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Journal of Chromatography B, 837 (2006) 59–68
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Screening anion-exchange chromatographic matrices for isolation of onco-retroviral vectors

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Received 19 December 2005; accepted 30 March 2006
Available online 11 May 2006

Abstract

The adsorption kinetics of retroviral vectors to several chromatographic media, DEAE FF, Streamline™ Q XL and CHT™ Ceramic Hydroxyapatite, in batch mode was investigated. The effects of buffer type, pH and operational temperature were studied. A mathematical model describing viral adsorption kinetics that considers viral degradation in solution was developed. The best results, either in terms of speed and extent of adsorbed infectious particles, were obtained with DEAE FF and Streamline™ Q XL. Fixed-bed chromatography was further investigated using DEAE FF, Q XL and Q FF, for validation of the batch adsorption process. Fixed-bed DEAE FF and Q XL proved to be good candidates for purification of MoMLV derived vectors due to resulting high yields, $53 \pm 13\%$ and $51 \pm 7\%$, respectively, while removing more than 99% of protein and 90% of the DNA contaminants.

Keywords: Retroviral vectors; Purification; Anion-exchange chromatography; Adsorption kinetics; Gene therapy

1. Introduction

Retroviral vectors are one of the most commonly used vectors in ongoing clinical trials [1,2]. They allow efficient transfer and integration of the therapeutic gene in the target cells, have low immunogenicity and are only capable of infecting proliferating cells, which makes them suitable candidates for the treatment of cancer and a variety of genetic disorders [1,3].

The difficulty of producing high titer supernatants from packaging cell lines, whose production in general vary from 10^6 to 10^7 IP/ml [4], and the inherent instability [5] of the Moloney Murine Leukaemia Virus (MoMLV), poses some challenges for the downstream process. It is therefore necessary to develop highly efficient and fast purification protocols, allowing maintenance of viral infectivity with high recoveries of infectious particles, removal of contaminating proteins and inhibitors of transduction and also concentration of the viral supernatants.

Retroviral vectors (RV) are quite complex assemblies with an average diameter of 100 nm and composed by 60–70% protein, 30–40% lipid (derived from the plasma membrane of the packaging cell line), 2–4% carbohydrate and 1% RNA [6]. Traditional methods of purification and concentration of retroviral vectors include ultracentrifugation through cesium chloride or sucrose cushion and gel filtration chromatography [6–8]. These methods are time-consuming, difficult to scale up and, in the case of ultracentrifugation, present very low recoveries in terms of infectious particles (less than 1%), due to viral vector degradation upon pressure forces [7,9]. Separation of virus particles from cellular contaminants, by sucrose density gradient centrifugation, is used after initial concentration. However, infectious retroviral activity is sensitive to both ultracentrifugation and osmotic shock due to suspension in sucrose [8]. Also the co-precipitation of impurities, derived from the culture media and packaging cell lines, limits the use of these methods for production of clinical-grade retroviral vectors [10].

The use of chromatographic methods, like anion-exchange chromatography (AEXc), for the purification of proteins is

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doi:10.1016/j.jchromb.2006.03.061

widespread; it is a suitable technique for the purification of retroviral vectors due to their overall negative charge at neutral pH [11]. The majority of the chromatographic media for preparative purification has small pores, only accessible to proteins. Large particles, like viruses, cannot enter the pores and interact only with charged groups at the surface of the beads, resulting in low retention of the viral particles. New macroporous adsorbents [12] and tentacle media [13], are being explored for the purification of these complex nano-particles. Anion-exchange chromatography has been used for the purification of VSV-G pseudotyped lentiviral vectors, using HiTrap™ Q HP media ($N^+(CH_3)_3$ charged group), with 50% total infectious particles recovery [14], and of inactivated HIV-1 particles, using Fractogel® TMAE [15]. So far, the purification of MoMLV derived vectors, currently used in a number of relevant clinical applications [16–18], using anion-exchange chromatography has not been successful. Reports using the tentacle chromatography media with DEAE ligands [13], show recoveries of less than 5% of the initial VSV-G pseudotyped infectious particles loaded onto the column. Other chromatographic strategies exploring the different characteristics of retroviral vector's have also been tested, namely heparin affinity [13], streptavidin–biotin affinity (using biotinylated vectors) [12,19] and immobilized metal affinity [20] (using his-tagged retroviral vectors). Although retroviral vectors can be sensitive to high salt concentrations, needed for elution from AEXc media, this option offers milder conditions for the purification of retroviral vectors without the need to modify the vector itself, either by biotinylation or by his-tagging of the *env* proteins. Heparin affinity offers good infectious particles yields, circa 60% [13]. However, many cellular and serum contaminant proteins also have affinity to heparin; hence, the need for incorporation of another purification step to remove contaminants [13,21]. Immobilized metal affinity produces also high recoveries (56% [20]) but uses imidazole as a desorption agent which is difficult to remove from the preparations, reduces viral infectivity and is toxic. Even lower infectious particles recoveries are obtained with streptavidin–biotin affinity, 8% [12] and 16.7% [21], also due to the large degradation of the retroviral vectors in the presence of the desorption agents (d-biotin).

Initial scouting of the best conditions for viral adsorption, including matrix type and buffer, is essential. Batch adsorption was selected due to the practical advantages that it offers, as it allows testing several experimental conditions simultaneously, is simple to execute and constitutes a source of valuable information regarding adsorption kinetics, essential preliminary knowledge to define column chromatographic parameters.

This paper describes the batch adsorption of MoMLV derived vectors to several commercial ion exchange chromatographic media. A simple mathematical model capable of describing the adsorption kinetics of the MoMLV derived vectors to the chromatographic media and the vector's degradation in solution was developed. The adsorption kinetics results were validated using fixed-bed chromatography, with three different chromatographic media, DEAE FF, Q XL and Q FF, being tested.

2. Materials and methods

2.1. Cell lines and culture conditions

Retroviral vectors were obtained from supernatant of human TE FLY A7 packaging cell line, derived from TE 671 cells (ECACC no. 89071904) transformed with the plasmid pMFGSnlsLacZ [22,23] that produces murine leukaemia virus-based vectors with a 4070A envelope protein. The HCT 116 adherent cell line was used for quantitation of infectious particles (ATCC no. CL-247). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) supplemented with 4.5 g/l of glucose (Merck, Darmstadt, Germany), 6 mM of glutamine (Gibco), 5% FBS (Gibco), all final concentrations. For the production of retroviral vectors DME Base (Gibco) supplemented in the same way as DMEM was used. Additionally, the HCT 116 cell line was cultured in the presence of 0.1 mg/ml penicillin/streptomycin (Gibco) to prevent contaminations during vector titration.

2.2. Retroviral vectors production

Retroviral vectors were produced by TE FLY A7 packaging cell line in 175 cm^2 T-flasks (NUNC, Roskilde, Denmark) with an inoculum concentration of 2×10^4 viable cells/cm^2 (determined by trypan blue exclusion) in DMEM. After 48 h of growth, the supernatant was discarded, the cells rinsed with 4 ml of PBS and the medium replaced by 25 ml of DME Base medium. Twenty-four hours after the medium exchange, the supernatant was collected, filtered through 0.45 μm pore sterile filter (Starstedt, Newton, USA) to remove cells and cellular debris, and frozen at −85 °C.

2.3. Quantitation of infectious particles

For the determination of the concentration of retroviral infectious particles the β-gal assay was performed: HCT 116 target cells were seeded in 96-well, flat-bottomed plates (Sarstedt) at a density of 3.6×10^5 cells/ml (100 μl per well) and incubated during 24 h at 37 °C and 5% CO_2. Infections were then carried out by replacing the medium with 20 μl of dilutions (10^{-1} to 10^{-4}) of viral supernatants in DMEM containing 8 μg/ml of polybrene (Sigma, Steinheim, Germany) and 0.1 mg/ml penicillin-streptomycin (Gibco) followed by incubation at 37 °C for 3 h. After the 3 h incubation period, 180 μl of fresh medium was added to the plate followed by a 48 h incubation period. Then, the medium of each well was aspirated and the cells washed with 100 μl of PBS. After aspirating the PBS, 100 μl of fixing solution (0.75% formaldehyde at 37% (Merck, Darmstadt, Germany) and 5.1% of glutaraldehyde (Sigma) in PBS), was added to each well and left for 3 min before aspiration; each well was then washed with 100 μl of PBS. After removing the PBS, 100 μl of a dye solution, consisting of 5 mM $K_3Fe_8(CN)_6$ (Merck), 5 mM $K_4Fe(CN)_6$ (Merck), 1 mM $MgCl_2$ (Merck) and 200 mg/ml X-gal (Stratagene, La Jolla, USA) in DMF (Riedel de Haën, Seelze, Germany) was added to each well. Finally, the plate was incubated at 37 °C for 24 h. Viral titers

were determined by counting between 20 and 200 LacZ-positive (blue) cells in each well at the highest dilution. Average counts of at least three replicates were used and errors were calculated as the standard deviation of the mean of the replicate counts.

2.4. Batch adsorption

For the batch adsorption experiments three chromatographic media were used (Table 1): DEAE FF (Amersham Biosciences, Uppsala, Sweden), Macro-Prep CHT™ Ceramic Hydroxyapatite type I (CHT) 80 μm (Bio-Rad, Hercules, USA) and Streamline™ Q XL (Amersham Biosciences). In these experiments 20 mM sodium phosphate (at pH 7.0, 7.5 and 8.0) or Tris base (at pH 7.5 and 8.0) were tested. In all buffers 60 mM NaCl and 0.5 M sucrose (for preserving viral stability during storage at −85 °C) were used. One millilitre of each chromatographic media was first equilibrated with 10 ml of the buffer in question for 20 min, sanitized with 0.1 M NaOH for 20 min and re-equilibrated with the respective buffer. After the chromatographic media was sedimented, the buffer was removed and replaced by 20 ml of diluted defrosted retroviral supernatant (12 ml of defrosted supernatant in 8 ml of buffer solution). The control consisted of diluted retroviral supernatant in buffer solution with no adsorbent. All assays were performed in 100 ml polypropylene beakers, under constant mixing at 200 rpm in an orbital shaker at room temperature (21 °C), inside a laminar flow cabinet chamber or inside a conditioned room at 10 °C. During a 120 min period, 200 μl samples of the supernatant of each chromatographic media, and of the control were taken, centrifuged at 10,000 rpm for 30 s, and the supernatant stored at −85 °C.

2.5. Batch adsorption model

A simple model for the description of the infectious particles adsorption to the various chromatographic media tested was used. Since the retroviral vectors used in this work are very unstable, resulting in a significant loss in infectious titer with time, it was necessary to predict the infectious particles concentration in the supernatant, under conditions of saturation of the chromatographic media. This simple mathematical model involves three main equations. The first order kinetics in Eq. (1) represents the degradation of infectious particles in solution, in the absence of chromatographic media,

$$\frac{dV^{sol}}{dt} = -k_d V^{sol} \quad (1)$$

where V^{sol} is the concentration of infectious particles in solution (IP ml^{-1}) at time t (min) and k_d the infectious particles decay rate constant (min^{-1}) [24]. The infectious particles concentration in the presence of chromatographic media can be described by Eq. (2),

$$\frac{dV^{sol,media}}{dt} = -k_d V^{sol,media} - k_{ads}\left(V^{sol,media} - V^*\right)^n \quad (2)$$

where $V^{sol,media}$ is the concentration of infectious particles in solution in the presence of chromatographic media (IP ml^{-1}), k_{ads} the infectious particles adsorption rate to the chromatographic media (min^{-1}), V^* the concentration of infectious particles in solution at saturation of the chromatographic media (IP ml^{-1}) and n the adsorption kinetics order. The first term accounts for the inherent degradation of infectious particles and the second for the loss of infectious particles in solution due to adsorption. Therefore, the adsorption kinetics of infectious particles can be described by Eq. (3),

$$\frac{dV^{ads}}{dt} = k_{ads}\left(V^{sol,media} - V^*\right)^n \quad (3)$$

where V^{ads} is the concentration of adsorbed infectious particles (IP ml^{-1}). During the development of the mathematical model it was assumed that desorption of infectious particles from the chromatographic media is negligible. This assumption was made based on the fact that retroviral vectors adsorb strongly to anion-exchange matrices. The model parameters were estimated, for 95% confidence intervals, by fitting the ordinary differential equations (ODE) 1–3 to the experimental data. The software Scientist v2.0 (MicroMath, Inc., USA) was used for parameter estimation while Mathlab software (Mathworks, Inc., USA) was used for simulations.

2.6. Fixed-bed anion exchange chromatography

For the fixed-bed anion exchange chromatographic experiments, three pre-packed 1 ml columns (0.7 cm × 2.5 cm), DEAE FF, Q XL and Q FF from the HiTrap™ IEX Selection kit (Amersham Biosciences) were used (Table 1). The columns were connected to an ÄKTA™ Explorer 100 Air system (Amersham Biosciences) equipped with UV and conductivity detectors and a fraction collector FRAC-950 (Amersham Biosciences). Before each run the columns were sanitized with three column volumes (CV) of 0.5 M NaOH and equilibrated with loading buffer 20 mM sodium phosphate (Merck) with pH 7.5 and 150 mM

Table 1
Relevant characteristics of the chromatographic media used in batch adsorption and fixed-bed chromatography experiments

Adsorbent	Bead structure	Charged group	Bead size (μm)	Pore size
DEAE Sepharose FF	6% highly	$N^+(C_2H_5)_2H$		n.a.
Q Sepharose FF	cross-linked agarose	$N^+(CH_3)_3$	45–165	n.a.
Q Sepharose XL	6% highly cross-linked agarose with bound dextran	$N^+(CH_3)_3$		n.a.
Streamline™ Q XL	Macroporous cross-linked 6% agarose containing crystalline quartz core and bound dextran	$N^+(CH_3)_3$	100–300	n.a.
CHT™ Hydroxyapatite	Sintered hydroxyapatite at high temperatures	$Ca_{10}(PO_4)_6(OH)_2$	80	600–800 Å

n.a.: information not supplied by the manufacturers.

Table 2
Elution conditions for step elution profiles

AEX media	Elution buffer (%)		NaCl (mM)	
	DEAE	Q XL and Q FF	DEAE	Q XL and Q FF
Step 1	25	54	488	864
Step 2	60	88	960	1338
Step 3	100	100	1500	1500

NaCl. Both in gradient and step elutions, 18 ml of defrosted retroviral supernatant were diluted with 12 ml of loading buffer. From these, 20 ml were loaded into the column at a flow rate of 1 ml/min. After loading, the column was washed with 20 ml of loading buffer at the same flow rate and afterwards the elution profile was created. The elution buffer was similar to the loading buffer but with 1500 mM NaCl. Linear gradient elution profiles were performed from 150 to 1500 mM NaCl concentrations at a flow rate of 1 ml/min and 38.6 mM NaCl/min. After gradient elution a step elution process was designed for each chromatographic media, using similar loading and wash steps. The first step promotes the elution of contaminant proteins, the second is designed for the elution of the viral vectors and the third for washing tightly bound contaminants. Step elution profiles were performed at a flow rate of 1 ml/min using the NaCl concentrations described in Table 2. During the chromatography process, fractions of the column flowthrough were collected onto 15 ml falcon tubes (Sarstedt), inside a laminar flow chamber. After the end of the process all fractions and the initially diluted supernatant were titrated using the β-gal assay for determination of the infectious particles titer. All fixed-bed ion exchange chromatographic experiments were performed at 10 °C.

2.7. DNA and protein quantitation

Protein concentration was determined with the BCA™ Protein Assay Kit (Pierce, Rockford, USA) using BSA as standard protein. DNA concentration was determined using the Quant-iT™ DNA Assay Kit, High sensitivity (Pierce).

3. Results and discussion

3.1. Batch adsorption kinetics of MoMLV particles

3.1.1. Effect of the chromatographic media and buffer system

The chromatographic media studied herein have been extensively used in protein purification; however, there are few reports in the literature on their applicability for MoMLV vectors. Thus, in this work several tests were designed to evaluate the adsorption ability of retroviral vectors to the different chromatographic media under different experimental conditions. Two buffer systems, sodium phosphate (pH 7.0, 7.5 and 8.0) and Tris base (pH 7.5 and 8.0), were tested for the determination of the best pH and buffer conditions. The viral supernatant was diluted in buffer and then incubated with the media, at room temperature, for 90 min. Orbital shaking was used to promote mixing of the media with the supernatant and also to prevent deposition of the medium at the bottom of the beaker. As a control for the stability of the RV vectors the diluted supernatant, not incubated with media, was subjected to equal conditions. Supernatant samples contacting with each chromatographic media were taken over a period of 90 min, and their titer determined using the β-gal assay. The results are shown in terms of infectious particles (IP), in solution, and their percentage relative to the initial titer before mixing with the chromatographic media (Fig. 1). The observed decrease in the percentage of infectious viral particles in solution, after incubation with the chromatographic medium, follows an exponential trend for all media and buffer conditions tested. Both DEAE FF and Streamline™ Q XL show the fastest adsorption kinetics and the lowest percentage of infectious particles in solution throughout the duration of the test (Fig. 1A). CHT medium shows slower adsorption kinetics and lower adsorption capacity for viral particles (Fig. 1A).

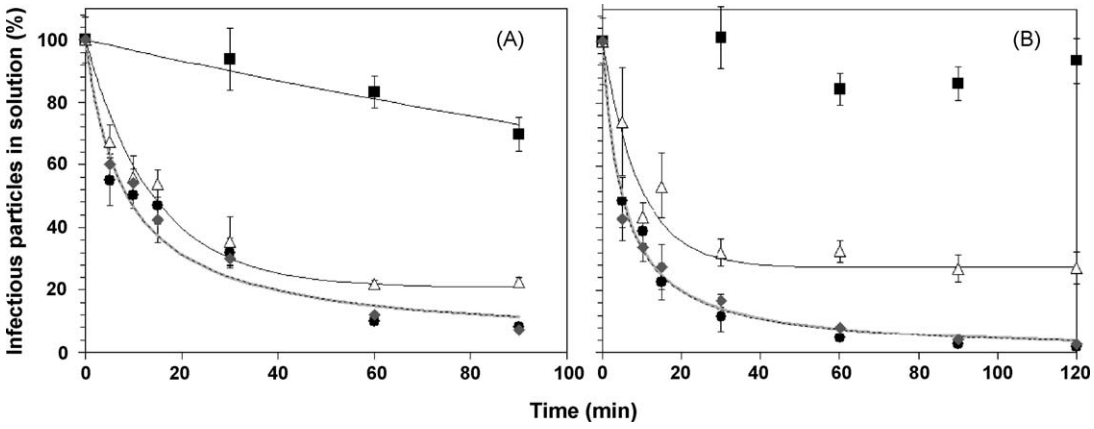

Fig. 1. Adsorption kinetics of infectious retroviral vectors in the presence of each chromatographic media studied. A control of diluted supernatant incubated under the same conditions but without any chromatographic media was used. The lines represent model simulations and the symbols represent the experimental values: (●) DEAE FF, (♦) Q XL, (▲) CHT and the control (■) that consists of retroviral supernatant not incubated with chromatographic matrix. Retroviral supernatant was diluted with 20 mM phosphate, pH 7.5, with 60 mM NaCl and 0.5 M sucrose. The studies were performed at room temperature (21 °C) (A) and at 10 °C (B). Fittings for the DEAE FF and Streamline™ Q XL (grey and black dashed line) overlap.

For all buffers and pH values tested at room temperature, viral stability decreased with incubation time, as indicated by the control of viral supernatant not incubated with chromatographic medium (Fig. 1A). Since viral stability depends on the buffer system used, for the same temperature, it is necessary to normalize the measured infectious particles concentration in solution with the degradation kinetics of the viral vectors; this was done using a simple model simulating the variation of the real adsorbed infectious particles concentration with time.

3.1.2. Model fitting to the experimental data

The changing concentrations of infectious particles in solution with time is a result of both thermal degradation of the vectors and adsorption to the chromatographic media. Thus, to predict the real variation of adsorbed infectious particles with time, it is necessary to account for the degradation of the vectors in solution. The developed model predicts the concentration of infectious particles adsorbed to the chromatographic media with time (V^{ads}), introducing a parameter that accounts for saturation of the chromatographic media, when it occurs (V^*). To simplify the model it was necessary to assume that viral degradation is negligible when the viral particles are adsorbed to the surface of the media; desorption of the viral particles was also considered to be negligible.

The infectious particles decay constant, k_d, for each buffer system, was determined by fitting Eq. (1) to the experimental data. Our results show that MoMLV particles follow a first order decay kinetics at room temperature (Fig. 1A); this observation has already been reported for this type of vectors [25]. The obtained values of k_d were then applied in Eq. (2) for estimation of k_{ads} and V^* (Fig. 1, Table 3). The variation of "unbound" infectious particles in solution with incubation time in the presence of CHT is best fitted with a first order equation, thus $n = 1$. For Streamline™ Q XL and DEAE FF this variation is best fitted with a second order adsorption kinetics ($n = 2$). Our model predicts with accuracy the experimental variation of the percentage of "unbound" viral particles with incubation time (Fig. 1A and B). The resulting model parameters are shown in Table 3. Estimated values of k_{ads} and V^* were further applied in Eq. (3) to simulate the percentage of adsorbed infectious particles with incubation time (Fig. 2).

Determination of the inactivation kinetics of buffered viral vectors is important in the establishment of a purification process. The type of buffer and pH of the solution influences viral stability and interaction with the chromatography media. The values for the infectious particles decay rate constant, k_d, obtained from the control curves, confirm this influence. k_d values obtained for supernatants buffered with Tris base buffer are higher when compared to phosphate buffer, for all pH values tested (Table 3). Thus, degradation of RV vectors is enhanced when the supernatants are buffered with Tris base. k_d values depend also on the buffer pH (Table 3). RV vectors are more stable at pH 7.5 and 8.0 for phosphate buffer and pH 8.0 for Tris base buffer. To our knowledge, studies on the effect of buffer type in liquid preparations have not been reported before, although the effect of the pH on the infectivity of MoMLV has been published [26,27]. It is important to use a buffer system that allows both the maintenance of viral stability and a good adsorption to the chromatographic media. For DEAE FF and Streamline™ Q XL phosphate buffer gave best results, in terms of adsorption kinetics, with higher k_{ads} and lower k_d than Tris buffer (Table 3, Fig. 2A and B).

Regarding Tris buffer, pH 8 shows better results in all chromatographic media studied (Fig. 2A), reaching higher k_{ads} (Table 3) as well as higher percentage of adsorbed infectious particles (close to 80%, Fig. 2A). At this pH, similar adsorption onto DEAE FF and Streamline™ Q XL in terms of adsorbed infectious particles at equilibrium were observed; DEAE FF showed faster adsorption kinetics, with three times higher k_{ads} than Streamline™ Q XL (Table 3). CHT Hydroxyapatite and Tris buffer are incompatible; for this same reason CHT was not used in our previous tests.

Comparing all chromatographic media studied using phosphate buffered supernatants, adsorption to CHT Hydroxyapatite is the worst, both in terms of kinetics and in terms of capacity, for MoMLV particles (Table 3, Fig. 2C and D). The highest adsorption of RV vectors for CHT Hydroxyapatite (Fig. 2C) is achieved at pH 7.0–7.5, with 70% of the initial infectious particles adsorbed to the matrix, whereas DEAE FF and Streamline™ Q XL reach values close to 90% depending on the buffer conditions and temperature (Fig. 2D). Adsorption onto CHT Hydroxyapatite follows a first order kinetics ($n = 1$), this occurring probably because this matrix has a different protein interaction mechanism comparing to conventional anion-exchangers. CHT is a complex ion exchanger since it can exchange both anions and cations, due to the existence of functional groups of positively

Table 3
Model parameters: estimated parameters for all the chromatographic media and buffer systems tested

Buffer	pH	k_{ads} (min^{-1}) × 10^{-8}		k_{ads} (min^{-1})	V^* (IP/ml) × 10^5			k_d (min^{-1}) × 10^{-3}
		DEAE	QXL	CHT	DEAE	QXL	CHT[3]	
Phosphate	7.0	12 ± 2	5.2 ± 0.7	0.15 ± 0.01	1.2 ± 0.5	0	4.4 ± 0.3	5.8 ± 0.8
	7.5	2.6 ± 0.4	2.5 ± 0.2	0.07 ± 0.01	0	0	9 ± 1	3.5 ± 0.4
	7.5[a]	11.4 ± 0.9	11.7 ± 0.8	0.11 ± 0.02	0	0	4.9 ± 0.6	≈0
	8.0	2.6 ± 0.1	2.4 ± 0.6	0.07 ± 0.01	0	0	15 ± 1	5 ± 1
Tris base	7.5	1.1 ± 0.5	1.7 ± 0.4	–	0	0	–	10 ± 2
	8.0	15 ± 3	4.4 ± 0.6	–	3.2 ± 0.6	0	–	6 ± 2

Errors within a 95% confidence interval.
[a] Experiment performed at pH 7.5 and 10 °C.

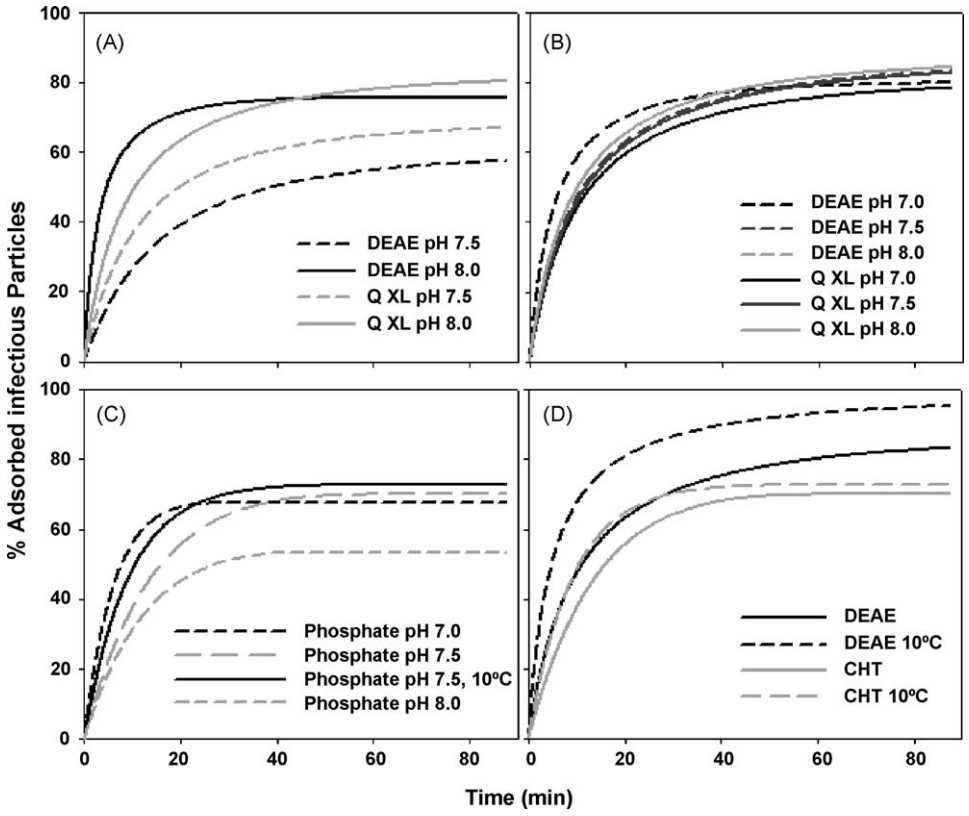

Fig. 2. Simulations of the percentage of adsorbed infectious viral particles to different chromatographic media with incubation time, different buffer and pH conditions: (A) supernatants buffered with 20 mM Tris base at pH 7.5 (dashed lines) and pH 8.0 (solid lines); (B) supernatants buffered with 20 mM phosphate buffer, at several pH values, and incubated with DEAE FF and Q XL media; (C) supernatants buffered with 20 mM phosphate buffer, at different pH values, and incubated with CHT Hydroxyapatite medium, at room temperature (dashed lines) and at 10 °C (solid line); (D) supernatants buffered with 20 mM phosphate buffer at pH 7.5 followed by incubation with DEAE FF, Q XL and CHT media at room temperature (solid lines) and at 10 °C (dashed lines). The simulations for DEAE FF and Streamline™ Q XL in part D, at both temperatures, overlap and for this reason the results for Streamline™ Q XL are not represented.

charged pairs of calcium ions and clusters of negatively charged phosphate groups. Positively charged zones in the viral vectors will be attracted by the phosphate groups and negatively charged zones will be attracted by the calcium ions, in opposition to strict anion adsorbers. Given this property the density of positively charged ligands to the surface of the CHT matrix is lower than for the other anion-exchangers, thus the total adsorption capacity is also lower. For supernatants incubated with CHT medium, the simulated percentage of adsorbed infectious viral particles (Fig. 2C) shows a plateau after 20–50 min incubation (i.e. the first derivative of the function approaches zero), depending on buffer and pH conditions. This is a clear indication that saturation of the CHT medium with contaminant proteins and viral particles is occurring. In fact, CHT has a lower binding capacity, around 13.7 mg lysozime/ml of adsorber, compared with the other anion-exchangers tested which can adsorb more than 100 mg BSA/ml adsorber. Determination of the capacity of CHT to adsorb MoMLV particles under the conditions studied is possible due to the determination of the percentage of the initial infectious particles that were adsorbed by the chromatographic matrix. The estimated value for the highest capacity of infectious particles adsorption by CHT is 5.1×10^7 IP/ml matrix, and was obtained for phosphate buffer at pH 7.5 and room temperature. Kuiper et al. [28] reported also batch adsorption studies with a similar CHT Hydroxyapatite matrix using 20 ml of 10-fold diluted MoMLV particles in DPBS (pH 7.4) contacting with 2 ml of the CHT matrix. They reported that 99% of the initial infectious particles ($\sim 9 \times 10^5$ IP/ml) were adsorbed after 15 min incubation at room temperature [28]. Through simple mathematical calculations, the estimated capacity reported was around 9×10^6 IP/ml, which is approximately 5.7-fold lower than that presented in this paper. The faster kinetics achieved was mainly because two-fold more CHT matrix was used, resulting in almost two-fold increase in adsorption velocity [28].

RV vectors buffered with phosphate show similar adsorption kinetics and equilibrium conditions when incubated with DEAE FF and Streamline™ Q XL (Table 3, Fig. 2B), within the studied pH range. This small variation in the simulated adsorption curves is an indication that the buffer pH will not have much influence in the adsorption kinetics of the RV vectors to the anion-exchange matrices. In the conditions studied, the maximum adsorption capacity of the mentioned matrices is not achieved ($V^* = 0$ for the majority of the conditions studied, Table 3). The matrix characteristics and the ligand type of DEAE FF and Streamline™ Q XL seem not to influence adsorption of the RV vectors. DEAE FF is a weak anion-exchanger with a weakly basic group $N^+(C_2H_5)_2H$ linked to an agarose matrix (Table 1). On the other hand, Streamline™ Q XL media is a strong anion-exchanger,

with the strongly basic amine group $N^+(CH_3)_3$ (Table 1), covalently bound to dextran molecules (tentacles) which are linked to the agarose matrix. In principle the physical properties of Streamline™ Q XL would favour viral particles adsorption since the ligands are covalently bound to dextran molecules (tentacles) and not directly to the surface of the matrix. Viral particles are quite large (~100 nm) and the use of tentacle technology could promote higher interaction of the sterically accessible ligands with the viral particles than just surface adsorption. This fact will be discussed further on, when presenting the validation of the batch results in fixed-bed chromatography. These two matrices are therefore good candidates for the purification of MoMLV particles. Any of the experimented phosphate pH values would be suitable for a chromatography-based process. But it is also important to consider retroviral inactivation during the process. Further studies on the effect of temperature upon adsorption kinetics and validation in fixed-bed mode were then performed using phosphate buffer at pH 7.5 due to the low k_d value obtained at this pH (Table 3).

3.1.3. Effect of temperature upon adsorption kinetics

The results obtained in batch mode presented so far were performed at room temperature (21 °C). To investigate if a lower temperature would benefit adsorption of RV vectors to the anion-exchangers the supernatants were buffered with phosphate at pH 7.5 and incubated with DEAE FF, Streamline™ Q XL and CHT Hydroxyapatite at 10 °C. It is difficult to work at low temperatures with fixed-bed chromatography due to increased viscosity of the solutions and higher backpressure so 10 °C was chosen over the more commonly used temperature of 4 °C.

As occurred at room temperature, depletion of infectious viral particles in the supernatant, contacting the chromatographic media, follows a second order kinetics (Fig. 1B). Degradation of viral particles at 10 °C is very low during the time frame tested and can be considered negligible for model fitting (Fig. 1B). Since $k_d = 0$, the model is simplified and the depletion of infectious viral particles in solution will be considered due only to adsorption to the chromatographic media. Once more, the model fits well to the experimental data (Fig. 1A). Comparing both temperatures, a slight increase in terms of adsorption rate at 10 °C and in the percentage of adsorbed RV vectors at equilibrium is observed (Fig. 2D). Higher k_{ads} values are obtained at 10 °C for all studied chromatographic matrices, comparing to room temperature conditions (Table 3). This can be attributed to the existence of more infectious particles available for adsorption due to longer maintenance of viral stability at 10 °C. Since the results obtained in the batch adsorption tests, at a lower temperature, improved both retroviral stability and higher adsorption properties to the chromatographic media, all fixed-bed AEX chromatography tests were performed at 10 °C.

3.1.4. Fixed-bed anion exchange chromatography

Fixed-bed anion exchange chromatography was assessed in order to validate the results obtained with batch chromatography. The media used were DEAE FF, Q XL and Q FF packed into 1 ml columns (Table 1). DEAE FF and Q XL matrices were used in the batch experiments and Q FF was tested to determine the influence of using tentacle technology in the purification of RV vectors (Table 1). These matrices are available in 1 ml packed columns under similar conditions and with the same geometry. The best buffer conditions found in the batch experiments were used, namely 20 mM phosphate buffer with pH of 7.5 at 10 °C. As the elution profiles are unknown for each chromatographic matrix, linear gradient elutions were performed with increasing salt concentration (NaCl) to determine the separation capability of the media and the conductivity at which the viral vectors would elute from the columns. In all the three resulting elution profiles (Fig. 3A–C), the infectious particles were eluted at a conductivity higher than the majority of the protein contaminants (shown as the first peak of 280 nm absorbance profiles), mostly coming from the foetal bovine serum present in the culture media used for retroviral vectors production. The separation of the infectious particles from the contaminant proteins was well achieved, in all the three media tested but is best with the strong anion exchangers Q FF and Q XL, where the infectious particles elute at an average conductivity of 73 and 79 mS/cm, respectively, in contrast with 55 mS/cm with DEAE FF (Fig. 3A–C). This is due to a stronger interaction between the $N^+(CH_3)_3$ charged group from Q FF and Q XL (Table 1) and the infectious viral particles. Thus anion exchange chromatography provides excellent separation of the infectious particles from contaminating proteins, due to strong binding of the particles to the chromatographic media. The viral peaks are very small, as in the case of DEAE FF (Fig. 3) and sometimes imperceptible (Q FF and Q XL) indicating a very high degree of purity in terms of protein contamination (Table 4). Most of the contaminant proteins do not bind to the studied chromatographic matrices, as depicted by the high percentages of the flowthrough peak area to the total peak area, measured at 280 nm: 75% for Q FF, 66% for Q XL and 87% for DEAE FF. This indicates that most contaminant proteins are neutral or positively charged. Affinity adsorbents, for example, heparin [13], are negatively charged and the separation of positively charged contaminant proteins is more difficult.

When comparing pooled fractions obtained by gradient elution, applying a titer constraint of 2×10^6 IP/ml, the results show (Table 4) that DEAE FF and Q XL columns produce similar yields ($57 \pm 6\%$ and $53 \pm 9\%$, respectively), far superior to the obtained with the Q FF column ($25 \pm 3\%$) (also valid for the step elutions). This result is in agreement with the data obtained from the batch adsorption studies: in the presence of sodium phosphate buffer at pH 7.5, DEAE FF and Streamline™ Q XL have similar adsorption kinetics. Although the Streamline™ Q XL media tested has a higher density and particle size due to the crystalline quartz core (suited for expanded-bed chromatography) the outer part of the particles has the same characteristics as the packed Q XL media: the charged group $N^+(CH_3)_3$ is bound to dextran chains which are bound to 6% cross-linked agarose (Table 1). Hence it can be assumed that the interaction between these media and the infectious particles will be similar. Q FF media has also the $N^+(CH_3)_3$ charged group but it is distributed on the surface of the beads and not bound to dextran molecules (tentacles). Therefore, capture of the viral par-

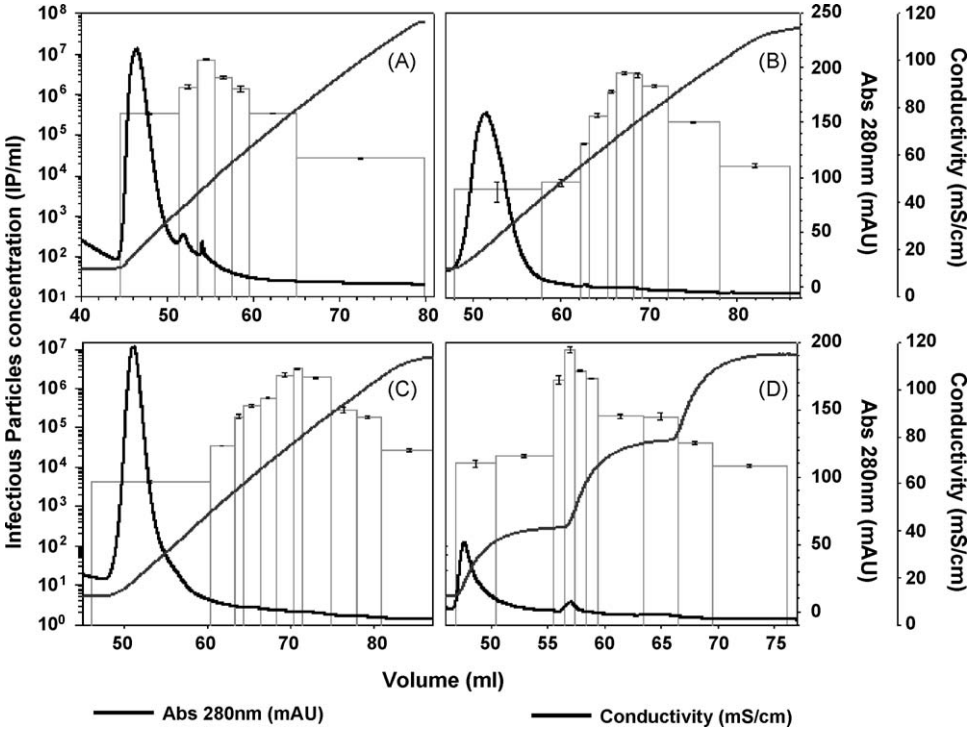

Fig. 3. Linear gradient and step elution profiles obtained after loading each 1 ml column with 20 ml of diluted supernatant in 20 mM phosphate buffer at pH 7.5 with 150 mM NaCl: (A) gradient elution profile using DEAE FF column; (B) gradient elution profile using Q FF column; (C) gradient elution profile using Q XL column; (D) step elution profile using DEAE FF column. Bars correspond to the infectious particles concentration of recovered fractions. Titration data is the mean ± S.D. of at least triplicate wells of a representative experiment and is shown on a logarithmic scale. All the runs were performed at 10 °C using a flow rate of 1 ml/min.

ticles by Q FF is not so efficient because the interaction occurs directly at the surface of the matrix particles; as a consequence, the resulting yield is approximately half of that obtained with Q XL. From the results reported here, expanded-bed chromatography is a promising process for the purification of retroviral vectors, since both Streamline™ Q XL and Q XL showed good performance in the batch adsorption and fixed-bed studies, respectively. Yields obtained for DEAE FF and Q XL are quite similar to the ones obtained by other chromatographic processes, 61.1 ± 2.1% for heparin affinity and 56% for immobilized metal affinity chromatography [13,20], reinforcing the applicability of AEX chromatography for the purification of retroviral vectors.

After determination of the gradient elution profiles, step elution could be designed since the separation between contaminant proteins and infectious particles is well achieved. Step elution enables separation and concentration of peaks while reducing process time. Overall, step elution using the three tested columns resulted in a small increase in the concentration factor and this was attributed, in part to the low amount of total protein loaded into the columns (∼20 mg of total protein), far from the total capacity of these media, and also to the not so steep salt steps produced by the chromatographic equipment. Both the pool and total yields decreased slightly when using step elution (Table 4). This is an indication that some degradation of the viral parti-

Table 4
Fixed-bed chromatography results

Elution	Titer[a] (10^6 IP/ml)	$Yield_{pool}$ (%)	V_{pool} (ml)	CF_{pool}[b]	Total yield[c] (%)	$(A_{pool}/A_{total})_{280\,nm}$ (%)
Linear gradient						
DEAE	3.8 ± 0.2	57 ± 6	6.0	1.9	77 ± 8	–
Q XL	2.2 ± 0.2	53 ± 9	6.5	1.6	68 ± 12	–
Q FF	2.3 ± 0.2	25 ± 3	6.8	0.8	28 ± 3	–
Step elution						
DEAE	4.0 ± 0.6	53 ± 13	4.0	2.6	59 ± 15	0.30
Q XL	3.3 ± 0.4	51 ± 7	4.9	2.1	60 ± 8	0.28
Q FF	2.2 ± 0.4	16 ± 3	3.0	1.0	28 ± 5	0.08

Values presented are the mean ± S.D. of at least triplicate analysis.

[a] Pooled fractions have at least 2×10^6 IP/ml.

[b] CF is the concentration factor defined as the ratio between the concentration of infectious particles in the pool and the concentration of the loaded diluted supernatant.

[c] Sum of the yield of all eluted fractions, including flowthrough and wash steps.

cles occurs when forced to desorb from the beads by steeper increases in salt concentration.

There is no detectable loss of non-adsorbed particles in the columns flowthrough for all media tested here. The mass balance between the total infectious particles loaded into the column and the total recovered in the flowthrough and eluted fractions does not close in any tested columns, as should be expected. This observation is especially relevant for the Q FF media because around 70% of the initial infectious particles seem to loose their infectivity, compared to less than 40% for DEAE FF and Q XL (Table 4). Extensive degradation of the viral particles might take place when they flow through the column or when they are eluted from it. Why this degradation occurs is difficult to explain because in principle the flow properties between Q XL and Q FF are similar, as the same conditions were used. Shedding of the viral envelope due to the direct interaction of the viral particles with the matrix could be happening but then similar results would be expected for DEAE FF.

Most of the protein contaminants that bind to the AEX matrices are eluted with low salt concentrations, thus, step elution allowed for efficient separation from retroviral vectors, as demonstrated by the low virus pool peak area to total peak area ratio, which ranges between 0.08 and 0.30%. Therefore, between 99.70 and 99.92% of the initial protein is removed. Produced supernatants have total protein concentrations ranging between 2.3 and 2.4 mg/ml. After simple calculations it can be determined that the concentration in the pools should range between 7.5 and 21 μg/ml; this is confirmed by the failed attempt to determine protein concentration with a commercial BCA kit from Pierce which has a detection range of 20–2000 μg/ml. DNA concentration of the eluted viral peaks was also assessed. All the matrices studied show potential in removing more than 90% of the initial DNA present in the supernatant (Table 5). In addition to high infectious particles yields, the AEX matrices studied also show high potential in removal of protein and DNA contaminants to a large extent.

The comparison between batch adsorption and fixed-bed adsorption kinetics is straightforward. In fixed-bed chromatography the viral particles are forced into contact with the matrix particles and adsorption occurs at higher velocities because the probability of interaction is also higher. Thus it is expected that lower contact times are needed in fixed-bed chromatography to achieve the same percentage of adsorbed infectious particles. Around 77% of total recovery in terms of infectious particles was achieved with DEAE FF in gradient elution mode for 20 min contact time (duration of the loading step). In batch mode, after 20 min, only 65% of infectious particles in solution had been adsorbed and it took 50 min to reach 77% of adsorbed infectious particles, thus confirming faster adsorption kinetics in packed column than in batch mode.

Table 5
DNA analysis of the viral peaks obtained by step elution

Matrix	DNA initial (μg)	Viral peak	
		DNA (μg)	DNA (%)
DEAE	4.65	0.29	6.2
Q XL	7.80	0.29	3.8
Q FF	4.46	<0.05[a]	<1.1

[a] Value below the lower detection limit of the method.

4. Conclusions

From the results obtained and reported herein, it is possible to conclude that anion-exchange chromatography is a good candidate for MoMLV purification due to the possibility to achieve high infectious particles yields and high protein and DNA contaminants removal.

DEAE FF and Q XL matrices were found to be the best adsorbers in the batch adsorption studies, both in terms of kinetics and extent of viral adsorption. From all experimental conditions studied, adsorption and maintenance of viral stability was favoured at 10 °C using phosphate buffered supernatants, at pH 7.5. Under these conditions DEAE FF and Q XL matrices proved to have a very high capacity for retroviral vectors: 95% of initial infectious particles were adsorbed after 2 h incubation. Additionally we could observe that DEAE ligands provide not only higher capacity for adsorption but also increased adsorption kinetics of viral particles and that the existence of helper molecules (dextran tentacles) to which the ligands are linked improves the capture performance of the AEX matrices, as opposed to conventional media. This is mainly due to the fact that MoMLV particles cannot access the pores, thus, adsorption occurs at the surface of the chromatographic beads.

Fixed-bed chromatography using DEAE FF and Q XL matrices validated the results obtained in the batch experiments and showed good separation performance between retroviral vectors and contaminant proteins. Thus, DEAE FF and Q XL constitute good candidates for purification of MoMLV vectors recovering high infectious particles yields, 53 ± 13% and 51 ± 7%, respectively; furthermore, they remove more than 99% of protein and 90% of the DNA contaminants.

Acknowledgements

The authors acknowledge the financial support received from the European Commission (QLK3-CT-2002-01949) and the *Fundação para a Ciência e Tecnologia*, Portugal (POCTI SFRH/BD/13771/2003). We would like also to thank Dr. Otto Merten from Généthon, France, for kindly providing the TE FLY A7 cell line, Eng. Cristina Peixoto for all the support given with the chromatographic system and Eng. Marlene Carmo for the support with the analytical methods.

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