Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 846 (2007) 124–131
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Exploiting heparin-binding properties of MoMLV-based retroviral vectors for affinity chromatography

María de las Mercedes Segura [a,b], Amine Kamen [b], Marie-Claude Lavoie [a], Alain Garnier [a,*]

[a] *Department of Chemical Engineering and Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines, Université Laval, Que., Canada G1K 7P4*
[b] *Biotechnology Research Institute, NRC, 6100 Royalmount Avenue, Montreal, Que., Canada H4P 2R2*

Received 8 March 2006; accepted 21 August 2006
Available online 12 September 2006

Abstract

Chromatography is deemed the most promising technology for large-scale purification of viral vectors. The authors have previously shown that heparin affinity chromatography could be successfully employed for the purification of VSV-G pseudotyped Moloney murine leukemia virus (MoMLV)-derived vectors giving excellent results in terms of recovery of active particles, reproducibility and selectivity. In this study, the authors examined whether the ability of retrovirus particles to specifically bind to heparin ligands is restricted to VSV-G pseudotypes produced by 293-based packaging cells. It is shown that VSV-G deficient retrovirus particles are captured by a heparin chromatography column as efficiently as VSV-G containing particles. Most strikingly, RD114 pseudotyped retrovirus particles derived from a HT1080-based cell line were found to bind heparin with the same affinity as 293-derived VSV-G pseudotypes. RD114 pseudotyped retrovirus particles were successfully isolated using heparin affinity chromatography obtaining good recoveries of functional particles (43%). These results indicate that heparin affinity chromatography can be extended to the purification of retroviral vectors produced by different packaging cell lines independently of the Env-protein used for pseudotyping.

Keywords: Retrovirus; Gene therapy vectors; Purification; Heparin affinity chromatography; Pseudotypes

1. Introduction

Retroviral vectors constitute a valuable tool for gene transfer technology. The wide clinical application of these vectors for gene therapy will depend on the availability of efficient large-scale manufacturing procedures useful for the production and purification of various vector pseudotypes. Although important progresses have been made in retroviral vector design and production processes, these improvements have not been paralleled by the development of purification methods, which is lagging behind [1,2]. Retroviral vectors are labile enveloped viruses that require the strategic design of gentle purification processes to avoid vector inactivation.

Centrifugation processes have traditionally been used for isolating retroviruses in small quantities. Typically, retrovirus particles are first separated from the bulk of contaminating serum proteins present in the growth medium by high speed centrifugation. The resulting pellet is resuspended in a small volume of buffer allowing simultaneous purification and concentration of the virions. However, virus pelleting techniques lack resolving capacity and are usually coupled with sucrose equilibrium density gradient ultracentrifugation. An interesting alternative is the use of rate zonal ultracentrifugation that results in highly pure preparations suitable for virus characterization studies [3]. However, an important limitation of ultracentrifugation procedures is that ultra-high speed rotors currently in use generally have small volume capacities [4–7]. Alternatively, several centrifugation methods for the concentration and partial purification of retroviruses by precipitation with additives (i.e. charged polymers or calcium phosphate) have been described [8–10]. The advantage of using additives to induce virus precipitation is that following the treatment, virus pellets can be obtained at low centrifugation speeds in a short time. Furthermore, using low-speed rotors, larger volumes of supernatant can be processed per run. However, a major disadvantage with the use of polymers is that they interact irreversibly with retrovirus particles to form a virus-polymer complex that cannot be dissociated for further processing [8].

* Corresponding author. Tel.: +1 418 656 3106; fax: +1 418 656 5993.
E-mail address: alain.garnier@gch.ulaval.ca (A. Garnier).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.08.032

Chromatography plays an important role in the purification of high value bioproducts since it enables fast, scaleable, reproducible and selective separations. Not surprisingly, chromatography is becoming the method of choice for the large-scale purification of most gene therapy vectors including retroviral vectors [11–17]. Chromatography separates retroviruses from impurities contained in the vector supernatant by exploiting physical and biochemical features of retrovirus particles. For instance, using size exclusion chromatography scientists can take advantage of the large size of retroviruses (∼100 nm) for its separation from contaminating proteins and other low molecular weight impurities [7,18,19]. Additionally, the negatively charged surface of retroviruses can also be exploited for purification purposes by utilizing positively charged functional groups, such as those found in anion exchange (or hydroxyapatite) resins, that strongly bind retrovirus particles [20–24]. On the other hand, the specific recognition of molecular structures on the viral membrane by affinity ligands would allow the selective isolation of retrovirus particles using affinity chromatography. Due to its high resolution, affinity chromatography offers the potential to reduce the number of purification steps increasing product yields and decreasing process costs. However, to take full advantage of this technology it is important to identify stable, inexpensive and versatile affinity ligands that specifically bind retrovirus particles. Unfortunately, little is known about the composition of the retroviral membrane, which complicates the selection of appropriate affinity ligands. Retroviral vectors are frequently genetically modified to contain Env-proteins of other viruses giving rise to a variety of vector pseudotypes. In addition, MoMLV particles are known to randomly incorporate various host-derived proteins on their membrane [25] that remain largely unidentified. Therefore, the composition of the viral membrane is expected to vary to some extent depending on the cell line used for vector production. As a consequence, it seems difficult to find an affinity ligand useful for the purification of all retroviral vectors, which would be highly desirable to simplify and unify vector manufacturing procedures.

In principle, retroviral vectors could be purified by immunoaffinity chromatography by relying on the specific interaction between immobilized antibodies and the viral Env-protein. However, the high costs associated with antibody purification and immobilization, the low stability of these ligands towards sanitizing agents and the harsh conditions usually required to break antibody–antigen interactions do not favor the use of this method for large-scale purification of retroviral vectors [26]. Moreover, depending on the Env-protein used to pseudotype the vector, chromatography columns and protocols should be specifically designed for each individual case. Another possibility is to engineer vectors to contain affinity tags inserted on the surface of the virus to facilitate their purification. Hexahistidine affinity tags have been inserted into the MoMLV ecotropic Env-protein to allow purification by immobilized metal affinity chromatography (IMAC) [27]. Additionally, chemically biotinylated retrovirus particles have shown to bind streptavidin coated adsorbents in batch experiments [28]. However, engineering vectors by inserting tags or chemically modifying the Env-protein without reducing or eliminating the viruses' ability to transduce cells has proved to be a difficult task as demonstrated by many unsuccessful efforts to alter the structure of Env-proteins for targeting purposes [29–31].

An attractive alternative is to explore the natural ability of these viruses to bind commercially available affinity ligands or immobilized viral receptors. Heparin is a relatively inexpensive and stable affinity chromatography ligand used to purify a variety of biomolecules and viruses. Heparin structurally mimics the widely distributed heparan sulfate cell surface proteoglycan which has been recognized as a receptor for attachment of numerous viruses including herpes simplex virus (both HSV-1 and HSV-2) [32–34], foot and mouth disease virus (FMDV type O) [35,36], dengue 2 virus [36] and adeno-associated virus (AAV-2) [37]. For these viruses, heparin affinity chromatography constitutes a valuable tool for purification and serves to study virus–heparin interactions [38–40]. It is interesting to note that for most viruses, including the ones mentioned above, the heparin-binding domains on the virus responsible for virus–heparin interaction were found to be localized on viral-encoded proteins [36,41–44]. Heparan sulfate proteoglycan has also been implicated as a receptor for some retroviruses, namely human immunodeficiency virus (HIV-1) and Friend murine leukemia virus (F-MuLV) for which heparin-binding sites responsible for the virus–heparin interaction were also identified within specific domains of the wild-type Env-protein [45–49].

Previous studies have shown that heparin affinity chromatography was a useful method for the purification of VSV-G pseudotyped retroviral vectors derived from 293 producer cells giving excellent results in terms of yield, selectivity and reproducibility [17]. Elution of retrovirus particles from heparin affinity columns was achieved under mild conditions (neutral pH and 0.35 M NaCl) resulting in high recoveries of infective particles (61%). However, the extended applicability of heparin affinity chromatography to the purification of different retroviral vector pseudotypes or vectors produced by different cell lines remained unclear. To further characterize retrovirus–heparin interactions, the authors examined the ability of VSV-G deficient retrovirus particles as well as RD114 pseudotyped particles produced by a different packaging cell line, the FLYRD18 which is a HT1080-based cell line, to bind immobilized heparin ligands.

2. Materials and methods

2.1. Packaging cell lines and retroviral vectors

Two packaging cell lines that produce Moloney murine leukemia virus (MoMLV) vector particles psedudotyped with the envelope glycoproteins of either vesicular stomatitis virus VSV-G (293GPG) or cat endogenous virus RD114 (FLYRD18) were used. The 293GPG packaging cell line, derived from 293 human embryonic kidney cells [50], was stably transfected to generate a retroviral vector encoding a fusion protein that links the simplex virus thymidine kinase protein (TK) with the green fluorescent protein (GFP) [51]. This cell line, a generous gift from Dr. J. Galipeau (Lady Davis Institute for Medical Research, Montreal, QC, Canada), was adapted to grow in suspension

culture (Ghani et al., submitted for publication). The stable FLYRD18 packaging cell line derived from HT1080 human fibrosarcoma cells [52] produces GFP3 vector [53]. These cells and the 143B target cells were graciously provided by Dr. M. Caruso (Centre de recherche en cancérologie de l'Hôtel-Dieu, Université Laval, Québec, QC, Canada). Cells were maintained in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated foetal bovine serum (FBS; HyClone, Logan, UT) at 37 °C, 100% humidity and a 5% CO_2 atmosphere. 293GPG culture medium additionally contained tetracycline (1 μg/mL; Fisher Scientific, Nepean, ON, Canada) to repress the expression of VSV-G gene.

2.2. Retroviral vector production

VSV-G pseudotyped vector production was carried out in a 250 mL shake flask (50 mL working volume) inoculated at 2×10^5 293GPG cells/mL. Cells were grown in calcium free DMEM supplemented with 10% FBS and tetracycline until the cell density reached 2×10^6 cells/mL. At this point VSV-G expression was induced by entirely removing the tetracycline-containing medium by centrifugation of the cell culture ($420 \times g$, 10 min). Cells were washed with phosphate-buffered saline pH 7.4 (PBS) and the cell pellet was resuspended in fresh tetracycline-free medium, re-introduced into the shake flask and incubated at 37 °C during 48 h. In parallel, the production of Env-protein deficient retrovirus particles was carried out following the same protocol with the exception that the cells were resuspended in fresh tetracycline-containing medium. Retrovirus containing supernatants were harvested every 24 h during 5 days by centrifugation of the cell culture ($420 \times g$, 10 min) and replaced with fresh medium. RD114 pseudotyped vector particles were produced in 175 cm^2 tissue culture flasks (35 mL working volume) by FLYRD18 adherent cells. Cells were seeded at a density of 4×10^5 cells/mL and grown for 48 h in High Glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The vector production phase was initiated at ~80% confluence (~8×10^5 cells/mL) by washing the cells with PBS and replacing the medium with 35 mL of fresh medium. Retrovirus containing supernatant was harvested every 24 h during 5 days and replaced with fresh medium. Harvested retrovirus supernatants were clarified using 0.45 μm pore size syringe-mounted filters (Millipore, Bedford, MA) and concentrated 20-fold using a 76 mm diameter Omega™ polyethersulfone membrane disc filter with a molecular weight cut-off of 300,000 (Pall Gelman Sciences) in a 400 mL stirred cell ultrafiltration unit (Amicon 8400; Millipore, Etobicoke, ON, Canada) as previously described [17]. Retrovirus-enriched retentate was diafiltered against cold heparin affinity adsorption buffer (150 mM NaCl in 20 mM Tris–HCl buffer, pH 7.5). Virus stocks were aliquoted and stored at −80 °C.

2.3. Infective retroviral vector titer determination

Quantification of infective particles by GFP expression assay and flow cytometric analysis has been previously reported [17]. Briefly, 293 (for HT1080-derived vector particles) or 143B (for 293GPG-derived vector particles) target cells were seeded in six-well plates and exposed to 1 mL aliquots of serial dilutions of virus in DMEM containing 8 μg/mL of polybrene during 3 h at 37 °C. After addition of DMEM containing 20% FBS (1 mL), the cells were incubated for 48 h at 37 °C under 5% CO_2 atmosphere. Transduced cells were washed with PBS, detached with trypsin-EDTA, fixed with 2% formaldehyde and resuspended in 1 mL of PBS. Samples were then subjected to fluorescent-activated cell sorting (FACS) analysis and viral titers were calculated as follows:

$$\text{Titer(IVP/mL)} = \frac{(\%\text{GFP}^+\text{cells}) \times (\text{number of cells at time of exposure}) \times (\text{dilution factor})}{(\text{sample volume})}$$

Samples from a given experiment were analyzed in a single titration assay to avoid inter-assay variability and all samples were processed in duplicate or triplicate to minimize intra-assay variability (R.S.D. <10%). Only virus dilutions that resulted in %GFP^+ cell values ranging from 3 to 20% were selected for titer calculations.

2.4. Quantitation of retrovirus particles by immunofluorescence microscopy

VSV-G pseudotyped and VSV-G deficient retrovirus particles were quantitated by immunofluorescence microscopy using a method adapted from Pizzato et al. [54]. Virus samples were mixed with 100 nm red fluorescent carboxylate-modified microspheres (Molecular probes, Eugene, OR) at a final concentration of 5.4×10^8 spheres/mL. Mixtures (5 μL) were spread on a 1 cm^2 area of glass slide and air dried at room temperature for 30 min. Virus particles were fixed with 2% formaldehyde during 15 min, washed 5 times with PBS and permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Slides were washed once with PBS, blocked for 15 min with 10% FBS in PBS and washed 3 times with PBS. Gag and VSV-G immunofluorescence staining was performed for each sample separately. Samples were incubated for 45 min at room temperature with primary antibodies, either rat polyclonal anti-Gag in house antibody or a monoclonal antibody against the envelope protein (mouse Mab anti-VSV-G; Roche Diagnostics, Indianapolis, IN). Slides were washed 3 times with PBS and incubated with appropriate secondary antibodies conjugated with fluorophores, either Alexa Fluor 488 goat anti-rat antibody (Molecular probes) or FITC F(ab′)2 goat anti-mouse antibody (Serotec, Oxford, UK). After 45 min of incubation with secondary antibodies at room temperature, the slides were washed once with PBS, air dried and mounted with slow-fade mounting solution (Molecular Probes). Pictures were taken using a Princeton Instruments CCD camera mounted on a Leitz Aristoplan upright fluorescence microscope. The Gag+ or VSV-G + particle concentration were estimated based on the ratio between immunostained virus particles and fluorescent microspheres.

2.5. Effect of soluble heparin on VSV-G retrovirus transduction to 143B target cells

Equal volumes of retrovirus supernatant were incubated for 30 min at 37 °C in the presence of various concentrations of heparin (Sigma). A negative control was incubated without heparin. Following virus treatment with heparin, titers were determined for triplicate experiments at each concentration of heparin.

2.6. Heparin affinity chromatography

Chromatography was performed using a low-pressure liquid chromatography system (GradiFrac; GE Healthcare, Uppsala, Sweden) at room temperature and monitoring protein elution by UV absorbance at 280 nm. All samples were filtered with a 0.45 μm GHP Acrodisc filter membrane (Pall Gelman Sciences) prior to chromatography. The ability of 293-derived VSV-G deficient retrovirus particles to bind immobilized heparin ligands was investigated using a previously defined heparin affinity chromatography step-wise elution strategy [17]. Briefly, a 1-mL Fractogel® EMD Heparin (S) column was pre-equilibrated with 150 mM NaCl in Tris–HCl buffer, pH 7.5 and loaded with 3 mL of sample. A step-wise NaCl elution strategy consisting in a wash step at 150 mM NaCl (19.5 column volumes [CV]), an elution step at 350 mM NaCl (13 CV) and a high stringency final wash step at 1200 mM NaCl (7.5 CV) was applied. The running linear flow rate was 153 cm/h. Fractions from each peak were pooled and analyzed by immunofluorescence microscopy. The binding aptitude and affinity of 293-derived VSV-G pseudotyped and HT1080-derived RD114 pseudotyped retrovirus particles was compared using a linear NaCl gradient elution strategy. Briefly, the same protocol described above was followed with the exception that following the wash step at 150 mM NaCl, a linear gradient was applied from 150 to 1150 mM NaCl in Tris–HCl buffer, pH 7.5, at a rate of 50 mM NaCl/min and a linear flow rate of 92 cm/h. Fractions of 2.5 mL were collected throughout the run and immediately tittered. In addition, the recovery of HT1080-derived RD114 pseudotyped retrovirus infective particles using the step-wise strategy was estimated by flow cytometry analysis of the pool of fractions from each peak.

3. Results

3.1. The effect of heparin on VSV-G retrovirus transduction to target cells

The first indication of retrovirus heparin-binding activity was provided by experiments showing that soluble heparin and other glycosaminoglycans (GAGs) were able to inhibit retrovirus infection [55,56] and retroviral vector transduction [45,57–60]. These observations were confirmed in our laboratory for the model vector used. VSV-G pseudotyped retrovector transduction to 143B target cells was significantly inhibited in the presence of soluble heparin in concentrations of 1.5 U/mL or greater (Fig. 1). Treatment of MoMLV particles with heparin in concentration higher than 2 U/mL almost completely abolished the

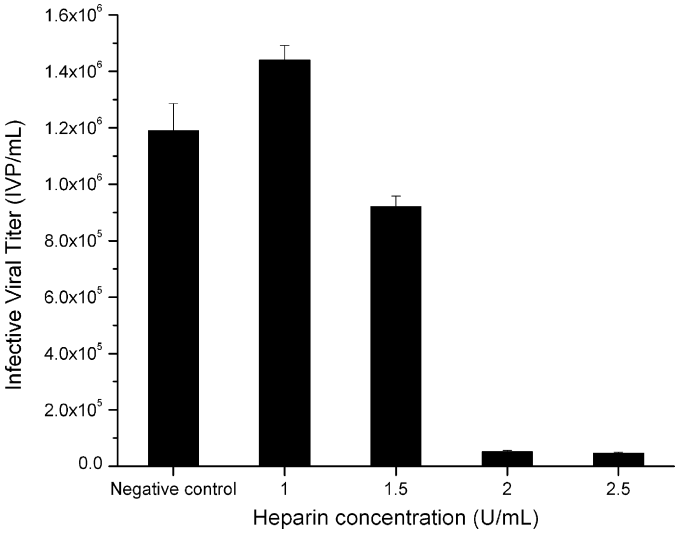

Fig. 1. The effect of heparin on VSV-G retrovirus transduction to target cells. Retrovirus supernatants were incubated in the presence of various concentrations of heparin as described in Section 2. A negative control was incubated without heparin. Infectious titers were determined by flow cytometric analysis for GFP expression. Titer values presented are the mean ± standard deviation of triplicate samples. Abbreviations: IVP; infective virus particles.

virus ability to transduce target cells with a 96% inhibition of transduction.

Although these experiments suggest a possible interaction between the virus and the heparin molecule, they are not conclusive. Polybrene and other polycations are generally believed to exert their enhancing effects on retrovirus transduction by reducing the electrostatic repulsion between retroviruses and cells; thus, increasing retrovirus binding to target cells. Since heparin is a highly sulfated linear polysaccharide, it could be mistakenly inferred that the inhibitory effect heparin has on vector transduction results from electrostatic interference as a consequence of its polyanionic nature that would repel both, the negatively charged viruses and cells. However, heparin affinity ligands attached to a chromatography matrix have shown to efficiently capture retrovirus particles while the same matrix carrying anionic sulfate groups failed to do so, which clearly indicated that a specific interaction between the virus and heparin is taking place [17].

Interestingly, treatment of MoMLV particles with low concentrations of heparin (1 U/mL) enhanced transduction by 21% compared to non-treated virus particles. A similar concentration dependent dual effect (enhancement and inhibition) of soluble heparin on the infectivity of F-MuLV has previously been reported [45]. The authors explained this effect of heparin by presenting a model in which the heparin molecule serves as a molecular bridge between the heparin-binding domains identified on this virus and heparin-binding structures at the cell surface. According to this model, low concentrations of soluble heparin are expected to enhance virus infectivity by acting as a bridge between the virus and the cells whereas high concentrations of heparin would inhibit virus infection by blocking binding sites on the virus and the cells. Another likely explanation for this phenomenon would be that the increase in transduction observed is due to flocculation of retrovirus

particles by the combined addition of oppositely charged polymers, namely the heparin and the polybrene used for virus titration in this work. Small increases in transduction activity were first observed when low doses of glycosaminoglycans were added to retrovirus stocks that contained polybrene [59]. Subsequently, several reports clearly showed that the increase in transduction at low doses of glycosaminoglycans (in the presence of polybrene or other cationic polymers) was due to the formation of polyelectrolyte-virus complexes that sediment onto the cells, increasing the rate of virus binding and transduction [8,61].

3.2. VSV-G deficient retrovirus particles interact with heparin ligands

The authors have recently reported that VSV-G pseudotyped particles can be efficiently purified using heparin affinity chromatography [17]. To further explore the nature of retrovirus–heparin interaction and determine the value of heparin affinity chromatography for the purification of other vector pseudotypes, the ability of VSV-G deficient retrovirus particles to bind immobilized heparin ligands was tested (Fig. 2). Production of either VSV-G containing or deficient particles was performed by taking advantage of the 293GPG packaging cell line inducible system for VSV-G expression. The number of Gag+ and VSV-G+ particles in cell culture supernatants was quantified by immunofluorescent microscopy. Induced and non-induced producer cell culture supernatants contained similar amounts of Gag+ particles (5.15×10^9 and 4.95×10^9 particles/mL, respectively). As expected, no VSV-G+ particles were detected in non-induced 293GPG culture supernatants whereas the concentration of VSV-G+ particles in induced cell culture supernatants was estimated at 7.66×10^9 particles/mL. A greater amount of VSV-G+ particles than Gag+ particles would indicate the presence of contaminating VSV-G loaded cell membrane vesicles in vectors supernatants [62]. Equal volumes of VSV-G pseudotyped and VSV-G deficient retrovirus stocks were separately loaded onto a heparin column and peak fractions eluting at 350 mM NaCl were pooled and analyzed for the presence of Gag+ particles. The amount of Gag+ particles in eluted fractions was comparable (1.56×10^9 and 1.33×10^9 particles/mL for VSV-G pseudotyped and VSV-G deficient particle stocks, respectively) showing that both types of particles were able to bind heparin with similar efficiency and affinity. Therefore, the VSV-G does not seem to be required for effective retrovirus–heparin interaction. Based on these results, the authors hypothesized that other retrovirus vector pseudotypes may also bind heparin ligands.

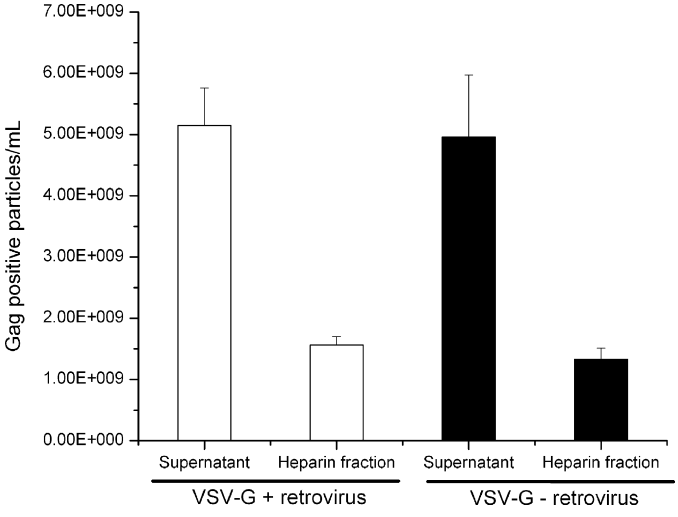

Fig. 2. Binding of Env-protein deficient particles to heparin ligands. Supernatants were produced in parallel with or without the addition of tetracycline generating comparable concentrations of VSV-G deficient retrovirus particles and VSV-G containing particles, respectively. Concentrated virus stocks were loaded onto a Fractogel® EMD Heparin (S) column and elution was carried out using a step NaCl gradient as described in Section 2. Heparin purified fractions eluting at 350 mM NaCl were collected, pooled and Gag+ particles were quantified by immunofluorescence microscopy. Values presented are the mean ± standard deviation of five counts.

3.3. 293 and HT1080-derived retrovectors bind heparin with the same affinity

Retroviral vectors are produced by a variety of packaging cell lines. However, there is a growing tendency is to use human cell lines because they offer numerous advantages over the earlier murine packaging systems [1]. The most common human cell types used for vector production are HEK 293 and HT1080 cells [1]. To further evaluate the usefulness of heparin affinity chromatography for the purification of retroviral vectors and test our hypothesis, HT1080-derived retrovectors carrying a different Env-protein (RD114) were challenged to bind heparin. Both RD114 pseudotyped and VSV-G pseudotyped retrovector particles were loaded separately onto a heparin column and eluted using a linear NaCl gradient. Fig. 3 shows the percentage of transduced cells obtained by titration of the fractions eluted from the heparin column throughout the run. As for VSV-G pseudotyped vectors, most infective RD114 pseudotyped vector particles were efficiently captured by the heparin column and eluted at 350 mM NaCl. For both viruses, only a small amount of viral particles did not successfully bind the column and was lost in the initial wash at 150 mM NaCl (Fig. 3). These results demonstrate that HT1080-derived RD114 pseudotyped retrovectors are also capable of interacting with heparin. Moreover, these vector particles displayed the same affinity as VSV-G pseudotyped 293-derived vector particles for the heparin column requiring the same salt concentration to disrupt heparin–virus interactions.

3.4. Purification of RD114 pseudotyped vectors by step-gradient heparin affinity chromatography

The ability of RD114 pseudotyped vector particles to bind heparin was exploited for purification purposes. Given that both RD114 and VSV-G pseudotyped vectors showed identical affinity for heparin ligands, recovery of RD114 pseudotyped vector particles from the chromatographic column was accomplished using the step gradient elution protocol originally designed for VSV-G pseudotyped vectors [17]. Chromatography was performed at room temperature and completed within 1.5 h. The

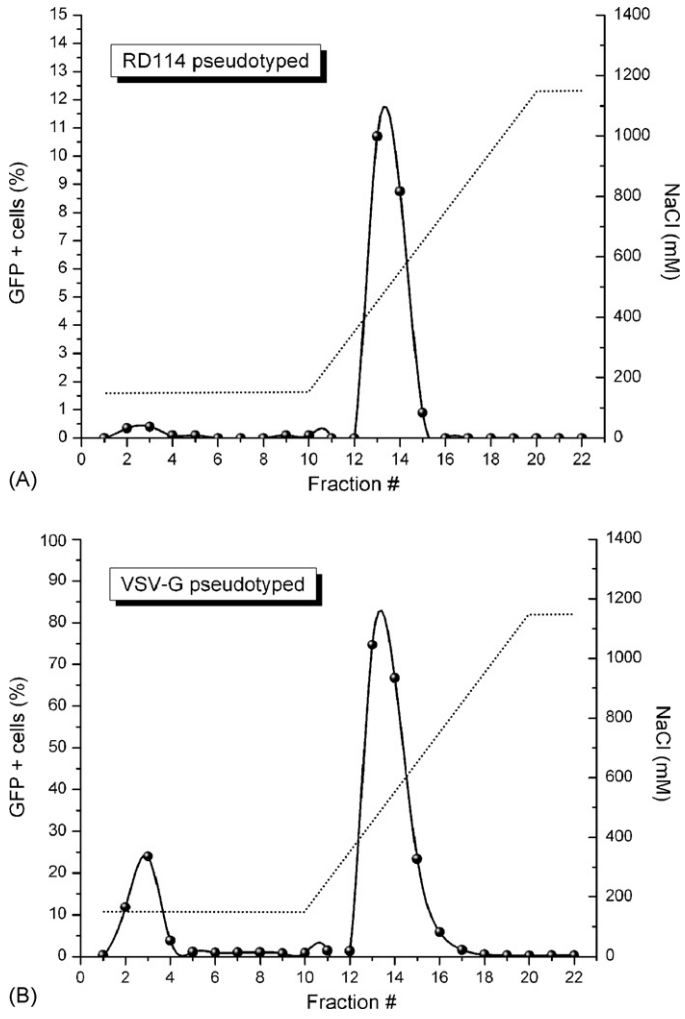

Fig. 3. Binding affinity of RD114 (A) and VSV-G (B) pseudotyped retrovirus particles to heparin affinity ligands. Concentrated VSV-G and RD114 pseudotyped virus stocks were loaded onto a Fractogel® EMD Heparin (S) column. Elution was carried out using a linear NaCl gradient (150–1150 mM) as described in Section 2. Eluted fractions collected throughout the run were diluted 1/10 in DMEM containing 8 μg/mL of polybrene and subjected the GFP expression assay and flow cytometric analysis as described in Section 2. GFP^+ cell values presented are the mean ± standard deviation of duplicate samples.

elution profile shows that the majority of serum protein contaminants did not interact with the heparin column and were easily washed off the column at low salt concentrations (150 mM NaCl) (Fig. 4). Bound virus particles are recovered by elution at 350 mM NaCl in a defined peak. The mean recovery of infective particles in this peak was 42.6 ± 1.2% (Table 1). Only a very small amount of infective particles were lost in the flowthrough and the high salt wash fractions (∼2%). The described chromatographic behavior comparable to that observed for VSV-G pseudotyped particles [17].

4. Discussion

Previous studies from our laboratory have shown that VSV-G pseudotyped MoMLV-derived vectors stably interact with heparin. In this work we demonstrate that both VSV-G deficient and RD114 pseudotyped retrovirus particles can also bind heparin with similar efficiency and affinity as VSV-G pseudotyped particles. Therefore, these results indicate that the viral VSV-G Env-protein is not required for retrovirus–heparin interactions. Thus, in principle the method can be extended to the purification of 293 and HT1080-derived retrovectors regardless of the Env-protein carried by the virus. Although the heparin-binding component responsible for the virus–heparin interaction remains unknown, most likely unidentified cellular component(s) on the virus surface play a role for the observed heparin-binding activity. Moreover, the fact that both 293 and HT1080-derived retrovectors bind heparin with identical affinity suggests that the heparin-binding activity probably derives from a common component ubiquitously distributed in different packaging cell types. In this case, the host-derived component would be incorporated into various vectors opening the possibility of extending the use of heparin affinity chromatography to the purification of potentially all MoMLV-derived vectors regardless of the cells from which they were derived or Env-protein used for pseudotyping. The usefulness of heparin affinity chromatography for the purification of AAV vectors has been severely compromised by the fact that unlike AAV-2 which stably interacts with heparin, other AAV serotypes (i.e. AAV-1, AAV-4 or AAV-5) lack heparin-binding activity [14,16,63]. In view of this limitation with AAV vectors, the possibility of extending the method to the purification of all MoMLV-derived vectors is very attractive.

Retrovirus–heparin interaction is stable but reversible requiring relatively low salt concentrations for dissociation. This is important considering the susceptibility of retroviruses to osmotic pressure [17,26,64]. The recovery of infective particles was higher for VSV-G pseudotyped vectors (61.1%) than for RD114 pseudotyped vectors (42.6%). This result could reflect

Table 1
Heparin affinity chromatography recoveries for RD114 pseudotyped vector

Fraction	Volume (mL)	Titer (IVP/mL)			Average titer (IVP/mL)	Average recovery ± S.D. (%)
		Run 1	Run 2	Run 3		
Load	3	2.18E+06	2.18E+06	2.18E+06	2.18E+06	100
Flowthrough (0.15 M NaCl)	6	1.74E+04	1.34E+04	1.46E+04	1.51E+04	1.4 ± 0.2
Elution (0.35 M NaCl)	4.5	6.37E+05	6.03E+05	6.15E+05	6.18E+05	42.6 ± 1.2
Wash (1.20 M NaCl)	1.5	2.02E+04	2.58E+04	3.75E+04	2.78E+04	0.6 ± 0.2

Recovery of RD114 pseudotyped retroviral vector in fractions eluted from the Fractogel® EMD Heparin (S) column using the presented step-wise elution strategy. Titer values presented for each fraction are the mean of duplicate determinations. Average titer and recovery values for the three runs are shown in the table. Abbreviations: IVP, infective virus particles; S.D., standard deviation.

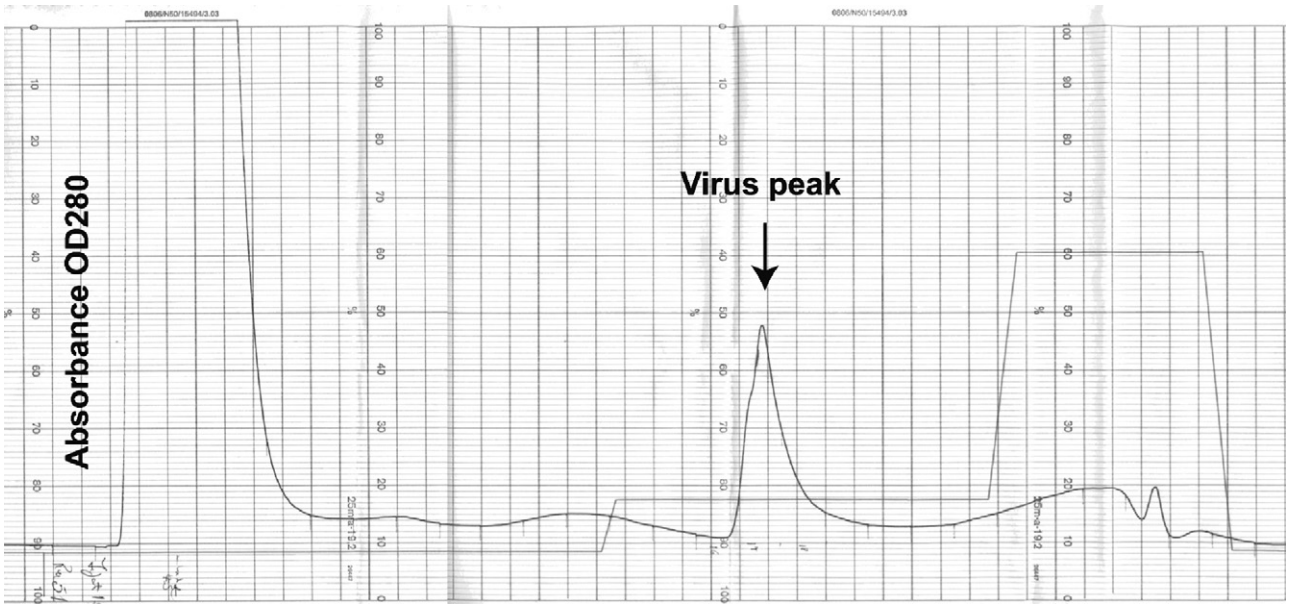

Fig. 4. Heparin affinity chromatography step gradient elution profile for RD114 pseudotyped vector. Three mL of a 20-fold concentrated virus containing 2.2×10^6 IVP/mL were loaded onto a 1 mL Fractogel® EMD Heparin (S) column. The virus was eluted by addition of 350 mM NaCl into the mobile phase. A similar chromatographic behavior was previously observed for VSV-G pseudotyped particles. Retrovirus particles were recovered in a defined peak (4.5 mL) containing 6.2×10^5 IVP/mL. Abbreviations: IVP; infective virus particles.

the poorer stability of the RD114 Env-protein that leads to unavoidable inactivation of infective retrovirus particles during chromatography [4,5]. Nevertheless, we would like to point out that to date heparin affinity chromatography has given the highest recoveries of infective particles for MoMLV-derived vectors; possibly because other adsorptive chromatography methods require harsher conditions for the elution of virus particles from the chromatography columns including the addition of noxious desorption reagents (d-biotin and imidazole) and the use of higher ionic strengths, all of which were shown to affect the vectors' stability [2,17,23,27,28].

The authors found 7.66×10^9 particles/mL of VSV-G+ particles, but only 5.15×10^9 particles/mL of Gag+ particles in virus supernatants, which suggests that 1/3 of the particles produced by the virus producer cells are not actual virus particles, but are probably lipid-VSV-G complexes. Similarly, large amounts of contaminating host protein-laden membrane vesicles (between 2- to 4-fold more vesicles than virions) were found in density-gradient purified HIV-1 preparations from lymphoid cells by electron microscopy [65,66]. Previous studies have also shown that a significant amount of VSV-G vesicles are released by 293 cells expressing VSV-G into the culture medium [62]. Complete removal of contaminating cell membrane vesicles is difficult to accomplish since these particles show important similarities in morphology, composition and physical characteristics with the virions. Thus, most chromatography methods, including heparin affinity chromatography, are unlikely to remove cell membrane vesicles. One possible way to remove these vesicles would be to employ immunoaffinity chromatography provided that a surface protein is found to be exclusively incorporated into either in the virions or the vesicles [67]. However, the poor characterization of MoMLV particles and limited knowledge about its membrane composition make this approach impossible at the present time. Moreover, low recoveries of active particles are predictable upon elution using this method since it usually requires stringent elution conditions to break antibody–antigen interactions. Another strategy to remove these vesicles would be to employ rate zonal ultracentrifigation as the polishing step following heparin affinity chromatography. Previous studies have shown that rate zonal ultracentrifugation render highly pure retrovirus preparations with no evident cell membrane vesicle contamination [3]. Testing and optimizing this approach is very attractive since it offers the possibility of combining both, the scalability of chromatography and the high resolution of rate zonal ultracentrifugation. In addition, rate zonal ultracentrifugation has shown great potential to separate defective vector forms, which is another closely-related vector structure, very difficult to remove using most available retrovirus purification techniques.

In conclusion, the heparin-binding properties of MoMLV-based retrovirus vectors can be exploited for downstream processing purposes. Heparin affinity chromatography proved to be a useful tool for the purification of retroviral vectors from different cellular origins carrying alternative Env-proteins, showing that it is possible to have a common scaleable affinity chromatography method for all retrovirus vectors. Moreover, this affinity purification strategy does not require the alteration of Env-protein with tags that can affect the virus ability to transduce cells. Additionally, the general ability of retrovirus vectors to bind heparin and probably heparin-related structures (i.e. heparan sulfate) at physiological pH and ionic strength as described herein may have important implications in the mechanism of virus attachment to target cells and the design of targeted retrovirus vectors.

Acknowledgements

The authors wish to thank Dr. Manuel Caruso, Normand Arcand and Gavin Whissell for careful review of this manuscript and helpful discussions. We appreciated the help of Pierre Trudel with fluorescence microscopy, Andre Migneault with image files and Lucie Bourget with FACS analysis. This work was supported by a NSERC Strategic Project grant and the NCE Canadian Stem Cell Network.

References

[1] O.W. Merten, J. Gene Med. 6 (Suppl. 1) (2004) S105.
[2] M.M. Segura, A. Kamen, A. Garnier, Biotechnol. Adv. 24 (2006) 321.
[3] M.M. Segura, A. Garnier, A. Kamen, J. Virol. Methods 133 (2006) 82.
[4] W.R. Beyer, M. Westphal, W. Ostertag, D. von Laer, J. Virol. 76 (2002) 1488.
[5] J.C. Burns, T. Friedmann, W. Driever, M. Burrascano, J.K. Yee, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 8033.
[6] J. Gatlin, M.W. Melkus, A. Padgett, P.F. Kelly, J.V. Garcia, J. Virol. 75 (2001) 9995.
[7] J. Transfiguracion, D.E. Jaalouk, K. Ghani, J. Galipeau, A. Kamen, Hum. Gene Ther. 14 (2003) 1139.
[8] J.M. Le Doux, N. Landazuri, M.L. Yarmush, J.R. Morgan, Hum. Gene Ther. 12 (2001) 1611.
[9] L. Pham, H. Ye, F.L. Cosset, S.J. Russell, K.W. Peng, J. Gene Med. 3 (2001) 188.
[10] B. Zhang, H.Q. Xia, G. Cleghorn, G. Gobe, M. West, M.Q. Wei, Gene Ther. 8 (2001) 1745.
[11] N. Arcand, A. Bernier, J. Transfiguracion, D. Jacob, H. Coelho, A. Kamen, Bioprocessing J. 2 (2003) 72.
[12] A.M. Davidoff, C.Y. Ng, S. Sleep, J. Gray, S. Azam, Y. Zhao, J.H. McIntosh, M. Karimipoor, A.C. Nathwani, J. Virol. Methods 121 (2004) 209.
[13] D. Debelak, J. Fisher, S. Iuliano, D. Sesholtz, D.L. Sloane, E.M. Atkinson, J. Chromatogr. B Biomed. Sci. Appl. 740 (2000) 195.
[14] N. Kaludov, B. Handelman, J.A. Chiorini, Hum. Gene Ther. 13 (2002) 1235.
[15] R.H. Smith, C. Ding, R.M. Kotin, J. Virol. Methods 114 (2003) 115.
[16] S. Zolotukhin, M. Potter, I. Zolotukhin, Y. Sakai, S. Loiler, T.J. Fraites Jr., V.A. Chiodo, T. Phillipsberg, N. Muzyczka, W.W. Hauswirth, T.R. Flotte, B.J. Byrne, R.O. Snyder, Methods 28 (2002) 158.
[17] M.M. Segura, A. Kamen, P. Trudel, A. Garnier, Biotechnol. Bioeng. 90 (2005) 391.
[18] M. McGrath, O. Witte, T. Pincus, I.L. Weissman, J. Virol. 25 (1978) 923.
[19] V. Slepushkin, N. Chang, R. Cohen, Y. Gan, B. Jiang, E. Deausen, D. Berlinger, G. Binder, K. Andre, L. Humeau, B. Drupolic, Bioprocessing J. 2 (2003) 89.
[20] C. Prior, P. Bay, B. Ebert, R. Gore, J. Holt, T. Irish, F. Jensen, C. Leone, J. Mitschelen, M. Stiglitz, C. Tarr, R.J. Trauger, D. Weber, M. Hrinda, BioPharmacology 8 (1995) 25.
[21] M. Scherr, K. Battmer, M. Eder, S. Schule, H. Hohenberg, A. Ganser, M. Grez, U. Blomer, Gene Ther. 9 (2002) 1708.
[22] K. Yamada, D.M. McCarty, V.J. Madden, C.E. Walsh, Biotechniques 34 (2003) 1074.
[23] M. Kuiper, R.M. Sanches, J.A. Walford, N.K. Slater, Biotechnol. Bioeng. 80 (2002) 445.
[24] R.G. Smith, S.A. Lee, Anal. Biochem. 86 (1978) 252.
[25] M. Hammarstedt, K. Wallengren, K.W. Pedersen, N. Roos, H. Garoff, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 7527.
[26] S.T. Andreadis, C.M. Roth, J.M. Le Doux, J.R. Morgan, M.L. Yarmush, Biotechnol. Prog. 15 (1999) 1.
[27] K. Ye, S. Jin, M.M. Ataai, J.S. Schultz, J. Ibeh, J. Virol. 78 (2004) 9820.
[28] S.L. Williams, D. Nesbeth, D.C. Darling, F. Farzaneh, N.K. Slater, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 820 (2005) 111.
[29] G. Palù, C. Parolin, Y. Takeuchi, M. Pizzato, Rev. Med. Virol. 10 (2000) 185.
[30] M. Katane, E. Takao, Y. Kubo, R. Fujita, H. Amanuma, EMBO Rep. 3 (2002) 899.
[31] C.K. Tai, C.R. Logg, J.M. Park, W.F. Anderson, M.F. Press, N. Kasahara, Hum. Gene Ther. 14 (2003) 789.
[32] M.T. Shieh, D. WuDunn, R.I. Montgomery, J.D. Esko, P.G. Spear, J. Cell Biol. 116 (1992) 1273.
[33] P.G. Spear, M.T. Shieh, B.C. Herold, D. WuDunn, T.I. Koshy, Adv. Exp. Med. Biol. 313 (1992) 341.
[34] D. WuDunn, P.G. Spear, J. Virol. 63 (1989) 52.
[35] T. Jackson, F.M. Ellard, R.A. Ghazaleh, S.M. Brookes, W.E. Blakemore, A.H. Corteyn, D.I. Stuart, J.W. Newman, A.M. King, J. Virol. 70 (1996) 5282.
[36] Y. Chen, T. Maguire, R.E. Hileman, J.R. Fromm, J.D. Esko, R.J. Linhardt, R.M. Marks, Nat. Med. 3 (1997) 866.
[37] C. Summerford, R.J. Samulski, J. Virol. 72 (1998) 1438.
[38] A.A. Navarro del Cañizo, M. Mazza, R. Bellinzoni, O. Cascone, Appl. Biochem. Biotechnol. 61 (1996) 399.
[39] R.S. O'Keeffe, M.D. Johnston, N.K. Slater, Biotechnol. Bioeng. 62 (1999) 537.
[40] S. Zolotukhin, B.J. Byrne, E. Mason, I. Zolotukhin, M. Potter, K. Chesnut, C. Summerford, R.J. Samulski, N. Muzyczka, Gene Ther. 6 (1999) 973.
[41] S.R. Opie, K.H. Warrington Jr., M. Agbandje-McKenna, S. Zolotukhin, N. Muzyczka, J. Virol. 77 (2003) 6995.
[42] E.E. Fry, S.M. Lea, T. Jackson, J.W. Newman, F.M. Ellard, W.E. Blakemore, R. Abu-Ghazaleh, A. Samuel, A.M. King, D.I. Stuart, EMBO J. 18 (1999) 543.
[43] B.C. Herold, D. WuDunn, N. Soltys, P.G. Spear, J. Virol. 65 (1991) 1090.
[44] A. Kern, K. Schmidt, C. Leder, O.J. Muller, C.E. Wobus, K. Bettinger, C.W. Von der Lieth, J.A. King, J.A. Kleinschmidt, J. Virol. 77 (2003) 11072.
[45] A. Jinno-Oue, M. Oue, S.K. Ruscetti, J. Virol. 75 (2001) 12439.
[46] G. Roderiquez, T. Oravecz, M. Yanagishita, D.C. Bou-Habib, H. Mostowski, M.A. Norcross, J. Virol. 69 (1995) 2233.
[47] J. Cladera, I. Martin, P. O'Shea, EMBO J. 20 (2001) 19.
[48] I. Mondor, S. Ugolini, Q.J. Sattentau, J. Virol. 72 (1998) 3623.
[49] R.R. Vives, A. Imberty, Q.J. Sattentau, H. Lortat-Jacob, J. Biol. Chem. 280 (2005) 21353.
[50] D.S. Ory, B.A. Neugeboren, R.C. Mulligan, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 11400.
[51] A. Paquin, D.E. Jaalouk, J. Galipeau, Hum. Gene Ther. 12 (2001) 13.
[52] F.L. Cosset, Y. Takeuchi, J.L. Battini, R.A. Weiss, M.K. Collins, J. Virol. 69 (1995) 7430.
[53] J. Qiao, V. Roy, M.H. Girard, M. Caruso, Hum. Gene Ther. 13 (2002) 881.
[54] M. Pizzato, S.A. Marlow, E.D. Blair, Y. Takeuchi, J. Virol. 73 (1999) 8599.
[55] M. Baba, R. Snoeck, R. Pauwels, E. de Clercq, Antimicrob. Agents Chemother. 32 (1988) 1742.
[56] D. Schols, E. De Clercq, J. Balzarini, M. Baba, M. Witvrouw, M. Hosoya, G. Andrei, R. Snoeck, J. Neyts, R. Pauwels, M. Nagy, J. Gyorgyi-Edelenyi, R. Machovich, I. Horvath, M. Low, S. Gorog, Antivir. Chem. Chemother. 1 (1990) 233.
[57] G.H. Guibinga, A. Miyanohara, J.D. Esko, T. Friedmann, Mol. Ther. 5 (2002) 538.
[58] J.M. Le Doux, J.R. Morgan, R.G. Snow, M.L. Yarmush, J. Virol. 70 (1996) 6468.
[59] J.M. Le Doux, J.R. Morgan, M.L. Yarmush, Biotechnol. Prog. 15 (1999) 397.
[60] S.J. Walker, M. Pizzato, Y. Takeuchi, S. Devereux, J. Virol. 76 (2002) 6909.
[61] N. Landazuri, J.M. Le Doux, J. Gene Med. 6 (2004) 1304.
[62] A. Abe, A. Miyanohara, T. Friedmann, J. Virol. 72 (1998) 6159.
[63] S. Zolotukhin, Hum. Gene Ther. 16 (2005) 551.
[64] M. Aboud, M. Wolfson, Y. Hassan, M. Huleihel, Arch. Virol. 71 (1982) 185.
[65] J.W. Bess Jr., R.J. Gorelick, W.J. Bosche, L.E. Henderson, L.O. Arthur, Virology 230 (1997) 134.
[66] P. Gluschankof, I. Mondor, H.R. Gelderblom, Q.J. Sattentau, Virology 230 (1997) 125.
[67] C.M. Trubey, E. Chertova, L.V. Coren, J.M. Hilburn, C.V. Hixson, K. Nagashima, J.D. Lifson, D.E. Ott, J. Virol. 77 (2003) 12699.