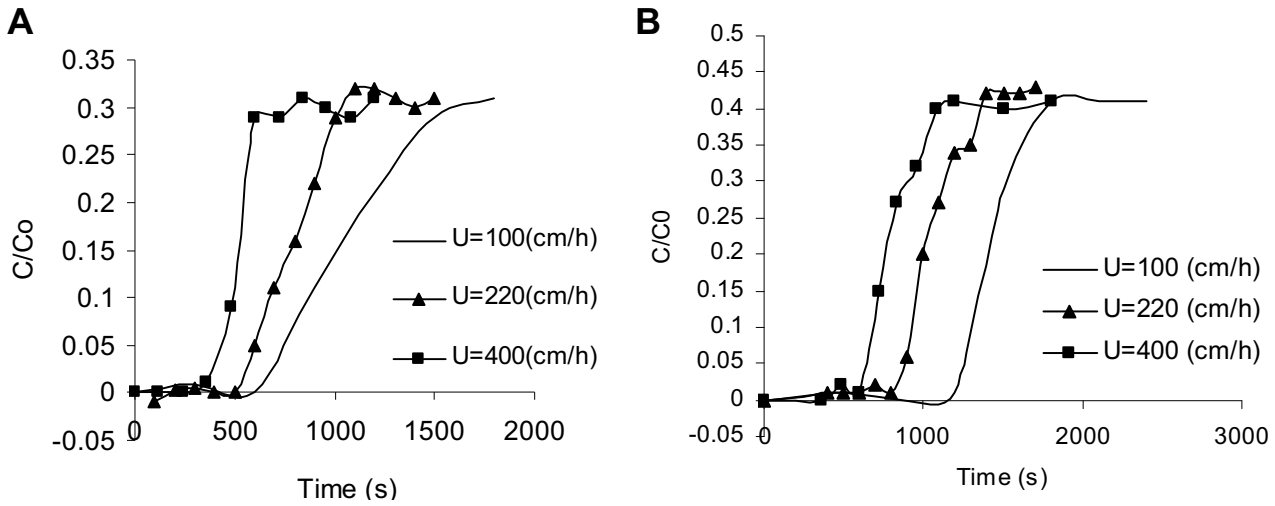

Fig. 3 Nanobioparticle breakthrough curves in the expanded bed of the streamline DEAE adsorbent. (**A**) BSA nanoparticle BTCs. (**B**) plasmid DNA nanoparticle BTCs.

buffer, and then the flow hydrodynamics of the beds was determined. The dynamic capacity, Q_{dyn}, was calculated using the estimates of adsorbent volume and void volume obtained from the RTD experiments (Tong *et al*. 2001). Dynamic binding capacity (DBC) is usually represented as the amount of NBPs bound in a column by the amount of adsorbent when the product concentration in the column effluent reaches (Bruce and Chase 2001).

RESULTS AND DISCUSSION

Dynamic adsorption of nanobioproducts by expanded bed (breakthrough curves)

In order to characterize the adsorption behavior of NBPs, breakthrough curves (BTCs) were measured (Fernandez-Lahore *et al*. 2000; Bruce and Chase 2001) and adsorption of two nanobioproducts, BSA NP (**Fig. 3A**) and pDNA (**Fig. 3B**), were studied. The breakthrough curves (BTCs), obtained at different velocity during the adsorption of BSA NP and pDNA on Streamline DEAE, and examined to determine the role of different flow velocity on overall adsorption performance. Breakthrough curves measured within the expanded bed column (expanded bed height = 7.8, 9.6, 12 cm and diameter =1.3 cm) were used to calculate the total amount of protein and pDNA NPs bound in the column at various extents of adsorbate breakthrough. As can be seen in these figures, BTCs of NBPs for u = 400 (cm h^{-1}) was steeper than other superficial flow velocity. Here, the plasmid DNA BTCs likely sharpen more than the BSA NPs BTCs because plasmid DNA has a more favourable isotherm (the dissociation constant K_d = 9.98 µg mL^{-1} for plasmid DNA, compared to 0.95 mg mL^{-1} for BSA NPs). These figures also show that the exit concentration of BSA NPs also approached the load concentration (C_0) much more gradually than plasmid DNA NPs. This is probably because BSA NP molecules begin to bind to previously adsorbed BSA NP molecules (forming dimers, etc.), which caused BSA NPs to continue to be removed from solution even though all binding sites on the streamline DEAE had been filled. Dynamic capacities calculated per unit settled volume of adsorbent in EBA column. Increasing the flow velocity results in a significant decrease of dynamic binding capacity. This indicates that the residence time for diffusion of NBPs into the adsorbent is shortened with increasing flow velocity and resulted in some of the NBPs being washed out of the column before diffusion in to adsorbent pores. Results are shown in **Table 2**.

Table 2 Parameters of BTC curve and DBC for nanobioparticles to streamline DEAE.

U (cm/h)	Plasmid DNA DBC at C/C_0=0.1 (µg mL^{-1} adsorbent)	BSA nanoparticle DBC at C/C_0=0.1 (mg mL^{-1} adsorbent)
100	58.95	8.59
220	50.75	7.60
400	46.23	6.66

Purification of NBPs from unclarified feedstock in EBA format

In pDNA purification after the optimal condition (physical and chemical) was achieved (Shahavi *et al*. 2008) the purification experiments were carried out in column with 1.3 cm in diameter and a settle bed height of 6.0 ± 0.2 cm. 42 ml of feedstock were injected and a linear NaCl gradient was performed. The streamline DEAE elution profiles also yielded two peaks (**Fig. 4**) whereas the clarified lysate loaded at a sufficiently high salt concentration, avoid from an unnecessary adsorption of low charge density impurities. Thus most of the impurities (proteins, RNA) were found in the flowthrough, while open circular (OC), supercoiled plasmid (SC), high molecular weight RNA, genomic DNA, and other plasmid isoforms were retained, and subsequently eluted as a single peak at 880–940 mM NaCl. No proteins were detected in this peak. Using this methodology, pDNA could be purified with 88% yield of recovery in a single step from unclarified lysate using the anion exchanger streamline DEAE.

In purification of BSA NPs with an average size of 150 nm (**Fig. 5**) from clarified solution the mode of expanded bed elution could result in the volume increase of the eluted

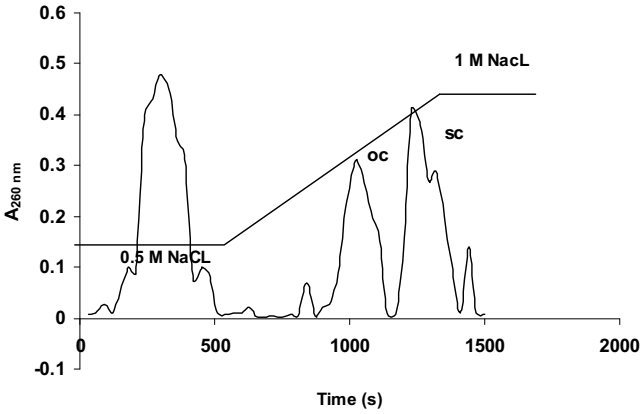

Fig. 4 Adsorption of the pDNA from unclarified lysate by expanded bed technique on streamline DEAE as adsorbent (column with 13 mm in diameter).

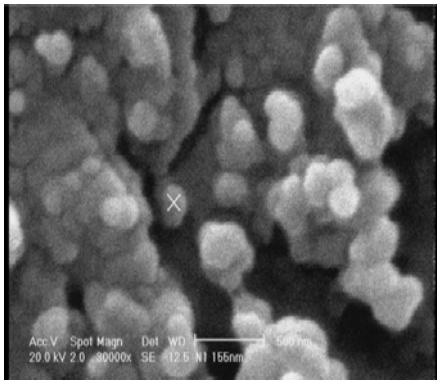

Fig. 5 Scanning electronic microscopy of the outer surface of the BSA nanoparticles as feedstock used in EBA.

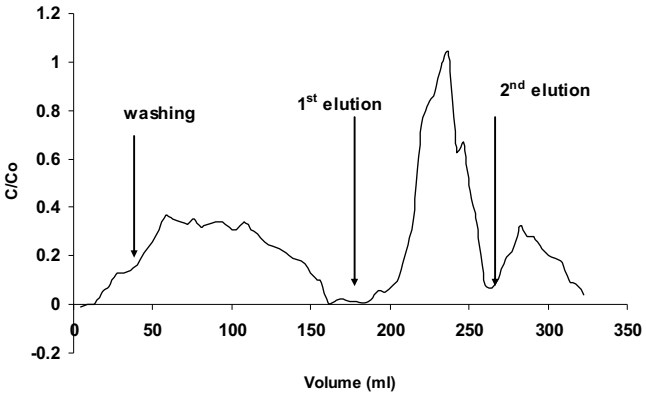

Fig. 6 Expanded bed recovery of BSA nanoparticle with streamline DEAE in column with 1.3 cm in diameter.

peak compared with the packed elution (Hjorth 1999). The time courses of BSA NP purification by EBA on the NBG column are shown in (**Fig. 6**). Since the anion exchange adsorbents have a high adsorption capacity for BSA NP in the Tris–HCl buffer containing 0.1 mol L^{-1} NaCl, this solution was used as the first elution buffer. In this case 80% yield of recovery was obtained.

CONCLUSION

Expanded-bed chromatography is a solid/liquid fluidized-bed technique with reduced mixing of adsorbent particles. It is an integrated method, combining particle separation, product concentration (Anspach *et al*. 1999). In this study it was shown that EBA system is successfully used to purify NBPs in terms of higher recovery, less process time and less solvent used, compared with the conventional process. The recovery yield increased more than 80%, showing that the expansion bed carries out the best nanobiomolecules recovery; it occurs due to more particles–nanobiomolecules interaction in expanded bed than in fixed bed by increase to bed voidage influence. However in order to characterize the adsorption behavior of these NBPs in column, BTCs were measured. Result showed that increasing the flow velocity results in a significant decrease of dynamic binding capacity. There is clearly much scope for the development of this method for recovery of nanoparticulate bioproducts.

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