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Purification of recombinant adenovirus type 3 dodecahedric virus-like particles for biomedical applications using short monolithic columns

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ABSTRACT

Adenovirus type 3 dodecahedric virus-like particles (Ad3 VLP) are an interesting delivery vector. They penetrate animal cells in culture very efficiently and up to 300,000 Ad3 VLP can be observed in one cell. The purification of such particles usually consists of several steps. In these work we describe the method development and optimization for the purification of Ad3 VLP using the Convective Interaction Media analytical columns (CIMac). Results obtained with the CIMac were compared to the already established two-step purification protocol for Ad3 VLP based on sucrose density gradient ultracentifugation and the Q-Sepharose ion-exchange column. Pure, concentrated and bioactive VLP were obtained and characterized by several analytical methods. The recovery of the Ad3 VLP was more than 50% and the purified fraction was almost completely depleted of DNA; less than 1% of DNA was present. The purification protocol was shortened from five days to one day and remarkably high penetration efficacy of the CIMac-purified vector was retained. Additionally, CIMac QA analytical column has proven to be applicable for the final and in-process control of various Ad3 VLP samples.

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1. Introduction

Virus-like particles (VLP) represent an interesting biomolecular tool for use in the field of biomedical applications. VLP are used for production of vaccines, as delivery systems, as well as in other fields of nanotechnology applications [1–7]. VLP are formed when recombinant structural viral proteins spontaneously self-assemble in baculovirus-transfected cells. Most VLP have an icosahedral structure, however, in the case of the influenza virus, non-symmetrical VLP can be formed as well [5,8,9].

Human adenoviruses are non-enveloped viruses causing respiratory infections. Their icosahedral capsid contains a 36 kbp dsDNA genome and consists of three major proteins: the hexon protein, the penton base and the fiber protein [10–12]. The two latter proteins form the penton complex, responsible for virus penetration. Twelve pentons of adenovirus serotype 3 can spontaneously self-assemble into VLP particles, called penton-dodecahedra that can be observed in infected cells. Such dodecahedra formed from pentons can be expressed in a baculovirus/insect cell system. The baculovirus system can also be employed for the expression of VLP formed only from penton bases [6,7,10–12], which are called here adenovirus type 3 dodecahedric virus-like particles (Ad3 VLP).

The baculovirus system is widely used for VLP production. Most commonly Sf9 (*Spodoptera frugiperda*) and High Five^{TM} (*Trichoplusia ni*) cells are employed and for the latter three times higher yields of VLP have been reported [3,5,13–15]. Since extract from expressing cells contains not only VLP but also cellular DNA and proteins, VLP purification represents a great challenge for the downstream processing. To obtain pure complete VLP several purification steps and a combination of various methods have to be employed. Ultracentrifugation methods such as CsCl or sucrose density gradients and various microfiltrations are commonly used, often followed by one or more chromatographic steps [5,16–20]. Such a purification procedure does not, however, provide large batches of sufficiently homogenous and pure material [5]. Traditional column chromatography can successfully remove host cell DNA and other production system impurities, but results in low VLP yields [5,16–19]. On the other hand, chromatography matrices such as hydroxyapatite, cellufine sulfate, and Q-Sepharose have been

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reported to improve yield and purity of the final product [3,5]. An alternative to conventional particle based chromatographic resins are the novel monolithic supports. Monoliths are continuous stationary phases cast as homogenous columns in a single piece [21]. Methacrylate monoliths are highly porous polymers with a distinctive structure. Pores of monoliths form a network of highly interconnected channels with diameters larger than 1.5 μm. Since all active sites are in these flow-through channels, mass transport is based on convection rather than diffusion [22–24]. These characteristics make Convective Interaction Media (CIM) monolithic supports appropriate for fast separations of macromolecules and nanoparticles. Monoliths exhibit high dynamic binding capacities for large molecules and low pressure drops at high volumetric flow rates. As a consequence of enhanced mass transfer properties the resolution and the dynamic binding capacity are flow independent [25–27]. CIM monoliths have already been successfully applied for purification of large biomolecules such as proteins, viruses and nucleic acids [28–33], however their use for the purification of VLP has not yet been reported in the literature. Recently, new monolithic columns (CIMac analytical columns) with a special design have been introduced, intended mainly for analyses of biomolecules. These columns have already proven to be of great value for the analyses of Adenovirus type 5 (Ad5), where a method for in-process control of the Ad5 purification process has been developed [34].

In this work, CIMac analytical columns were examined for purification and HPLC analyses of Ad3 VLP. Ad3 VLP have shown to be a very efficient vector for delivery of the anticancer antibiotic bleomycin (BLM) – use of Ad3 VLP resulted in over 100 fold improvement of BLM bioavailability [6]. The current purification process of Ad3 VLP consists of an ultracentrifugation step followed by ion-exchange chromatography on a Q-Sepharose column [6]. In this paper we first examined whether the CIM monolithic column was comparable to the Q-Sepharose column. Therefore, an ultracentrifugation purified sample was used for screening of anion and cation-exchange CIMac columns. In the next step, a filtered crude cell lysate sample was applied directly onto the column. We wanted to test whether Ad3 VLP could be purified directly from the cell lysate and if the ultracentrifugation step could be omitted. This would greatly improve the currently established Ad3 VLP purification process. The purity and bioactivity of the CIMac purified Ad3 VLP was determined and the recovery of the purification was estimated. CIMac columns were also examined for the in-process control of Ad3 VLP of samples from various purification steps.

2. Materials and methods

2.1. Expression of adenovirus type 3 dodecahedric VLP

Adenovirus type 3 dodecahedric VLP were expressed from a full-length human Ad3 penton base gene in the baculovirus system and purified as described earlier [6,10]. Virus amplification was performed in monolayers of Sf21 cells, maintained in TC-100 insect medium supplemented with 5% (v/v) fetal calf serum (both from Lonza, Belgium). For Ad3 VLP expression, High-Five (HF) cells grown in suspension in Express Five SFM medium (Invitrogen) with gentamicin (50 mg/l) and amphotericin B (0.25 mg/l) (both from Invitrogen) were transfected with the recombinant baculovirus at multiplicity of infection of 4 pfu/cell. After 48 h cells were collected.

2.2. Crude cell lysate Ad3 VLP (lysate sample) and pre-purified Ad3 VLP (pre-purified sample) preparation

Expressing cells were collected by centrifugation at 3000 rpm for 5 min, suspended in hypotonic lysis buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA) containing protease inhibitors (Roche) and lysed by three rounds of freezing (in liquid nitrogen) and thawing (in 37 °C water bath). The crude cell lysate was centrifuged at 13 000 rpm for 3 min and the supernatant (lysate sample) was collected for further experiments. Alternatively, clarified lysates were fractionated on 15–40% sucrose density gradients as previously described by Fender [10]. Heavy sucrose density gradient fractions containing Ad3 VLP were pooled, and dialyzed against 20 mM Tris, pH 7.5, containing 1 mM EDTA and 5% glycerol (pre-purified sample).

2.3. Chromatographic equipment

All chromatographic experiments were carried out using a gradient chromatography workstation, consisting of two pumps, an autosampler with various sample loop volumes and an UV detector (Knauer, Berlin, Germany) set to 280 nm. For data acquisition and control ChromGate 3.1.6 software (Knauer) was used.

2.3.1. CIMac™ and Q-Sepharose analytical columns

CIMac™ Convective Interaction Media analytical monolithic columns (5.2 mm I.D. × 5 mm; *V*: 0.1 ml) with the following chemistries: quaternary amine (QA), diethylamine (DEAE), sulfate (SO3) and ethylenediamine (EDA), were provided by BIA Separations (Ljubljana, Slovenia). A Q-Sepharose column was obtained by packing the Q-Sepharose™ XL resin (GE Healthcare, Uppsala, Sweden) into a stainless steel housing (4 mm I.D. × 30 mm, *V*: 0.38 ml).

2.3.2. Chemicals

All chemicals were obtained from Merck (Darmstadt, Germany), except for glycerol and EDTA which were purchased from Kemika (Zagreb, Croatia). All buffers were filtered through 0.22 μm PES membrane filters from TPP (Trasadingen, Switzerland).

2.3.3. Optimization of chromatography conditions for purification of Ad3 VLP (pre-purified sample and lysate sample)

Screenings on QA, DEAE, SO3, EDA monolithic columns and on the Q-Sepharose column were performed with the pre-purified sample in a gradient elution mode. The optimal buffering system was found to be the Tris buffer. A 20 mM Tris, pH 7.5, containing 1 mM EDTA and 5% glycerol was applied as the loading buffer (mobile phase A), and mobile phase A containing 1 M NaCl was applied as the elution buffer (mobile phase B). Further stepwise elution experiments with the pre-purified and lysate sample were performed with the same buffer system. Prior to separation, samples were diluted in mobile phase A three to five times and filtered through a 0.45 μm filter (Chromafil CA-45/25, Machery-Nagel). All experiments were performed with the 1 ml/min flow rate using various linear or stepwise gradients, as depicted in the respective figures.

2.3.4. Dynamic binding capacity determination

The dynamic binding capacity (DBC) for Ad3 VLP in the pre-purified sample was determined by continuously pumping the pre-purified sample (twice diluted with mobile phase A) through the column. Flow-through fractions were collected and analyzed by SDS-PAGE for the presence of bands characteristic for Ad3 VLP. These appeared in the collected fractions only when Ad3 VLP were no longer able to bind to the CIMac column because the DBC of the column was exceeded. The retention time of the last fraction not containing Ad3 VLP was used for the DBC calculation using the following equation:

$$K_d \quad [\text{particles/ml}] = \frac{(\Phi \times t_R - V_m) \times C_{VLPs}}{V_{colsumn}} \tag{1}$$

where K_d stands for the dynamic binding capacity (particles/ml), Φ is the flow rate (ml/min), t_R is the retention time at the breakthrough (min), V_m is the dead volume of the column (ml), V_{column} is the column volume (ml), and C_{VLP} is the concentration of the Ad3 VLP in the loaded sample (Ad3 VLP particles/ml).

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, agarose electrophoresis

Protein content of the collected fractions was examined by SDS-PAGE, using a Mini Protean II electrophoresis Cell (Bio-Rad, Hercules, CA, USA) and 4–20% PAGE Gold gradient gels (Cambrex, Rockland, ME, USA) or 4–20% RunBlue gels (Expedeon, San Diego, CA, USA). Electrophoresis was carried out under reducing conditions according to the manufacturer's instructions; gels were run at 200 V for 60 min. Protein bands were visualized by silver staining (GE Healthcare) or with the Coomassie-based Instant Blue Stain (Expedeon). A 10–200 kDa molecular weight standards were used (Fermentas Life Sciences, Burlington, Canada). For Western blots, proteins resolved by SDS-PAGE were electroblotted onto a PVDF membrane (Millipore, Billerica, MA, USA) and revealed using rabbit anti-Dd serum (prepared in the laboratory) at 1:40,000, followed by incubation with horse radish peroxidase (HRP)-labeled anti-rabbit secondary Ab (Sigma, St Louis, MO, USA) diluted 1:160,000. Ad3 VLP were visualized using the ECL detection system (GE Healthcare).

The presence of DNA was examined by agarose electrophoresis using a Wide mini sub cell II (Bio-Rad) electrophoresis cell. Agarose gels (0.8%), containing 50 mM Tris and 200 mM glycine pH 8.0, were run at 75 V at room temperature. Staining was performed with ethidium bromide (Merck).

2.5. Transmission electron microscopy (TEM) analysis

To analyze the integrity and morphology of the Ad3 VLP, electron microscopy was performed as follows: first, the 400 MESH copper grid was placed on the sample drop for 5 min. The sample excess was blotted away from the grid and 3–5 drops of sterile water were dropped on a grid and blotted away to wash off the unbound sample. Subsequently four drops of 1% uranyl acetate were placed on the grid. After uranyl acetate was blotted away, the grid was dried at room temperature and examined at 80 kV with a Philips CM 100 transmission electron microscope (Royal Philips Electronics, Amsterdam, The Netherlands) connected to a Bioscan CCD camera. For the additional analyses of photomicrographs the Digital Micrograph (Gatan, Pleasanton, CA, USA) software was used.

2.6. Protein concentration and DNA content

The concentration of proteins was determined with the Bradford Ultra Assay (Expedeon), according to the manufacturer‘s instructions. A calibration curve was obtained with serial dilution of BSA (Thermo Scientific, Wilmington, DE, USA) diluted in the equilibration buffer. Absorbance was measured at 595 nm with a Sunrise microplate reader from Tecan (Männedorf, Switzerland). For the quantification of DNA, Quant-iT PicoGreen dsDNA Assay kit (Invitrogen) was used. DNA concentration was measured with the NanoDrop 3300 Fluorospectrometer (Thermo Scientific).

2.7. Ad3 VLP recovery estimation

Aliquots of, the lysate sample, the pre-purified sample of Ad3 VLP and fractions purified with chromatography were loaded on SDS-PAGE gels, together with various amounts of BSA. Proteins were stained with Coomassie Brilliant Blue, gels were scanned and further analyzed using Image Quant software (GE Healthcare).

2.8. Ad3 VLP internalization assay and confocal microscopy

To examine whether the VLP purified on monolithic columns retained their biological function, cell internalization was assessed by Western blot and by immunofluorescence. The Western blot analysis was performed as follows. HeLa cells were cultured in EMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS), penicillin (50 IU/ml), and streptomycin (50 μg/ml) (all from Invitrogen) at 37 °C, in 5% CO_2 atmosphere. The cells were allowed to attach to the wells of 96-well plastic dishes (2×10^4 cells/well). The medium was removed and the purified, Ad3 VLP (4 μg/100 μl) were applied to cells in EMEM without FCS. After 90 min incubation at 37 °C, cells were washed with sterile PBS, detached from wells and lysed in Laemmli solution. Samples were run on SDS-PAGE and analyzed by Western blot using rabbit anti-Ad3 VLP serum (prepared in the laboratory) as described above. For observation by confocal microscopy, HeLa cells (5×10^4) were grown overnight on coverslips. Ad3 VLP were applied to cells in EMEM without serum. After 90 min incubation at 37 °C cells were rinsed with cold PBS and fixed in 100% cold methanol for 10 min. Fixed cells were incubated for 1 h at room temperature with the primary anti-Ad3 VLP serum (1:1000), rinsed with PBS and incubated for 1 h at room temperature with the FITC-conjugated goat anti-rabbit secondary Ab (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:200), and finally with DAPI (Applichem, 1 μg/ml solution, 5 min, room temperature). Images were collected with EZ-C1 Nikon CLSM attached to an inverted microscope Eclipse TE2000 E (Nikon) using objective 60×, Plan Apo 1.4 NA (Nikon), with oil immersion. DAPI and FITC fluorescence was excited at 408 and 488 nm, and emission was measured at 430–465 and 500–530 nm, respectively. Images show a single confocal scan averaged four times with 5 μs pixel dwell. All images were collected with 1024/1024 resolution and zoom 1.0 and processed with EZ-C1 Viewer (Nikon) and Photoshop 6.0.

2.9. HPLC analysis of different Ad3 VLP samples

CIMac QA analytical column was examined as a monitoring tool for Ad3 VLP samples. A 20 mM Tris, pH 7.5, containing 1 mM EDTA and 5% glycerol was applied as mobile phase A and the same buffer containing 1 M NaCl was used as mobile phase B. Samples were separated using a gradient from 0 to 100% mobile phase B within 8 min.

3. Results and discussion

The adenovirus type 3 dodecahedric virus-like particles (Ad3 VLP) are usually purified by ultracentrifugation in a sucrose density gradient followed by purification on an ion-exchange Q-Sepharose column [6]. In our work CIM monolithic columns were examined for various purposes. First the applicability of monoliths for chromatographic purification of the sample purified in a sucrose density gradient (pre-purified sample) was studied. Several ion-exchange monoliths were tested and conditions were optimized in order to separate the Ad3 VLP from the free penton bases and insect cell DNA and proteins. The obtained results were compared to the purification on the Q-Sepharose column. In the second step, we examined whether the ultracentrifugation step could be omitted and the Ad3 VLP from crude cell lysate (lysate sample) could be purified with the monolithic columns. Beside the use of CIM monoliths for process development designs, the feasibility of using monolithic columns for the analysis of purity of the Ad3 VLP was tested as well. All experiments were performed on CIMac analytical columns with 0.1 ml volume, since only a few milliliters of sample were available. The Ad3 VLP are composed of 12 pentameric penton bases.

When denaturated they yield ~63 kDa monomers of penton base protein, a building block of Ad3 VLP. The selectivity of the binding of Ad3 VLP and the purity of the chromatographic fractions was therefore examined by SDS-PAGE under reducing conditions.

3.1. Screening of various CIM monolithic columns; comparison to Q-Sepharose column purification

Initial screenings were performed using an Ad3 VLP sample that was pre-purified on the sucrose density gradient (pre-purified sample). The pre-purified sample contained a significant amount of insect cell DNA [6]. Three CIMac anion-exchange columns (QA, DEAE, EDA) and one cation-exchange column (SO3) were tested. The SO3 cation-exchanger was examined first, since it is negatively charged and should not bind DNA. However, SDS-PAGE analysis of the SO3 fractions revealed that not all of the Ad3 VLP bound to the column under the applied conditions. Approximately one third of Ad3 VLP were in the flow-through fraction (a semi quantitative estimation made on the basis of the thickness of bands on the electrophoresis gel-data not shown).

Table 1
Concentration of NaCl needed to elute Ad3 VLP and DNA from various monolithic anion-exchange columns.

CIM anion-exchange column	C_{NaCl} at elution [mol/ml]	
	VLP	DNA
QA	0.45	0.6
DEAE	0.6	0.65
EDA	1.0	1.2

The situation was different with anion exchangers (Table 1). In all cases Ad3 VLP bound to the columns completely; there was no Ad3 VLP in the flow-through according to SDS-PAGE (data not shown). DNA was also retained on anionic columns and eluted later on in the gradient as confirmed by the PicoGreen Assay (results not shown). The type of anion exchanger had a pronounced affect on the binding of both Ad3 VLP and DNA (Table 1). The binding of the Ad3 VLP to the EDA column was the strongest among the examined anion-exchangers; Ad3 VLP eluted only at 1 M NaCl (Table 1). However, the elution peak was shallow and not well

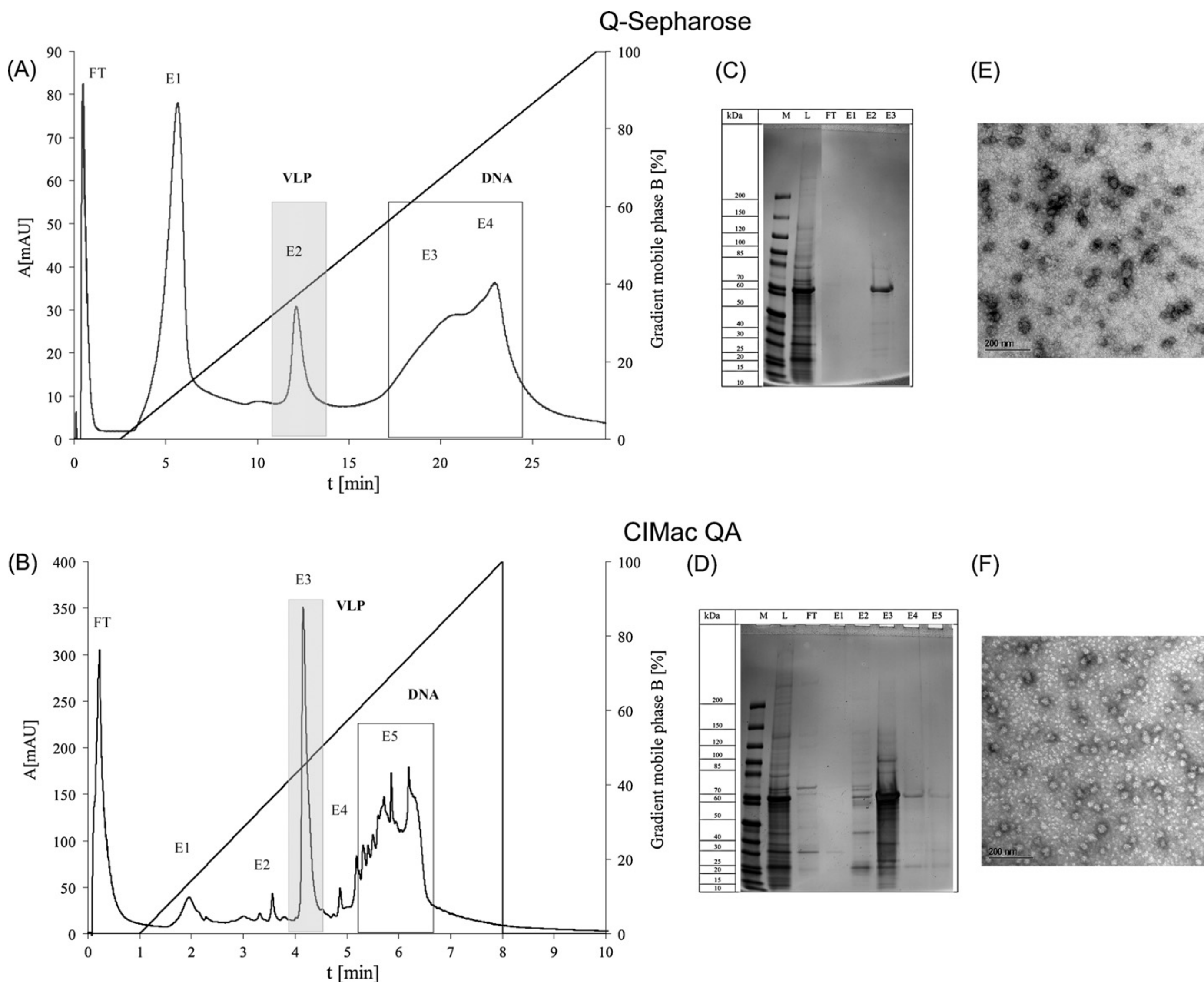

Fig. 1. Comparison of Ad3 VLP purification from pre-purified sample on a Q-Sepharose (A, C, E) and on a CIMac QA (B, D, F) columns. (A, B) Conditions: mobile phase A: 20 mM Tris, pH 7.5, containing 1 mM EDTA and 5% glycerol; mobile phase B: mobile phase A containing 1 M NaCl; injection volume: 200 μl of two times diluted pre-purified sample; method: linear gradient as shown in the figure; flow rate: 1 ml/min. (C, D) SDS-PAGE analyses of collected fractions; M: protein standards, L: loading sample; FT–E5: flow-through and eluted fractions. (E, F) Electron microscopy analyses of fractions containing VLP. Bar corresponds to 200 nm.

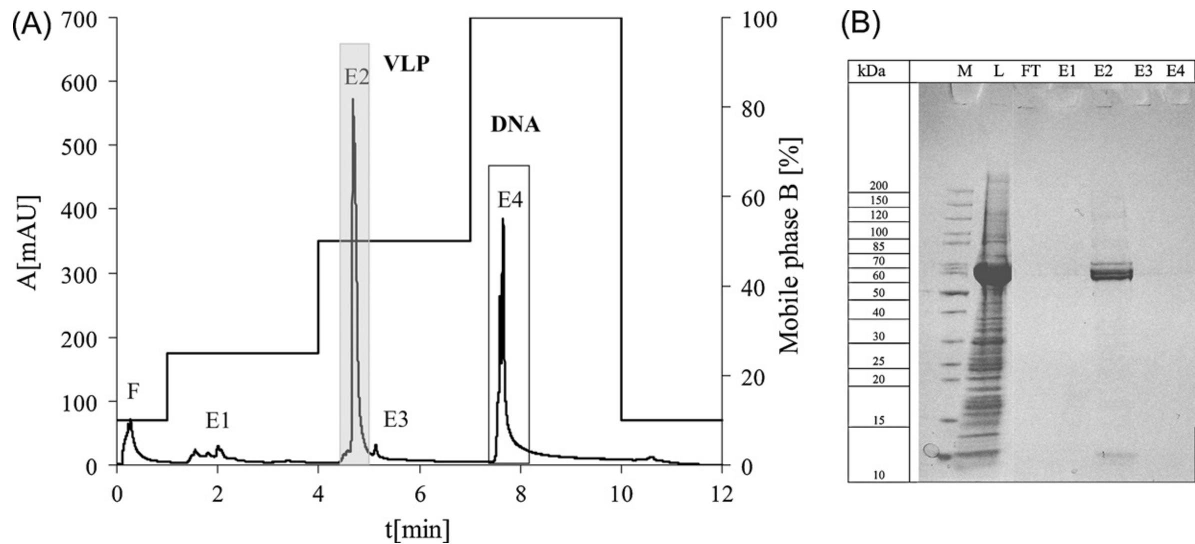

Fig. 2. Pre-purified sample fractionation on a CIMac QA column under stepwise gradient conditions. (A) Elution profile. Mobile phase A and B as described in Fig. 1. Injection volume: 200 μl of two times diluted pre-purified sample; method: stepwise gradient as shown in the figure, flow rate: 1 ml/min. (B) SDS-PAGE analysis of collected fractions; M: protein standards, L: loading sample; FT–E4: flow-through and eluted fractions.

resolved from DNA (results not shown). Ad3 VLP eluted from the DEAE column at 0.6 M salt (Table 1) and were practically coeluting with DNA. In the case of the QA column, the peak representing Ad3 VLP was high, narrow and very well resolved from DNA, which started to elute at 0.6 M NaCl (Fig. 1B). Ad3 VLP mainly eluted in one fraction (Fig. 1D, E3 fraction), they were not present in the flow-through and were well separated from DNA (Fig. 1B). The QA anion-exchange column was most suitable for purification of Ad3 VLP among the columns examined. We compared the performance of the QA column to the performance of the Q-Sepharose (Fig. 1). Both systems were examined under the same set of conditions. 200 μl of the two times diluted pre-purified sample were injected on the Q-Sepharose and CIMac QA column using a gradient from 0 to 100% mobile phase B in 70 column volumes. Comparison of chromatograms, their corresponding SDS-PAGE gels and TEM analysis showed similar results. Both columns had the Ad3 VLP peak well resolved from DNA (Fig. 1A and B) and in both cases Ad3 VLP eluted mainly in one fraction (Fig. 1C, E2 fraction and Fig. 1D, E3 fraction). According to the SDS PAGE results, the Ad3 VLP elution fraction from Q-Sepharose was purer (Fig. 1C, E2) than the one obtained from the CIMac QA column (Fig. 1D, E3 fraction). There was a difference in the run time of the analysis; one run took 30 min on the Q-Sepharose and only 10 min on the CIMac QA column. Nevertheless, material collected from both columns contained *bona fide* Ad3 VLP as confirmed by electron microscopy (Fig. 1E, F). In both cases Ad3 VLP preserved their structure and were not visibly damaged by the ion-exchange chromatographic steps.

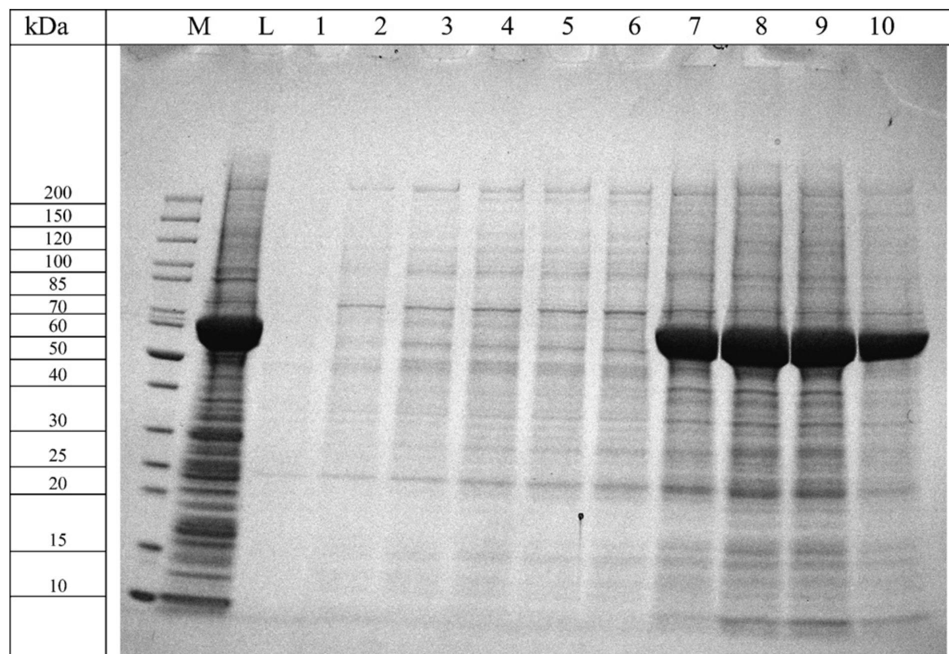

Fig. 3. Determination of the dynamic binding capacity of the CIMac QA column for the pre-purified sample. SDS-PAGE analysis of flow-through fractions; M: protein standards, L: loading sample, 1–10: collected flow-through fractions.

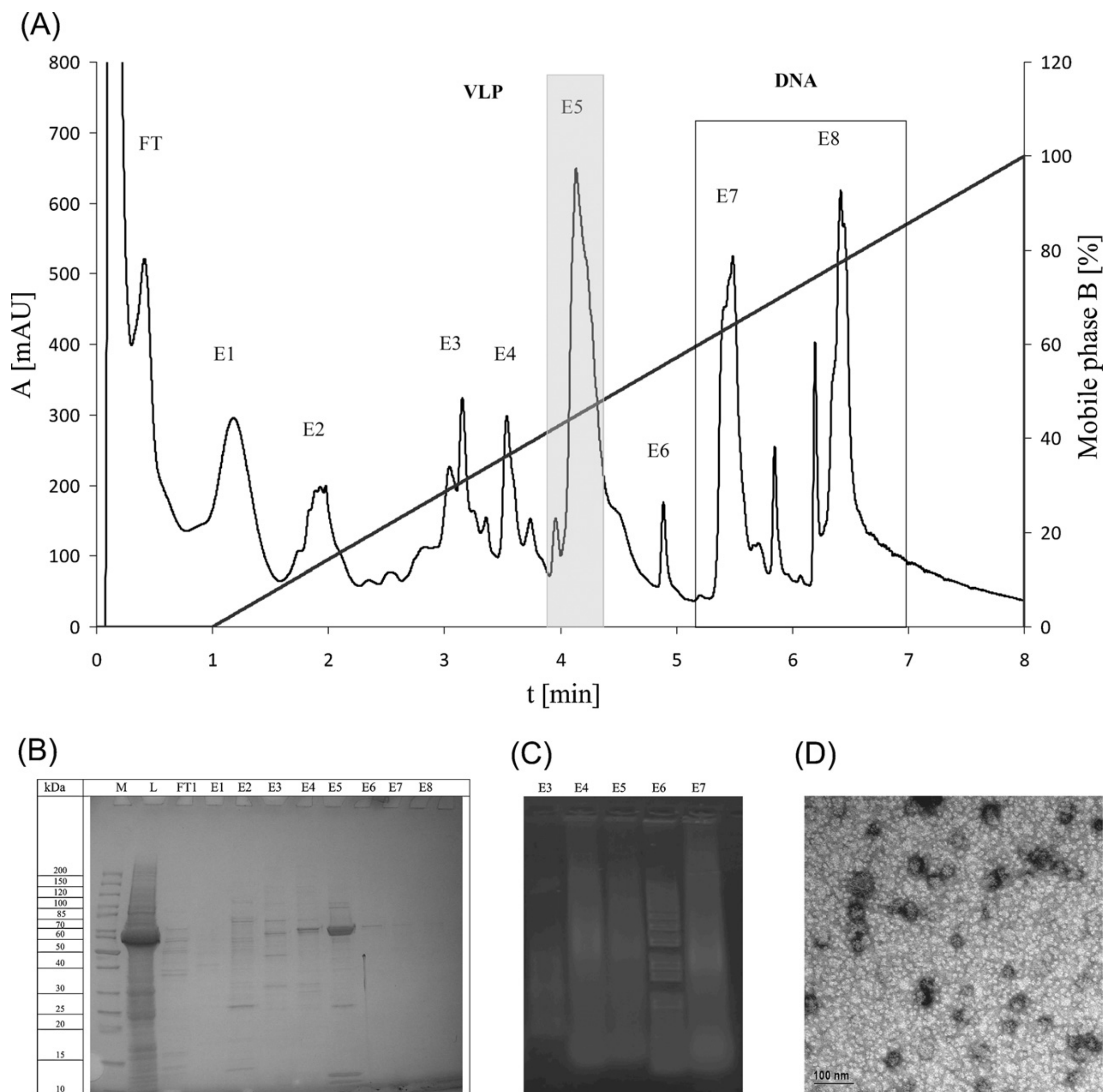

Fig. 4. Fractionation of the crude lysate (lysate sample) on a CIMac QA column. (A) Elution profile; mobile phase A and B as described in Fig. 1. Injection volume: 60 μl of three times diluted lysate sample; method: linear gradient as shown in the figure; flow rate: 1 ml/min. (B) SDS-PAGE analysis of collected fractions stained with Coomassie Brilliant Blue. M: protein standards, L: loading sample, FT–E8 flow-through and eluted fractions. (C) Agarose gel (0.8%) of collected fractions, stained with ethidium bromide. (D) TEM analysis of the main VLP peak obtained from CIMac QA, fraction E5; bar corresponds to 100 nm.

3.2. Purification with stepwise gradient and the determination of the dynamic binding capacity (DBC)

In order to simplify the purification protocol for the pre-purified sample, a stepwise gradient was designed and examined (Fig. 2A). Ad3 VLP eluted in one fraction, well separated from DNA and other impurities (Fig. 2B). To determine the amount of Ad3 VLP that can be loaded on the column, DBC for the pre-purified sample was evaluated. The CIMac QA column was equilibrated with mobile phase A. The pre-purified sample that was diluted two times with mobile phase A was continuously pumped through the column. To evaluate the breakthrough volume for Ad3 VLP, flow-through fractions were collected and analyzed by SDS-PAGE (Fig. 3). Ad3 VLP were not present in the first 6 flow-through fractions. However, the bend representing Ad3 VLP became pronounced in fraction seven and this was considered to be the breakthrough fraction. Therefore the retention time of fraction 6 was taken into account for the calculation of the DBC. The concentration of Ad3 VLP in the pre-purified sample was estimated according to the procedure described in Section 2.6 and was 16.2×10^{14} Ad3 VLP/ml. After inserting this and other experimental parameters into Eq. (1), the DBC for Ad3 VLP was calculated to be 1.38×10^{16} Ad3 VLP/ml. Such a DBC is higher than some published for particles such as bacteriophages [33], which suggests that we achieved highly efficient purification of VLP.

3.3. Analysis of the crude lysate (lysate sample)

The ultracentrifugation step used in the current purification procedure of the Ad3 VLP has many disadvantages, the main ones being low resolution and low capacity. The purification procedure of Ad3 VLP would thus gain a lot if this step could be omitted. Therefore,

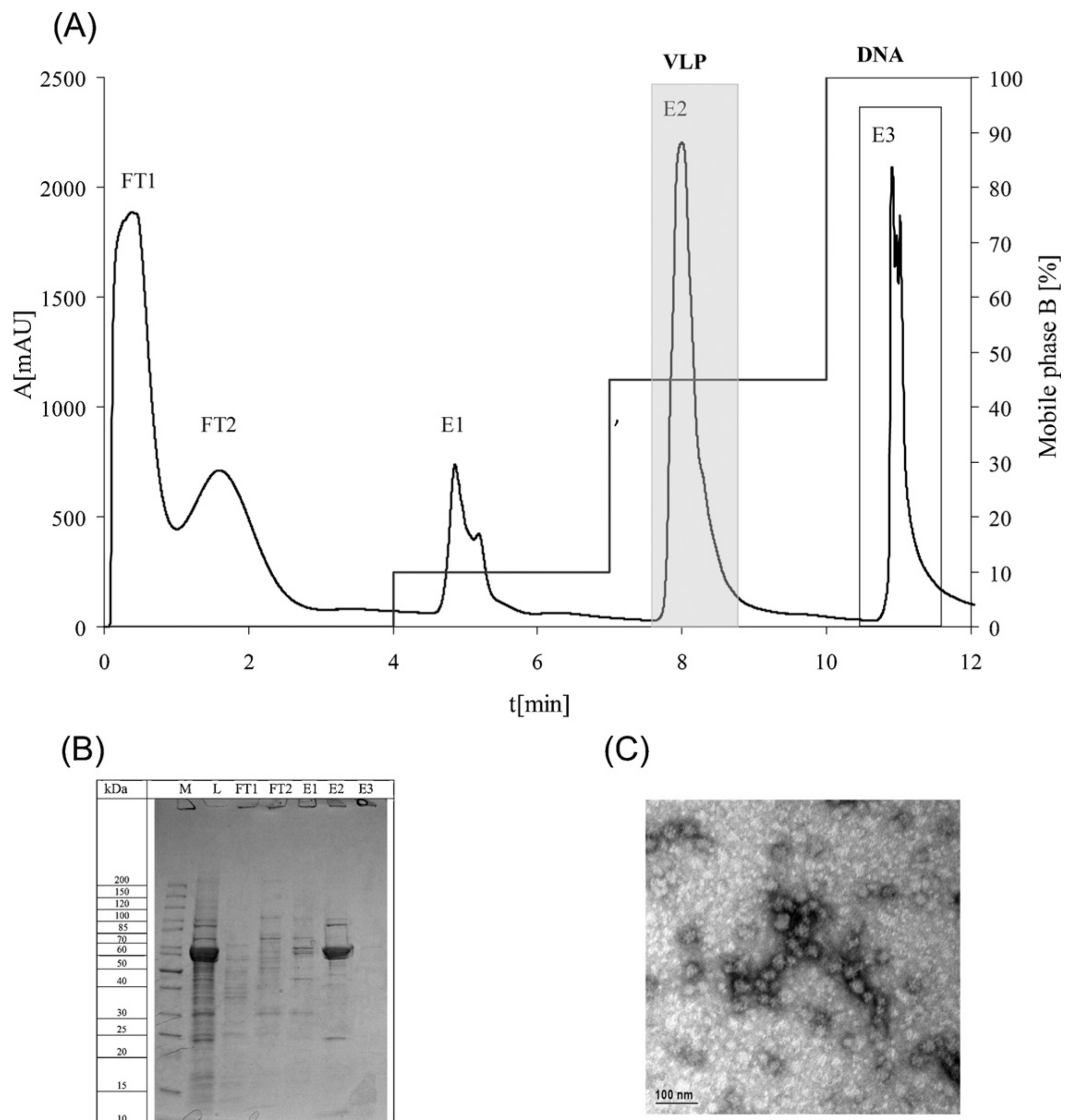

Fig. 5. Fractionation of the crude lysate (lysate sample) with a stepwise gradient on a CIMac QA column. Mobile phase A and B as described in Fig. 1. Injection volume: 500 μl of five times diluted sample, method: stepwise gradient as shown in the figure, flow rate: 1 ml/min. (B) SDS-PAGE analysis of the flow-through and eluted fractions (FT–E3); M: protein standards, L: loading sample. (C) TEM analysis of the main VLP peak from lysate sample (fraction E2 in A). Bar corresponds to 100 nm.

the CIMac QA column was examined for direct Ad3 VLP purification from the crude lysate (lysate sample). First, the same method as the one employed for purification of the pre-purified sample was applied (Fig. 4). Ad3 VLP eluted at approximately the same retention time (Fig. 4A, t_R: 4.14 min) as with the pre-purified sample (Fig. 1B; t_R: 4.16 min). SDS-PAGE analysis showed that Ad3 VLP were mainly present in the E5 fraction (Fig. 4A, B), devoid of DNA, which eluted later (Fig. 4C). The presence of Ad3 VLP was additionally confirmed by TEM analysis (Fig. 4D), showing again that the morphology of Ad3 VLP after chromatographic purification on the CIMac column remained unaltered.

Since a step-wise approach is more common, a stepwise elution method was designed (Fig. 5A). Results obtained from SDS-PAGE analysis and transmission electron microscopy showed that the Ad3 VLP were mainly present in the E2 fraction (Fig. 5B). The fraction was almost entirely depleted of DNA, less than 1% of total DNA was detected by the PicoGreen assay. The recovery of Ad3 VLP was evaluated to be approximately 52%, which is satisfactory at this point. It is comparable to the data published for other chromatographic VLP purification procedures, where recoveries below 50% have been reported [16–18]. Additionally, it is higher than in the case of the Q-Sepharose column where the recovery was around 30%.

3.4. Functional analyses of purified vector fractions

Functional analysis of biological activity for Ad3 VLP obtained from the Q-Sepharose and CIMac QA columns was carried out using the HeLa cell internalization assay. Ad3 VLP particles entry capacity was compared by the Western blot technique and in addition cell penetration was visualized by confocal microscopy. It is relevant that the Ad3 VLP undergo extensive proteolysis, which can be analyzed by Western blot with anti-Ad3 VLP antibody [6]. Similar proteolysis was observed here for the Q-Sepharose- and CIMac QA-purified Ad3 VLP (Fig. 6A, left panel). In addition, comparable amounts of intracellular Ad3 VLP were detected by Western blots in lysates of cells transduced with both preparations (Fig. 6A, right panel). Finally, the confocal microscopy images showed Ad3 VLP in

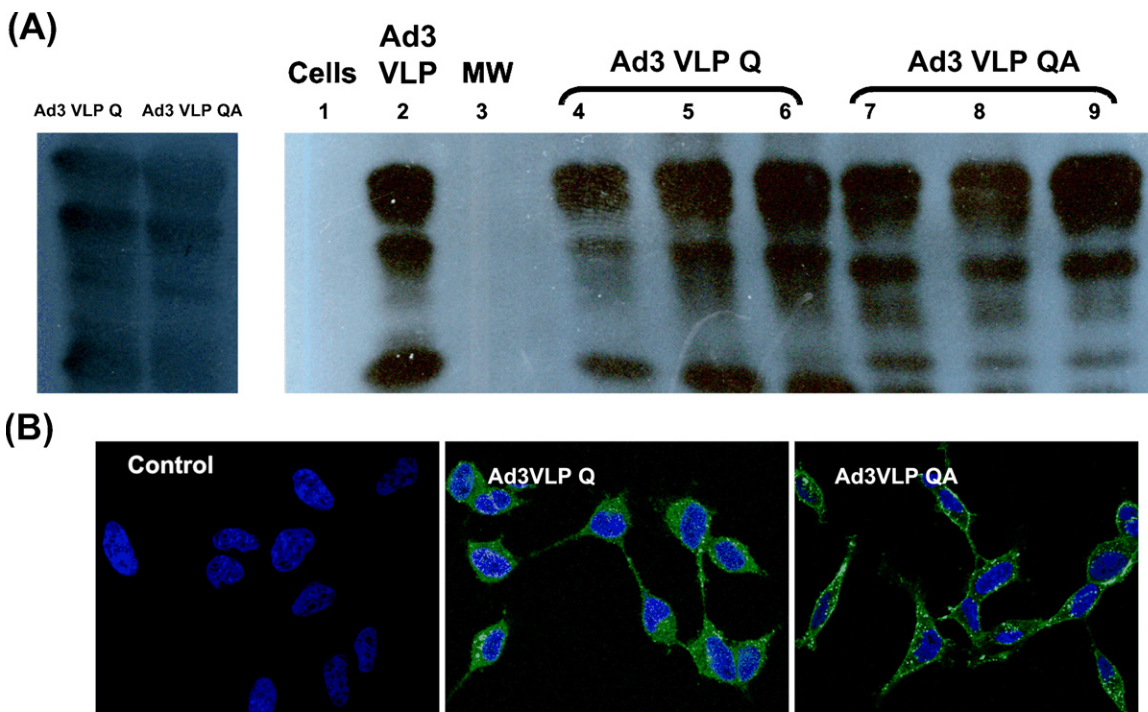

Fig. 6. Vector internalization. Ad3 VLP were applied onto HeLa cells and visualized by Western blot (A) or by confocal microscopy (B) as described in Section 2. (A) Western blot. Left panel shows the comparable quality of VLP samples obtained from Q-Sepharose and CIMac QA column, note the similar extent of proteolysis. The right panel shows Ad3 VLP in lysates obtained from vector-treated cells, visualized with anti-Ad3 VLP antibodies. Note the comparable level of intracellular Ad3 VLP. The first track contains untreated HeLa cells, the second – control vector preparation, and the third – molecular weight standards. (B) Confocal microscopy. Ad3 VLP were stained in green and cell nuclei were counterstained in blue with DAPI. The left panel shows untreated control cells, the next panels show cells with internalized Ad3 VLP vector purified by Q-Sepharose and CIMac QA, respectively.

the cytoplasm of all HeLa cells after 90 min of incubation with both preparations (Fig. 6B). These data showed that the entry potential of Ad3 VLP, that were purified by the CIMac QA monolithic column directly from the lysate sample, was not affected by the purification process. The purified VLP retained their remarkable cell penetration capacity.

3.5. In-process control of collected fractions

The CIMac QA columns are primarily intended for HPLC analysis and in-process control. The fractions collected from prior purifications were therefore further analyzed with the CIMac QA column. A short, less than 10 min long method was applied for the analy-

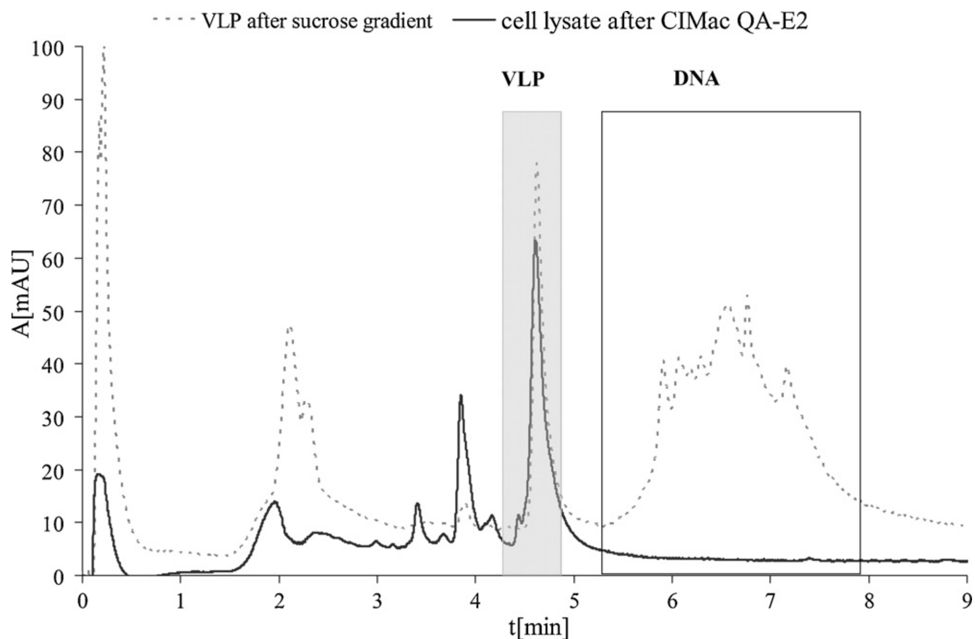

Fig. 7. Comparison of the profile of Ad3 VLP after sucrose density gradient (pre-purified sample) and the fraction E2 from the lysate sample purified with the CIMac QA column using a stepwise gradient (bold line), see Fig. 4. Conditions: mobile phase A and B as described in Fig. 1; injection volume: 200 μl of sample, five times diluted with mobile phase A; method: gradient from 0 to 100% mobile phase B in 8 min; flow rate: 1 ml/min.

sis of the pre-purified sample and the E2 fraction purified from the lysate sample (see Fig. 4A). The comparison shown in Fig. 7 indicates that the CIMac QA purified sample did not contain DNA. There was also a difference in the overall elution profile. The pre-purified sample chromatogram showed some additional peaks whereas the CIMac QA chromatogram contained mainly the Ad3 VLP peak. This demonstrates that the monolithic column can distinguish between samples containing only Ad3 VLP and samples containing DNA and other impurities.

4. Conclusions

The ion-exchange CIMac QA analytical column has proven to be an excellent option for the purification of Ad3 VLP expressed in the baculovirus expression system. The results obtained in this work were comparable to the results obtained with the currently used two-step Ad3 VLP purification procedure. QA monolithic columns have proven to be efficient in purifying Ad3 VLP from pre-purified samples as well as directly from crude cell lysate samples. With the use of the CIMac QA column the ultracentrifugation step could be omitted and the purification procedure became significantly shorter. Previously it took five days to purify Ad3 VLP from crude cell lysate, with CIMac QA the procedure was reduced to one day. The recovery of Ad3 VLP was 52% and Ad3 VLP were efficiently separated from cellular DNA and proteins. The morphology of the particles was not affected by the purification procedure on the column and the vector particles retained their biological cell penetration capacity. The CIMac QA column was utilized as a tool for designing a purification procedure as well as an analytical column for examining the purity of fractions during process development.

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