MARIA DE LAS MERCEDES SEGURA

DOWNSTREAM PROCESSING OF RECOMBINANT RETROVIRAL VECTORS

Thèse présentée
à la Faculté des études supérieures de l'Université Laval
dans le cadre du programme de doctorat en génie chimique
pour l'obtention du grade de Philosophiae Doctor (Ph.D.)

FACULTÉ DE SCIENCES ET DE GÉNIE
UNIVERSITÉ LAVAL
QUÉBEC

2006

Résumé

Les vecteurs rétroviraux dérivés du virus Moloney de la leucémie murine (MoMLV) ont été utilisés pour livrer des gènes depuis plus de 20 ans et ils continuent d'être le meilleur outil disponible pour transférer de façon stable et efficace des gènes thérapeutiques dans différents types de cellules. Bien que la plupart des études précliniques de thérapie génique utilisent des surnageants bruts ou concentrés de vecteurs rétroviraux, l'étape de purification, pour éliminer le sérum et les impuretés dérivées des cellules hôtes contenus dans ces préparations, est incontournable pour les applications cliniques. Cette thèse décrit le développement de stratégies de purification des vecteurs rétroviraux. Au cours de ce projet, deux procédés complets de purification (à partir d'un surnageant brut de rétrovirus jusqu'au virus de grade clinique) ont été établis, vérifiés, et leurs performances ont été analysées en détail. La filtration sur membrane a contribuée à la clarification, la concentration, à l'échange de tampon et à la purification partielle des particules retrovirales à partir de surnageants à l'état brut sans aucune perte significative d'infectivité virale. Deux nouvelles méthodes de purification, spécifiquement adaptées aux caractéristiques biochimiques et physiques des particules rétrovirales, ont été développées. La première méthode de purification des particules rétrovirales, utilise la chromatographie d'affinité sur colonne d'héparine suivie d'un tamis moléculaire. L'avantage principal d'utiliser les techniques de chromatographie pour la purification des virus, est d'offrir la possibilité de purifier à grande échelle les rétrovirus de façon sélective et efficace. De plus, la chromatographie d'affinité sur colonne d'héparine a donné lieu à des taux de récupération exceptionnels de particules infectieuses et s'est avérée utile pour la purification des vecteurs rétroviraux produits par différentes lignées cellulaires indépendamment de l'enveloppe protéique utilisée pour le pseudo-typage. La deuxième méthode de purification est basée sur la technique de centrifugation zonale transitoire utilisant l'iodixanol comme milieu pour former un gradient. La force de cette technique repose sur les hauts niveaux de pureté obtenus en une seule étape de purification et la capacité à séparer les particules virales des espèces proches telles que les vecteurs défectueux et / ou les vésicules membranaires, qui posent un sérieux défi dans les procédés de purification. Les récupérations finales en particules infectieuses (~38%) et le degré de pureté atteint (plus de 95%) étaient comparables avec l'une ou l'autre des stratégies de purification utilisées. Les

méthodes décrites dans cette thèse représentent une amélioration significative sur la méthodologie conventionnelle utilisant un gradient de densité de sucrose pour la purification des rétrovirus et contribuera certainement à l'avancement technologique dans le domaine de la thérapie génique.

Abstract

Retroviral vectors derived from the Moloney murine leukemia virus (MoMLV) have been used as gene delivery vehicles for more than two decades and continue to be the best available tool for stable and efficient transfer of therapeutic genes into various cell types. Although most gene therapy preclinical studies use crude or concentrated retroviral vector supernatants, purification to eliminate serum and host-derived impurities contained in these stocks is a must for clinical applications. This thesis describes the development of downstream processing strategies for retroviral vectors. During the course of this project, two complete multi-step purification schemes (from crude retrovirus supernatant to clinical-grade virus) were designed, tested and their performance analyzed in detail. Membrane filtration contributed to the clarification, concentration, buffer exchange and partial purification of retroviral particles from crude supernatants with essentially no loss in vector infectivity. Two novel purification methods specifically tailored to the biochemical and physical features of retroviral particles were developed. The first method consists of the chromatographic purification of retroviral particles by heparin affinity chromatography followed by size exclusion chromatography. The main advantage of employing chromatography technology for virus purification is that it offers the possibility to selectively and efficiently purify retroviruses on a large-scale. Moreover, heparin affinity chromatography resulted in exceptional recoveries of infective particles and proved to be useful for the purification of retroviral vectors produced by different packaging cell lines independently of the Env-protein used for pseudotyping. The second purification method is based on a rate zonal centrifugation technique using iodixanol as gradient medium. The power of this technique was revealed by the high levels of purity achieved in a single purification step and its potential to separate viral particles from closely-related species such as defective vector forms and/or cell membrane vesicles, all of which pose a serious challenge in downstream processing. The overall yield of infective particles ($\sim$38%) and level of purity achieved (over 95%) using either purification strategy was comparable. The methods described in this thesis represent a significant improvement over the conventional sucrose density gradient methodology used for retrovirus purification and will hopefully contribute to the technological progress in the field of gene therapy.

Foreword

The body of this thesis is composed of four chapters, prefaced by a general introduction and followed by a general conclusion and perspective section. Each chapter is based on a scientific article which at the time of the thesis submission was either published, accepted or in evaluation. The author of this thesis is also the principal writer of these articles and responsible for the planning, execution and analysis of the experimental work presented herein. It wouldn't have been possible to complete such an endeavor without the constant guidance and supervision of Dr. Alain Garnier at the Université Laval and Dr. Amine Kamen at the Biotechnology Research Institute of National Research Council of Canada (BRI-NRC) who are co-authors in all four articles.

The first chapter consists in a review article that provides all relevant background information about the state of the art in downstream processing of retroviral vectors. The methods currently described in the literature for clarification, concentration and purification of retroviral vectors are presented whereas problems associated with stability and quantitation of retroviral particles are critically analyzed. The review also covers aspects of lentiviral vectors purification given that the structural similarities between both types of particles permit them to share common purification strategies. This article has recently been accepted for publication by *Biotechnology Advances* (December 6th 2005).

In the second chapter, a complete scaleable purification strategy for retroviral vectors that utilizes membrane and chromatography technologies is presented. In this article, heparin affinity chromatography is introduced as a novel and convenient technique for the purification of retroviral particles. This article was published in *Biotechnology & Bioengineering,* 2005, 90: 391-404. Dr. Pierre Trudel, co-author of this article, participated in the analysis of the data and revision of the draft manuscript.

An alternative complete purification strategy is presented in the third chapter of this thesis. This strategy is based on a rate zonal ultracentrifugation using iodixanol as gradient medium. The development of this method as well as the thorough characterization of the purified product is presented in an article that has been accepted for publication by the *Journal of Virological Methods* (October 6th 2005). The method is mainly intended for use

in laboratories that may lack preparative liquid chromatography systems but are equipped with ultracentrifuges, which is typically the case in academic virology laboratories. The possibility of scaling-up such protocol will depend on the availability of high capacity ultracentrifuge equipment suitable for retrovirus purification purposes.

In the last chapter, the general applicability of heparin affinity chromatography to the purification of retroviral vectors produced by different cell lines and carrying different Env-proteins is assessed. Results obtained from these studies are presented in a fourth article. Co-author of this article Marie-Claude Lavoie, a graduate student at Université Laval, carried out RD114-pseudotyped vector production.

In addition, results from this project were communicated in the following conferences:

"Purification and characterization of recombinant retrovirus" Oral presentation. **Segura M.M.**, Garnier A., and Kamen A. Seventh Ontario-Quebec CSChE Biotechnology Meeting, Queen's University, Kingston, June 9-10, 2005. ***Best Ph.D. oral presentation award.***

"Retrovirus vectors-heparin interaction and its implications for gene therapy" Oral presentation. **Segura M.M.**, Kamen A., Trudel P. and Garnier A. 7ième colloque de l'Association de thérapie génique du Québec, Quebec, October 29, 2004.

"Are host proteins on the viral membrane responsible for retrovirus attachment to target cells?" Oral presentation. **Segura M.M**., Kamen A., Pierre T. and Garnier A. 54th Canadian Chemical Engineering Conference, Calgary, October 3-6, 2004.

 "Preparation of highly purified retroviral vectors by rate zonal ultracentrifugation" Poster. **Segura M.M.**, Garnier A. and Kamen A. Bioprocess Perspective Meeting-Biotechnology Research Institute, Montreal, September 24, 2004.

"Développement d'une nouvelle méthode pour la purification à grande échelle de vecteurs rétroviraux" Poster. **Segura M.M.**, Kamen A., Trudel P., Transfiguracion J. and Garnier A. 4º Symposium annuel du CRESFSIP, Quebec, May 10, 2004.

"A novel scaleable approach for retrovirus vector purification" Poster. **Segura M.M.**, Kamen A., Trudel P., Transfiguracion J. and Garnier A. Cell Culture Engineering IX, Cancún, March 7-12, 2004.

"Development of a novel protocol for the downstream processing of recombinant retroviral vectors". Poster. **Segura M.M.**, Kamen A., Trudel P., Transfiguracion J. and Garnier A. 6th colloque de l'Association de Thérapie Génique du Québec, Montreal, October 24, 2003.

"A novel approach for MuLV-derived retrovirus vector purification". Poster. **Segura M.M.**, Kamen A., Trudel P., Transfiguracion J. and Garnier A. Stem Cell Network Annual General Meeting, Vancouver, September 18-20, 2003. ***Best poster award.***

"Development of a scaleable method for recombinant retroviral vector purification". Oral presentation. **Segura M.M.**, Kamen A., Trudel P., Transfiguracion J. and Garnier A. Fifth Ontario-Quebec Biotechnology Meeting, University of Waterloo, Waterloo, June 12-13, 2003.

"Downstream processing of recombinant retroviral vectors" Poster. **Segura M.M.**, Kamen A., Trudel P., Transfiguracion J. and Garnier A. Stem Cell Network Annual General Meeting, Toronto, September 26-28, 2002. ***Best poster award.***

Acknowledgements

I want to express my gratitude to my family in Argentina for their unconditional love and support, particularly to my mother who taught me the most important lessons and, in spite of the distance, managed to closely accompany me in every step of my career and life. I am forever grateful to my friends, particularly those who I was fortunate enough to have close to me during my studies. Their moral support and encouragement enabled me to complete this thesis and have a wonderful time along the way. Finally, I thank Gavin for the many hours of stimulating discussions and editing during the preparation of this thesis, but above all for being a part of my life.

A mis seres queridos

Table of contents

List of abbreviations and symbols

AAV: adeno-associated virus

BSA: bovine serum albumin

CA: capsid protein

$CaPO_4$: calcium phosphate

CsCl: cesium chloride

Da: Dalton

DEAE: diethylaminoethyl

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

cDNA: complementary DNA

dsDNA: double-stranded deoxyribonucleic acid

DTT: 1,4-dithiotreitol

EBA: expanded bed adsorption

Env-protein: envelope protein

EDTA: ethylene diamine tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorting

FBS: foetal bovine serum

F-MLV: Friend murine leukemia virus

GAG: glycosaminoglycan

Gag: group specific antigen retroviral protiens

GFP: green fluorescent protein

HCl: hydrochloric acid

HEK 293: Human embryonic kidney 293 cell line

HIV-1: human immunodeficiency virus type 1

HPLC: high pressure liquid chromatography

HRP: horseradish peroxidase

HSC: hematopoietic stem cells

HTLV: human T-cell lymphotropic virus

IMAC: immobilized metal affinity chromatography

IN: integrase

IVP: infective virus particles

LDL: low density lipoprotein

MA: matrix protein

Mab: Monoclonal antibody

MoMLV: Moloney murine leukemia virus

mRNA: messenger ribonucleic acid

MWCO: molecular weight cut-off

NaCl: sodium chloride

NC: nucleocapsid protein

NSEM: negative stain electron microscopy

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PTA: phosphotungstic acid

RCV: replication-competent virus

RT-PCR: reverse transcriptase polymerase chain reaction

PR: protease

qPCR: quantitative polymerase chain reaction

qRT-PCR: quantitative reverse transcriptase polymerase chain reaction

RNA: ribonucleic acid

mRNA : messenger RNA

RT: reverse transcriptase

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC: Size-exclusion chromatography

SO3-: sulphoisobutyl chromatography ligands

SU: surface subunit

$t_{1/2}$: half-life

TK: thymidine kinase protein

TM: transmembrane subunit

VP: total virus particles

VSV: vesicular stomatitis virus

VSV-G: vesicular stomatitis virus glycoprotein

List of figures

List of tables

Introduction

In 1960, Moloney published the isolation of a strain of murine leukemia virus that now carries his name (Moloney, 1960). Since then, the Moloney murine leukemia virus (MoMLV) has been widely used in research and applied biotechnology.

The MoMLV belongs to the large Retroviridae family of enveloped RNA viruses. The hallmark of this family is their ability to reverse transcribe their genome from RNA to double stranded DNA and integrate into the genome of the host cell. With the discovery of reverse transcription, the first exemption to the unidirectional and presumably irreversible flow of genetic information from DNA to RNA to protein was found. The finding of an RNA-dependent DNA polymerase or reverse transcriptase in purified virions proved that the RNA could be transcribed into DNA and its discoverers, Baltimore and Temin, shared a Nobel Prize in Physiology and Medicine with their former mentor Dulbecco in 1975 (Baltimore, 1970; Temin and Mizutani, 1970). Today, the reverse transcriptase of the MoMLV is a standard enzyme used in molecular biology laboratories to generate complementary DNA (cDNA) copies of RNA. Unlike mRNA which is unstable, cDNA can be manipulated with relative ease and thus is used in a variety of molecular techniques including cloning, sequencing, RT-PCR, microarrays and serves as a specific hybridization probe (Skalka and Goff, 1993). The cDNA copies of cellular messenger RNA (mRNA) extracts represent the genes that are being expressed in a given cell.

The study of retroviral oncogenesis started at the turn of the century with the first evidence of retrovirus existence (Ellermann and Bang, 1908; Rous, 1911) and led to the discovery of viral oncogenes (v-oncogenes) (Huebner and Todaro, 1969). These were found to be cellular mutated genes picked up by tranducing oncogenic retroviruses which transfer them into new hosts inducing tumor development (Bishop, 1983; Varmus, 1984). These naturally occurring retroviruses not only helped lay the foundation for our current understanding of cancer but also inspired scientists to use genetically modified retroviruses to transfer a gene of choice to cells. The retrovirus genome can be divided functionally into *cis*-acting sequences that are required for encapsidation, reverse-transcription and integration and *trans*-acting sequences that code for the products of the 3 viral genes (*gag, pol* and *env*). After the retroviral core is formed, no further protein synthesis is required for

the events leading to retrovirus integration in the host cell genome. In fact all viral genes can be removed from the genome and replaced with a gene of interest without affecting its ability to be encapsidated, reverse-transcribed and integrated. This is the principle behind the retroviral vector system (Miller, 1997). In nature, most retroviruses carrying oncogenes are replication-defective and require the presence of non-defective "helper" viruses which provide virus proteins *in trans* to replicate. The development of packaging systems capable of producing replication-defective retroviruses started by mimicking this natural strategy (Shimotohno and Temin, 1981; Wei et al., 1981). However, the presence of helper virus was undesired for many applications. In 1983, Baltimore and Temin's research groups reported the development of the first packaging cells that supplied *in trans* all viral proteins supporting the replication of defective retroviruses in the absence of helper viruses (Mann et al., 1983; Watanabe and Temin, 1983). This represented a major advance in retroviral vector design.

The ability of retroviruses to stably integrate into the host cell genome, thereby providing the possibility of long-term expression in the transduced cells and their progeny, provided additional incentive for the development of retroviral gene transfer vectors. Retroviral vectors were the first vectors used for efficient and stable gene transfer into mammalian cells (Cone and Mulligan, 1984). The most extensively used retrovirus for the generation of vectors is the MoMLV partly because of the simplicity of its well-studied genome. For the last 20 years, advances in retroviral vector design were accompanied by the development of novel applications (Barquinero et al., 2004). Experimental applications now include among others the construction of cDNA libraries, the generation of transgenic animals, gene silencing by stable RNA interference, chromosome tagging and cell tagging for cell lineage and clonality studies (Barquinero et al., 2004; Miller, 1997).

Perhaps the most exciting application of retroviral vectors is human gene therapy. Gene therapy is a new therapeutic approach that involves the transfer of genes into a patient's cells. Retroviral vectors have long been recognized as ideal delivery vehicles for gene transfer and thus were among the first viral systems to be developed. In 1990, they were the first viral vector to be approved by the government of the United States for gene

therapy (Anderson et al., 1990). The aim of this first clinical protocol was to treat a 4-year old patient suffering from adenosine deaminase (ADA)-deficient severe combined immunodeficiency (SCID) by transducing her own T-lymphocytes with a retroviral vector expressing a normal ADA gene *ex vivo* and then re-infusing these modified blood cells into her circulation. Although results show relatively long-term persistence of modified T cells, ideally, one would like to transduce blood stem cells to provide a permanent solution for ADA deficiency since stem cells can continuously give rise to new modified lymphocytes. In further trials, successful retroviral-mediated transfer to bone marrow CD34+ cells was reported (Aiuti et al., 2002).

In 2000, scientists put theory into practice using MoMLV-based vectors in the first clinical trial that cured a disease demonstrating proof of principle for gene therapy. In this clinical protocol, conducted by Fischer (Paris), 9 out of 11 children suffering from X-linked severe combined immunodeficiency (X-SCID) showed a clear clinical improvement (Cavazzana-Calvo et al., 2000). The protocol consisted in transducing the patient's hematopoietic stem cells *ex vivo* with a retroviral vector carrying a normal cytokine-receptor gamma chain gene. Unfortunately, a few months following this treatment 3 out of the 11 patients developed T-cell leukemia likely due to retrovirus insertional mutagenesis near the *LMO2* locus. After considerable research, it has now been determined that this adverse event appears to be due to a uniquely high-risk situation using that specific vector construct in patients suffering from that particular disease (Barquinero et al., 2004; Berns, 2004; Sinn et al., 2005). In a further ongoing trial for patients with X-SCID, significant clinical benefit with no adverse effects was reported for the 4 patients treated (Gaspar et al., 2004). In addition to the treatment of genetic disorders, retroviral vectors are also attractive candidates for the treatment of cancer *ex vivo* and *in vivo*, because they can selectively target rapidly dividing cells (Gunzburg, 2003; Rainov and Ren, 2003). Cancer is the most frequently targeted disease by gene therapy (www.wiley.co.uk). With the initiation of more than 272 clinical trials since 1990, retroviral vectors have been the most widely used vectors in gene therapy to date.

Interest in the use of retroviral vectors for gene therapy applications continues to grow, although most scientists agree that progress toward controlled integration for increased biosafety must be done and further developments in large-scale manufacturing are needed (Barquinero et al., 2004; Gunzburg, 2003; Sinn et al., 2005). In spite of the extensive work done with retroviral vectors, advances in vector design and production systems did not parallel the lagging development of purification methods (Merten, 2004). Retrovirus purification still largely relies on the traditional method of sucrose equilibrium density gradient ultracentrifugation which typically results in poor recovery of infective particles and significant contamination with cell membrane vesicles (Vogt, 1997). Highly purified retrovirus preparations are required not only for gene transfer purposes but for characterization studies, immunological studies, and as "gold standards" for downstream processing quality control. These preparations are difficult to obtain, particularly in cases where viruses occur at low titers and the viral particles are unstable, which is the case for retroviruses. For retroviruses to be used as gene transfer vectors they need to be active, that is the ability to transduce target cells must be preserved during downstream processing. Furthermore, the development of scaleable purification strategies is necessary to ensure that sufficient quantities of high purity vector stocks are available for pre-clinical and clinical trials (Andreadis et al., 1999).

The main objective of this thesis was to develop purification procedures to obtain highly purified MoMLV-derived vector preparations for use in clinical and experimental applications. The finding that retroviruses can efficiently bind heparin ligands led us to the development of a novel affinity chromatography technique and to study retrovirus-heparin interactions. The lack of a purified standard to evaluate the purity of the vectors led us to the development of a powerful alternative purification strategy based on rate zonal ultracentrifugation. Finally, a detailed analysis of the purity of the preparations was performed.

Chapter I

Downstream processing of oncoretroviral and lentiviral gene therapy vectors

María de las Mercedes Segura [1,2], Amine Kamen [2], and Alain Garnier [1]*

1- Department of Chemical Engineering, Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines, Université Laval, Québec, Canada, G1K 7P4; phone : 418-656-3106; fax: 418-656-5993; e-mail: alain.garnier@gch.ulaval.ca

2- Biotechnology Research Institute, NRC, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

1 Résumé

Les vecteurs rétroviraux d'origines oncorétrovirale et lentivirale, ont un grand potentiel pour livrer des gènes étant donné leur intégration efficace dans le génome des cellules cibles, procurant la possibilité d'une expression génique à long terme. Plusieurs groupes de recherche ont déployé des efforts considérables pour le développement de procédés à grande échelle permettant d'obtenir des quantités de vecteurs rétroviraux nécessaires aux essais pré-cliniques et cliniques. Une attention particulière a été portée à la conception de systèmes de production optimisés capables de générer sécuritairement de grands volumes de vecteurs ayant des titres viraux satisfaisants. Cependant, la production de vecteurs de grade clinique pour la thérapie génique, spécifiquement pour les applications *in vivo*, requière également des stratégies de purification pouvant être mises à l'échelle, pour enlever les contaminants présents dans les surnageants récoltés, tout en préservant la fonctionnalité des vecteurs. Dans cet article, nous avons fait le bilan des récents progrès dans le domaine des procédés de purification des vecteurs retroviraux. Les méthodes courantes décrites dans la littérature concernant la clarification, la concentration et la purification des vecteurs rétroviraux seront présentées, avec une emphase spéciale sur les nouvelles méthodes de chromatographie qui donnent la possibilité de purifier les rétrovirus à grande échelle et ce de façon sélective et efficace. Les problèmes associés à la stabilité et la quantification des particules rétrovirales seront soulignés et les défis futurs seront discutés.

2 Abstract

Retroviral vectors from both oncoretroviral and lentiviral origin have a great potential as gene delivery vehicles because they integrate efficiently into the genome of the target cells providing the possibility of lifelong gene expression. A number of research groups have devoted considerable effort to the development of large-scale processing strategies for retroviral vectors to ensure that sufficient quantities of vector stocks are available for pre-clinical and clinical trials. Particular attention has been given to the design of optimized production systems able to generate large volumes of safe retroviral vector stocks in satisfactory viral titers. However, the manufacturing of clinical-grade vectors for gene therapy, especially for *in vivo* applications, additionally requires scaleable purification strategies to remove the contaminants present in the harvested supernatants while preserving the functionality of the vectors. In this article, we review recent advances made in the field of downstream processing of retroviral vectors. The methods currently described in the literature for clarification, concentration and purification of retroviral vectors will be presented, with special emphasis on novel chromatography methods that open up the possibility to selectively and efficiently purify retroviruses on a large-scale. Problems associated with stability and quantitation of retroviral particles will be outlined and future challenges will be discussed.

3 Introduction

Gene therapy is defined as the administration of genetic material in order to modify or manipulate the expression of a gene product or to alter the biological properties of living cells for therapeutic use. It is a developing technology that holds great promise for the treatment of inherited metabolic disorders as well as acquired diseases such as cancer, cardiovascular and some infectious diseases, cancer being the most frequently targeted disease.

According to the mode of gene delivery to the target cells, there are two major categories of somatic cell gene therapy. In the *ex vivo* approach, cells are removed from the body, incubated with the vector and genetically modified cells are returned to the body. This procedure is generally limited to a few cell types, such as blood cells, that are easy to remove and return. The second is the *in vivo* approach, where the vector is administered directly to the patient. The vector can be delivered either locally into the affected tissue or systemically into the bloodstream of the patient. The injection of a vector directly into a tumour mass is a good example of local administration.

Retroviral vectors have attracted the attention of gene therapy researchers for their ability to stably integrate the transgene of interest into the target cell genome providing the possibility of long-term gene expression and ultimately long-term therapeutic effect. Being the most popular viral vector used in clinical trials, vectors derived from the oncovirus Moloney murine leukemia virus (MoMLV) have demonstrated great potential as gene delivery vehicles. More recently, vectors derived from another well characterized member of the retrovirus family, the lentivirus human immunodeficiency virus type 1 (HIV-1), have been developed and approved for use in human clinical studies. While oncoretroviral vectors can only transduce dividing cells, lentivirus vectors can deliver genes to dividing as well as non-dividing cells. This is very convenient since many potential target cells including neurons, hepatocytes, myocytes, retinal photoreceptors, macrophages and hematopoietic stem cells (HSC) divide infrequently *in vivo*. However, the inability of oncoviruses to transduce non-dividing cells can be attractive to selectively target rapidly

dividing cells such as cancer cells (Rainov and Ren, 2003). For simplicity purposes, we will refer to oncoretroviral and lentiviral vectors as retroviral vectors in this review.

Major obstacles associated with retroviral vectors include the production of low-titer viral stocks and the instability of the viral particles produced. In the best cases, between 10^6 to 10^7 infective viral particles per mL of cell culture supernatant are produced by commonly used producer systems. These concentrations are high enough for certain *ex vivo* applications. However, concentration of vector stocks is required for most gene therapy applications in order to improve transduction efficiencies. Moreover, concentrated or not, viral stocks still contain contaminants that need to be removed to increase the potency and safety of the final product. Non-purified vector preparations contain contaminating molecules that are toxic to cells and reduce transduction efficiencies *ex vivo* (Yamada et al., 2003). These preparations also induce a systemic immune response and inflammation when injected *in vivo* (Baekelandt et al., 2003; Scherr et al., 2002). Impurities contained in the vector supernatant not only come from the supplements and reagents added to the culture (i.e. serum, plasmid DNA for transient transfection), but also are released by intact or disrupted producer cells (i.e. inhibitors of transduction, genomic DNA and host proteins).

The development of large-scale production and purification methods for the generation of high-titer clinical-grade retroviral vectors is critical to advances in gene therapy. Over the past several years considerable progress has been made in the fields of vector design and production systems. Using optimized bioreactor systems, production of large volumes of retroviral vector stocks is feasible (for review see McTaggart and Al-Rubeai, 2002; Merten, 2004; Zufferey, 2002). Less effort has been invested in developing and optimizing purification processes able to handle large volumes of vector stocks.

Candidate technologies for the downstream processing of oncoretroviral vectors were previously proposed (for review see Andreadis et al., 1999; Braas et al., 1996; Lyddiatt and O'Sullivan, 1998). Membrane separation and chromatography were deemed the most promising technologies for large-scale manufacturing of retroviral vectors. Additionally, the authors strongly encouraged the development of methods specifically

tailored to the unique biochemical and physical features of retroviral particles. As a result, various affinity chromatography strategies and new chromatography technologies for the purification of retroviral vectors have emerged in the past few years (Segura et al., 2005; Slepushkin et al., 2003; Williams et al., 2005a; Williams et al., 2005b; Ye et al., 2004). This review will provide the reader with an overview of the techniques recently made available for downstream processing of oncoretroviral and lentiviral vectors. Since both types of vectors share common structural and physical properties, we anticipate that it will be possible to rapidly adapt techniques originally developed for one vector to the other. It is the authors' hope that the information presented here benefits future developments.

4 The retroviral particle

Retroviruses comprise a family of RNA enveloped viruses broadly divided in two categories (simple and complex) according to their genome organization. All retroviruses contain at least 3 major coding domains: gag, pol and env. While simple retroviruses such as the MoMLV only carry these genes, complex retroviruses including lentiviruses present several accessory genes which regulate details in the virus replication cycle.

Retroviral particles are enveloped with a lipid membrane derived from the virus-producer cell (Figure 1). Embedded in this membrane is the viral encoded envelope protein that interacts with specific receptors on the cell surface. This protein is cleaved into transmembrane (TM) and surface (SU) subunits that remain attached to each other by noncovalent interactions. Retroviral vectors are usually pseudotyped; that is, they carry foreign virus envelope proteins that confer them beneficial properties for gene therapy. For instance, retrovirus pseudotypes bearing the VSV-G protein instead of the natural envelope protein have an extended host cell range and show increased physical stability (Burns et al., 1993). Gag, the most abundant protein in the virion, is cleaved during maturation into 3 individual structural proteins that form "layers" underneath the lipid membrane. The matrix (MA) forms the outer layer that surrounds the viral core. The core is delimited by a protein shell composed of capsid (CA) proteins and encloses the nucleoprotein complex that contains two identical positive strands of RNA genome complexed with nucleocapsid (NC) proteins. Infective retrovirus particles contain 3 virally encoded enzymes: reverse transcriptase (RT), integrase (IN) and protease (PR). Additionally, retroviruses incorporate several host cellular proteins on the surface and inside the virion, some of which are believed to play a role in the virus replication cycle (Ott, 2002). Overall, the retrovirus particles are composed of 60-70% protein, 30-40% lipid, 2-4% carbohydrate and 1-2% RNA (Andreadis et al., 1999).

Retroviruses share common physical characteristics. The particles are spherical and measure about 80-120 nm in diameter according to thin-section electron microscopy. They have a mass of ~ 2.5×10^8 Da (Vogt and Simon, 1999) and present a density of 1.16 g/mL in sucrose density gradients.
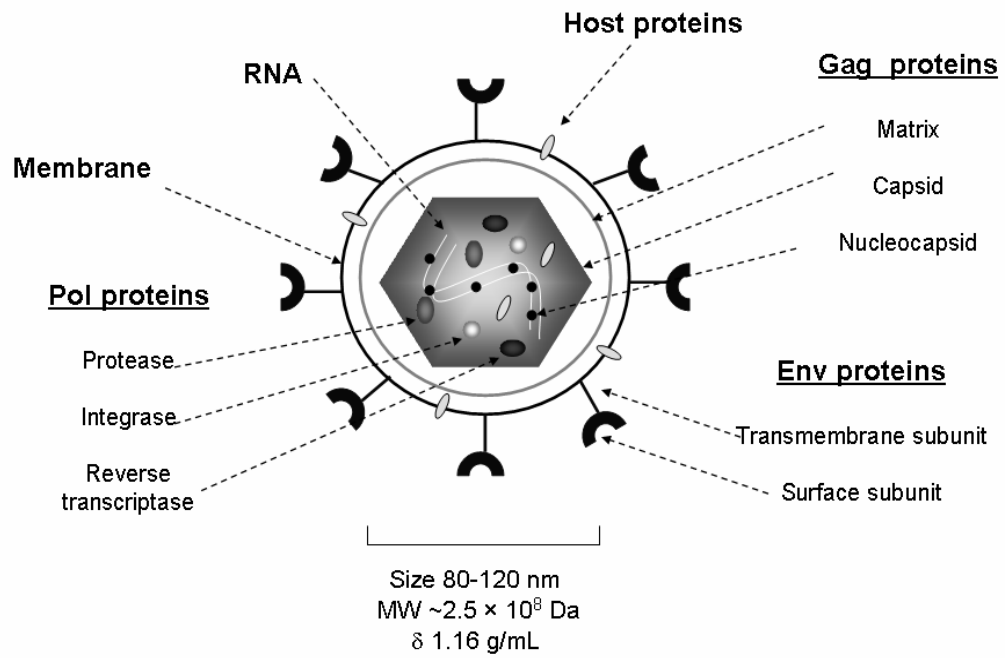
Figure 1 Retroviral particle structure

5 Stability of retroviral particles

Retroviral particles are extremely labile. From the downstream processing point of view, retrovirus instability is translated into low overall recoveries of infective viral particles. To minimize the loss of infective particles, it is important to have a good knowledge of the stability of the vector and its susceptibility to different factors (i.e. temperature, pH, ionic strength, shear stress) prior to designing downstream processing strategies. Ideally, stability studies of the vector in question to the environmental conditions to which the virus will be exposed during purification should be performed.

Retroviral vectors rapidly lose their activity at 37°C, the temperature at which the vectors are produced and titered, with a half-life between 5 to 8 h (Andreadis et al., 1997; Higashikawa and Chang, 2001; Le Doux et al., 1999; McTaggart and Al-Rubeai, 2002; Segura et al., 2005). As temperature decreases, retrovirus half-life increases. At room temperature, retroviral vectors present half-lives between 1 and 2 days (Higashikawa and Chang, 2001; Segura et al., 2005). The vectors' stability markedly improves at 4°C with half-lives over 8 days (Higashikawa and Chang, 2001). Retrovirus temperature stability was found to be dependent on the particular vector envelope protein and producer cell line type from which the viral lipid envelope was derived (Beer et al., 2003; Burns et al., 1993). The number of freeze-and-thaw cycles should be kept to a minimum during downstream processing. Retroviral vector stocks, both concentrated and nonconcentrated, lose half of their activity after the first 2 to 4 freeze-and-thaw cycles (Bowles et al., 1996; Burns et al., 1993). Therefore, in order to predict and correctly interpret the temperature-related inactivation that occurs during purification and rationally select the most convenient way to store vector stocks in between downstream processing operations, vector stability at room temperature, 4°C and the stability to freeze-and-thaw cycles should be determined in each case.

Studies of the effect of pH on the activity of VSV-G pseudotyped retroviral vectors revealed that the vectors are more stable at pH 7.0, 37°C, but their half-lives markedly dropped to less than 10 min at pH 6 or pH 8 (Higashikawa and Chang, 2001). Similar observations were reported by Ye and collaborators (Ye et al., 2003) who found that

ecotropic MoMLV remains infectious in a narrow pH range from 5.5 to 8.0. Virus inactivation beyond these limits of pH was fast and irreversible. Electron microscopy studies showed that the viral envelope was degraded at extreme pH as revealed by the penetration of the heavy metals used for staining.

Hyperosmotic conditions lead to the loss of water from organelles, vesicles, and enveloped virions. Loss of infective retroviral particles following salt precipitation and sucrose density ultracentrifugation were partly attributed to retrovirus sensitivity to osmotic pressure (Aboud et al., 1982; Andreadis et al., 1999). VSV-G pseudotyped oncoretroviral vectors infectivity was shown to be affected by increasing NaCl concentrations (Segura et al., 2005). The biological inactivation of the vector after NaCl treatment was irreversible and happened very rapidly. Just 1 h of exposure to 1M NaCl at room temperature was enough to inactivate 50% of the virus. Morphological changes and broken particles were observed after a 3 h treatment with high salt concentration.

Chemical compounds introduced at some stage during downstream processing may also affect retroviral vectors' ability to transduce. For example, oncoretroviral vectors were found to be sensitive to imidazole, a common desorption agent used for immobilized metal affinity chromatography (IMAC) (Ye et al., 2004). Recovery of infective particles was improved from 35% to 56% by using half the concentration of imidazole for vector elution. Similarly, oncoretroviral vectors were found to be susceptible to increasing concentrations of d-biotin which is used to elute bound proteins from streptavidin coated chromatography supports (Williams et al., 2005b). In addition, it has been demonstrated that exposure of retrovirus particles to denaturing agents (i.e. guanidine-HCl or urea), typically used to elute proteins from affinity matrices, results in 100% inactivation of the virus (Williams et al., 2005b). Susceptibility of the retroviral particles to EDTA used to re-dissolve retrovirus-calcium phosphate pellets has also been described (Pham et al., 2001).

Finally, shear forces encountered during ultracentrifugation also influence the stability of retroviral vectors. Due to the monomeric structure of the protein, VSV-G pseudotyped particles are more stable than those containing the widely used dimeric

amphotropic Env-protein and thus can be effectively concentrated generating high-titer vector stocks (Burns et al., 1993).

Given the instability of retroviral particles, the factors described above should be considered at the time of selecting appropriate virus purification methods in order to maximize recovery of infective retroviral particles. Density ultracentrifugation using highly hyperosmotic media, aqueous two-phase extraction using high salt concentrations, precipitation with salts and also adsorptive chromatography procedures that require the use of harsh conditions to elute viral particles are among the methods that could potentially have an impact on the stability of the virus particle.

6 Retrovirus quantitation methods

The availability of reliable tools to quantify retrovirus particles is critical for the development of downstream processing strategies. Although a variety of quantitation methods are being used, most suffer from known flaws. Ideally, a combination of two methods should be used to determine both active (transduction-competent) and total retrovirus particles. While direct quantitation of transduction-competent retroviral particles is carried out in assays involving the use of target cells, the total number of virus particles can be determined directly in vector supernatants (Figure 2).

The viral titer is usually defined as the number of transduction-competent retrovirus particles per mL of virus stock. Viral titers are typically quantitated by measuring transgene expression in target cells. For this purpose, most retroviral vectors used in developmental phases usually carry marker genes, such as GFP, lacZ or antibiotic resistance genes, which allow for rapid detection of transduced cells. The titration assays consist of overlaying serial dilutions of vector stocks onto target cells. Detection of transduced cells is carried out either by visual identification of marker protein expressing colonies (Chang and Zaiss, 2002; Srinivasakumar, 2002) or by flow cytometry (Dull et al., 1998; White et al., 1999). Although measuring marker transgene expression remains the most useful criterion to determine vector potency, the method has several limitations. Viral titers are influenced by specific transduction conditions used in the assay such as virus stock volume, time of virus exposure to target cells, the number and size of the target cells, polybrene concentrations and in the case of oncoretroviral vector the rate of cell growth. In addition, the assay is time consuming typically requiring 4 to 5 days for completion (Carmo et al., 2004) depending on the detection technique employed. Moreover, due to slow virus diffusivity and rapid virus decay only a small proportion of active virus particles in a stock (~10%) successfully transduces target cells (Andreadis et al., 2000). Hence, the method underestimates the number of transduction-competent retrovirus particles. Mathematical models that provide a better estimate of the initial concentration of active virus particles in a stock, independent of the specific conditions used in an assay, have been reported (Andreadis et al., 2000; Kwon and Peng, 2002).

Alternatively, viral titers can be determined by quantifying proviral DNA or transgene mRNA levels in the transduced target cells. The main advantage these methods offer over the traditional titration assay described above is that they do no rely on the presence of marker genes. Proviral DNA integration events can be determined using real time quantitative polymerase chain reaction (qPCR) (Pan et al., 2002; Sastry et al., 2002). However, since vector integration does not necessarily correlate with successful transgene expression, the method tends to overestimate viral titers (Sastry et al., 2002). A better approach is to measure transgene expression at the mRNA level by quantitative reverse transcriptase PCR (qRT-PCR) (Lizee et al., 2003). Semiquantitative Southern and Northern blotting could also be used to quantify proviral DNA and mRNA levels in transduced cells. However, these methods are time consuming, labor-intensive and have limited accuracy compared to PCR-based assays.

Several methods can be used to quantify total virus particles directly in vector stocks. These methods do not discriminate between active and inactive retrovirus particles and therefore provide little information concerning the potency of the vector preparation. Nevertheless, they are useful to study variations in total to active particle ratios and determine the quality of vector stocks at different stages of the purification process.

Negative stain electron microscopy is the gold standard for the quantitation of total retrovirus particles. Virus particles premixed with a known concentration of latex beads are typically stained with uranyl acetate or phosphotungstic acid and counted under transmission electron microscope (Alain, 1997). The method requires previous concentration and purification of vector supernatants since virus concentrations in vector supernatants are usually too low to be accurately quantified and impurities contained in the vector supernatant may prevent observation of virus particles (Kwon et al., 2003). Moreover, caution should be taken when examining samples containing high amounts of cellular membrane vesicles such as those obtained by sucrose density ultracentrifugation since these vesicles might be confused with retroviral particles (Bess et al., 1997; Gluschankof et al., 1997). High performance liquid chromatography (HPLC) also showed to be useful for the quantitation of total virus particles (Transfiguracion et al., 2004).

Retroviral particles were separated from protein contaminants using anion exchange chromatography and detected by absorbance at 260 nm. This method also requires concentration of vector supernatants and Benzonase® treatment due to contaminating DNA interference.

Another possibility is to estimate the number of total particles by measuring virus components. A variety of immunoassays can be employed to detect and quantify viral proteins including quantitative determinations of p24Gag (lentivectors) or p30Gag (oncoretrovectors) capsid protein content by enzyme-linked immunosorbent assay (ELISA) or semiquantitative Western blotting (Naldini et al., 1996; Rigg et al., 1996). Additionally, enzymatic assays for reverse transcriptase activity can be performed. Some of these procedures may be conducted using commercially available kits (Logan et al., 2004; Naldini et al., 1996). A method for single retrovirus particle visualization and enumeration using indirect immunofluorescence microscopy has also been described (Pizzato et al., 1999). A sensitive method to quantify RNA genome copies directly in vector supernatants using qRT-PCR has been described (Carmo et al., 2004). Although the method allows for accurate and rapid results, it only quantifies vector particles containing RNA. Due to the presence of defective retrovirus particles without RNA, the method underestimates total particle counts. On the other hand, the number of transduction-competent particles is overestimated by this method since defective particles with RNA are also present in vector stocks.
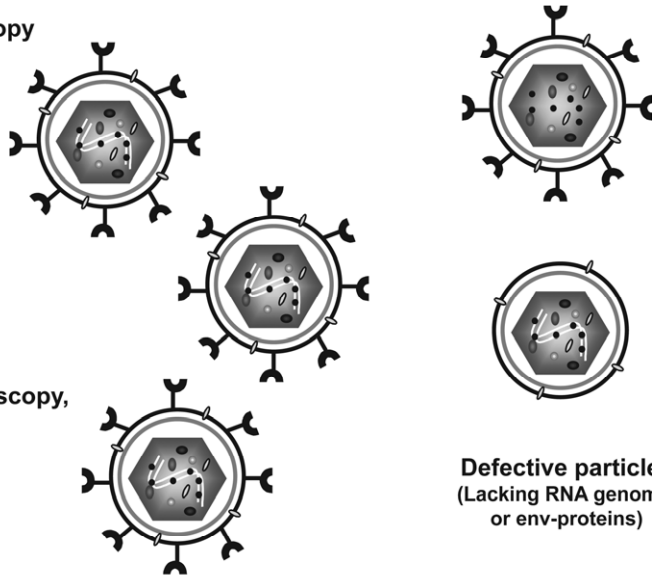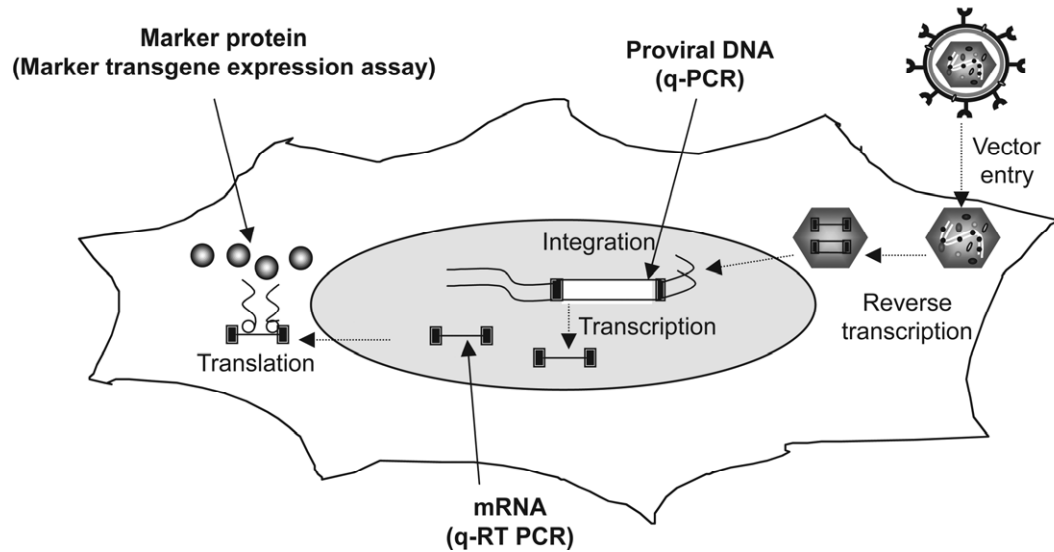
Figure 2 Assays used for the quantitation of total retrovirus particles in vector stocks and transduction-competent particles.

It is important to note that each purification step carries the risk of virus inactivation and the potential to separate active from defective particles, thus active to total particle ratios may change during purification. As a result, these ratios are not universal and can not be used blindly to determine the concentration of active virus particles based on a total particle count and vice-versa. Ratios for each particular situation could be established for use in routine quantitation of samples at the same purification stage, but this approach has less value for developmental phases.

Additionally, the use of in-house virus standards is highly recommended to avoid inter-assay discrepancies. Moreover, to validate each laboratory's in-house virus standards and assays and to facilitate inter-laboratory comparisons, it is necessary to normalize titer values to a common standard. As described for adenoviral and adeno-associated viral vectors, lentiviral and oncoretroviral vector reference standards are being established (Flotte et al., 2002).

Finally, a major concern for the safety of retroviral vector preparations is the presence of replication-competent viruses (RCV). Methods for detection of RCV are beyond the scope of this article and the reader is referred to the "Supplemental Guidance on Testing for Replication-Competent Retrovirus in Retroviral Vector-Based Gene Therapy Products and During Follow-up of Patients in Clinical Trials Using Retroviral Vectors" issued by the FDA's Center for Biologics Evaluation and Research (CBER) in October 2000 for useful information about this subject (CBER, 2001).

7 Downstream processing strategies

At the end of the production phase, harvested retroviral vector supernatants undergo a series of processing steps aimed at improving the potency of the vector preparation and eliminating the impurities contained in the vector supernatant.

```
┌─────────────────────────────────┐
│           Production            │
└─────────────────────────────────┘
                │
                ▼
┌─────────────────────────────────┐
│          Clarification          │
└─────────────────────────────────┘
                │      Producer cells, cell debris,
                │      organelles
                ▼
┌─────────────────────────────────┐
│          Concentration          │
└─────────────────────────────────┘
                │      Water, salts, serum and
                │      cell low molecular
                │      weight contaminants
                ▼
┌─────────────────────────────────┐
│           Purification          │
└─────────────────────────────────┘
                │      Host DNA and RNA,
                │      proteins and lipids
                ▼
┌─────────────────────────────────┐
│            Polishing            │
└─────────────────────────────────┘
                │      Remaining contaminants,
                │      defective vector forms
                │      and/or cell microvesicles
                ▼
┌─────────────────────────────────┐
│        Formulation/Storage      │
└─────────────────────────────────┘
```
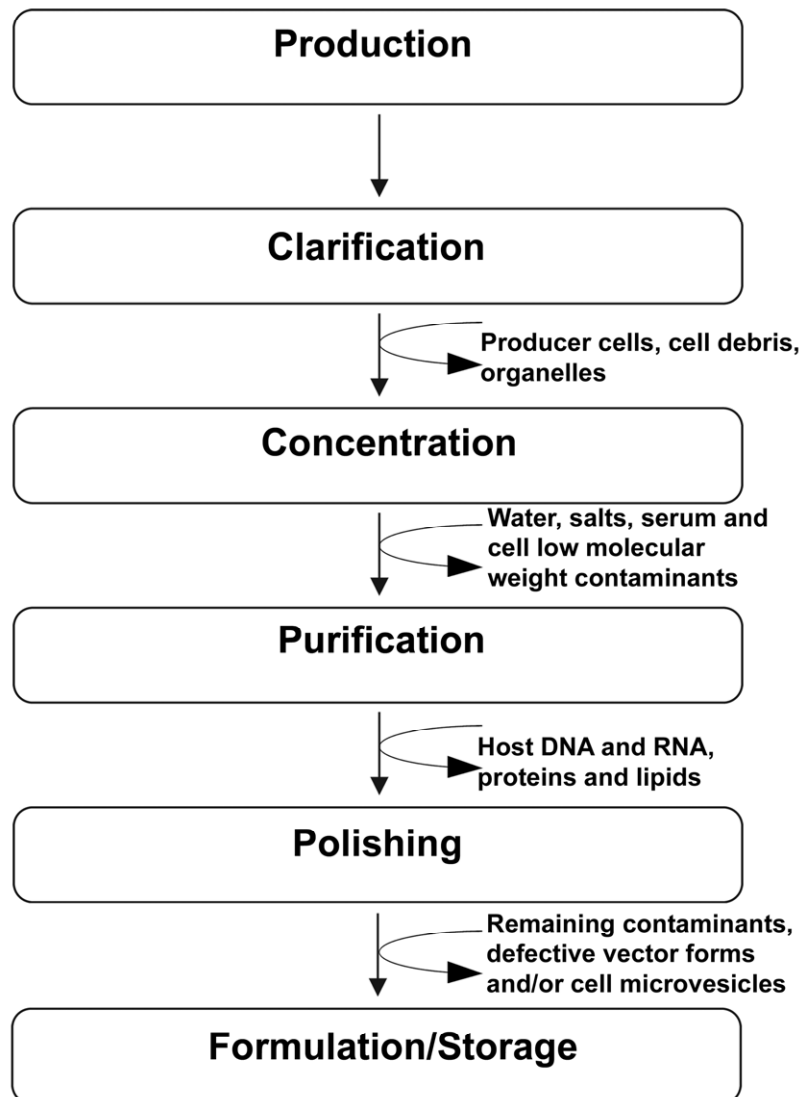
Figure 3 Flow chart for the downstream processing of retroviral gene therapy vectors. Contaminants eliminated in each process step are indicated.

Serum

Serum is the main source of contaminants in harvested supernatants. Serum supplementation increases the complexity, duration and cost of downstream processing operations and presents the risk of introducing biological contaminants. Naturally, the use of serum-free media for vector production facilitates downstream processing by dramatically decreasing the amount of contaminating proteins (i.e. bovine serum albumin, bovine transferrin and immunoglobulins) and lipids. Unfortunately, reports demonstrating successful production of retroviral vectors in serum-free media are scarce (McTaggart and Al-Rubeai, 2002). Alternatively, production of vectors in very low protein media helps reduce the chances of contamination (McTaggart and Al-Rubeai, 2000; Moy et al., 2000). Specific vector productivity is often higher in low protein media than at the 10% serum concentrations typically used (McTaggart and Al-Rubeai, 2002; Merten, 2004; Zufferey, 2002).

Inhibitors of transduction

The producer cell line itself could be a source of contamination. Producer cells release inhibitors of transduction such as proteoglycans, glycosaminoglycans and free envelope proteins into the supernatant that reduce the vectors' potential for efficient gene delivery (Le Doux et al., 1996; Le Doux et al., 1998; Slingsby et al., 2000)

Host proteins

In addition, disrupted producer cells release membrane fragments and impurities derived from the cell cytoplasm including large amounts of host proteins and genomic DNA. Pre-clinical studies with lentiviral vectors have shown the significant contribution of 293T producer cell-derived components to the immune response (Baekelandt et al., 2003).

DNA contaminants

Contaminating genomic DNA is also considered potentially hazardous. Moreover, it interferes with RCV detection by PCR-based methods (Chen et al., 2001). The levels of DNA contamination were found to continuously increase during production of VSV-G

oncoretroviral vectors probably due to VSV-G toxicity on producer cells (Segura et al., 2005). In the case of vector production by transient transfection, a large amount of plasmid DNA is added to cell cultures every time a vector lot is produced with the associated risk of introducing adventitious agents including endotoxins (a fever-producing byproduct of gram-negative bacteria commonly known as pyrogen). Removal of the plasmid DNA coding for the packaging functions may be desired to avoid the risk of transferring these functions to the target cells (Sastry et al., 2004).

Host cell-derived impurities and endotoxins are of particular concern for regulatory agencies and their removal beyond detectable limits is required for the production of clinical-grade vector preparations (Smith et al., 1996). Determining the optimum harvesting period is critical to avoid massive contamination with host cell impurities. In practice, sacrificing product yield for quality by discarding the last days of vector production can be worthwhile.

Both high molecular weight proteoglycans and DNA contaminants represent an important challenge for downstream processing. Due to their large size and strong negative charge they can co-purify with retroviruses when using common separation methods based on size or charge. Digestion steps using condroitinase ABC and DNase could be introduced in the downstream process to eliminate proteoglycans and DNA contaminants respectively (Le Doux et al., 1996; Le Doux et al., 1998; Sastry et al., 2004). However, subsequent removal of digested products and added enzymes would be required, increasing the duration of the process. Considering the instability of retroviral vectors, longer purification processes that may result in low overall recoveries of infective viral particles should be avoided whenever possible.

Strategic design and optimization of the procedures is critical to maximize yield and quality of the final product and ensure consistency of the manufacturing process (Figure 3). The selected methods for the clarification and concentration of retroviral particles should be amenable to handling large volumes of supernatant. These initial steps are primarily intended for removing cells, cell debris and water. Some degree of purification may also be

accomplished during concentration. However, high resolution at these early stages of the process is not as important as scalability (Table I). The main purification issues are left to be resolved during the purification stage itself. During this stage, retroviral particles are separated from most contaminants contained in the vector supernatant. Often more than one purification step is required to bring the product to the desired level of purity. The polishing step is further introduced to remove remaining impurities and/or closely-related species (i.e. defective vector forms and/or cell membrane vesicles). The final product should be specially formulated for long-term storage stability.

Table I Laboratory and large-scale methods for retrovirus purification

Process	Laboratory scale	Large scale
Clarification	Centrifugation Microfiltration	Microfiltration
Concentration	Pelleting Precipitation	Ultrafiltration Continuous flow centrifugation
Purification	Density gradient ultracentrifugation	Chromatography Continuous flow centrifugation

7.1 Clarification

Clarification, the removal of producer cells and cell debris from crude supernatant, is the first step of the downstream process. This step is performed immediately after vector harvest. At the laboratory scale, removal of cells and large cell debris is achieved by low speed centrifugation and microfiltration. The introduction of a centrifugation step before membrane filtration avoids membrane clogging. Microfiltration through 0.45 μm pore size

filters follows to achieve greater clarification. For working volumes exceeding 1L, clarification using a single step of membrane filtration is preferred. In this case, fast clogging of the pores with cell debris may occur, depending on the initial membrane pore size and quality of the crude stock, resulting in reduction of the membrane actual pore size and consequently virus rejection. Indeed, recovery of infective particles after microfiltration through 0.45 μm membranes was found to correlate with filtration rates which are associated with the extent of pore obstruction (Reeves and Cornetta, 2000). Therefore, it is crucial to limit the volume of supernatant to be passed per filter. It is also convenient to filter crude supernatants through a series of membranes with decreasing pore size to minimize membrane clogging. This strategy avoids the need for a prior centrifugation step and results in efficient supernatant clarification with minimum loss in vectors' titer (Moy et al., 2000; Reeves and Cornetta, 2000; Segura et al., 2005; Slepushkin et al., 2003)

7.2 Concentration

One of the main limitations with retroviral mediated gene therapy is that gene delivery rates are usually too low to achieve therapeutic effect for most *in vivo* applications. Transduction efficiencies can be improved by using concentrated doses of retroviral vectors. Several methods have been proposed for concentration of viral particles (Table II). Introducing this step in the early stages of downstream processing facilitates subsequent operations by reducing the volume of feed and consequently the size of the equipment and infrastructure required.

7.2.1 Centrifugation

Virus pelleting by centrifugation is traditionally employed to concentrate viruses. Both ultracentrifugation and long low-speed centrifugation methods (usually several hours) can efficiently pellet retroviruses. Using centrifugation, high concentration of the virus stocks (over 100-fold) can be easily attained by resuspending viral pellets in small volumes of resuspension buffer (Table II). However, transduction efficiencies usually do not increase proportionally with the concentration factor and often they do not increase at all compared to nonconcentrated virus stocks. This effect has been attributed to loss of active viral

particles due to shear stress or extended processing time and to co-concentration of viral particles with high molecular weight inhibitors of transduction (Bajaj et al., 2001; Burns et al., 1993; Le Doux et al., 1996; Transfiguracion et al., 2003). In addition, susceptibility of each particular pseudotyped retroviral vector to hydrodynamic shear varies depending on the stability of the env-protein (Burns et al., 1993). Another important limitation of ultracentrifugation procedures is that ultra-high speed rotors currently in use generally have small volume capacity (Table II).

7.2.2 Precipitation

Several methods for the concentration of retroviruses by precipitation with additives have been described. 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 (Table II). Charged polymers can be used to induce retrovirus precipitation. Cationic polymers enhance transduction efficiency and form virus-polymer complexes that can be pelleted by low-speed centrifugation. For instance, Zhang and collaborators (Zhang et al., 2001) reported a protocol for the concentration of 3 L of supernatant per round using poly-L-lysine, although recovery of active viral particles was only 26%. Curiously, while anionic polymers alone inhibit retroviral transduction, the addition of a mixture of anionic and cationic polymers to virus stocks improves transduction efficiencies and results in the formation of complexes that can easily be concentrated and purified by a rapid low speed centrifugation step (Le Doux et al., 2001). A major disadvantage with the use of these polymers is that they interact irreversibly with retrovirus particles to form a virus-polymer complex that cannot be dissociated for further processing. Alternatively, co-precipitation with calcium phosphate ($CaPO_4$) has been used to concentrate retrovirus particles (Morling and Russell, 1995). In this case, virus pellets can be re-dissolved by chelation using EDTA and re-concentrated (Pham et al., 2001). However, high concentrations of EDTA have been shown to affect virus stability. Moreover, the use of salts for virus precipitation may contribute to the loss of active viral particles due to changes in osmotic pressure. In order to minimize virus inactivation during the procedure, immediate dialysis of the concentrated

preparation was performed that resulted in satisfactory virus recoveries (50-60%) (Pham et al., 2001).

7.2.3 Ultrafiltration

Ultrafiltration is the preferred method for large-scale processing of retroviral particles because it allows gentle processing of large volumes of supernatant in a relatively short time (Table II). In contrast to the standard concentration methods discussed above, filtration processes involve no change of phase (liquid to solid) which may be traumatic enough to cause virus inactivation (Lyddiatt and O'Sullivan, 1998). Viral particles are enriched in the retentate while water and small molecular weight molecules are removed with the permeate. It should be noted that in order to keep viral particles in the retentate 100,000 or 300,000 molecular weight cut-off (MWCO) membranes are most often employed (Table II). Membrane processes offer the possibility of washing off impurities (ultra/diafiltration), thus achieving greater levels of purity. In addition, the retentate could be diafiltered against equilibration buffer used for chromatography.

Ultrafiltration can be carried out using a variety of filtration devices. At small scale, centrifugal filtration devices usually work well (Reiser, 2000). To process small to medium volumes of vector stocks (10 mL to 2 L) stirred cell tanks are ideal (Miller et al., 1996). Stability of retroviral particles using this method was found to be strongly dependent on the ultrafiltration operational parameters including pressure, stirring rate and process time (Cruz et al., 2000). By keeping these variables low we were able to concentrate vector supernatants 20-fold with excellent recovery of active particles and simultaneously remove significant amounts of serum proteins, degraded DNA fragments and inhibitors of transduction (Segura et al., 2005). Larger volumes of retroviral vectors (8 to 10 L) were concentrated by tangential-flow filtration achieving high recovery of active vector particles (Kotani et al., 1994). Tangential-flow hollow fiber filters were successfully employed for the concentration of wild-type retroviruses as well as retroviral vectors that resulted in 10 to 40-fold concentration of supernatants with good recovery of retrovirus activity (Makino et al., 1994; Paul et al., 1993). This strategy is currently being used for the concentration of lentiviral vectors for phase I clinical trials (Slepushkin et al., 2003).

Membrane fouling is the main problem faced during ultrafiltration since it causes the flow rate to decrease over time. To keep process time within reasonable limits, without increasing operating pressures that might affect the virus stability, it is often necessary to restrict the volume reduction.

CONCENTRATION	Retrovirus	Concentration Factor[a]	Concentration Parameters	Recovery[b]	Reference
CENTRIFUGATION					
Pelleting by ultracentrifugation	VSV-G oncoretrovector	100-300	Centrifuge 50,000xg for 1.5 h at 4°C (3-9 mL/tube), Beckman SW41 rotor	94-100%	Burns et al., 1993
Pelleting by low speed centrifugation	Oncoretrovector	10-100	Centrifuge 6,000xg for 16 h at 4°C (250 mL/bottle), Sorvall H6000A rotor	90-97%	Bowles et al., 1996
Pelleting by ultracentrifugation	Lentivectors	86	Centrifuge 110,000xg for 1.75 h at 15°C (30 mL/tube), Beckman SW28 rotor	81-116%	Reiser, 2000
Pelleting by ultracentrifugation	RD114-oncoretrovector	-	Centrifuge 100,000xg for 1.4 h	50-70%	Gatlin et al., 2001
Pelleting by ultracentrifugation	LCMV-oncoretrovectors	37-74	Centrifuge 110,000xg for 2 h at 4°C (37 mL/tube), Beckman SW28 rotor	103 ± 62%	Beyer et al., 2002
Pelleting by ultracentrifugation with sucrose cushion	VSV-G oncoretrovector	25-100	Centrifuge 100,000xg for 2 h at 4°C (34 mL/tube), Beckman SW28 rotor	45-89%	Transfiguracion et al., 2003
Pelleting by low speed centrifugation	RD114-oncoretrovector	100	Centrifuge 7277xg for 24 h at 4°C	37-80%	Neff et al., 2004
PRECIPITATION					
Co-precipitation with CaPO₄	Retroviral vectors	30	Incubate 30 min at 37°C; Centrifuge 2,000xg for 4 min (0.3 L/bottle), GS-3.4 rotor	50-60%	Pham et al., 2001
Complexation with Poly-L-lysine	VSV-G lentivector	500	Incubate 30 min at 4°C; Centrifuge 10,000xg for 2h at 4° (0.5 L/bottle), Sorvall GS-3 rotor	~26%	Zhang et al., 2001
Complexation with polybrene & Chondroitin sulphate C	Oncoretrovectors	8	Incubate 20 min at 37°C; Centrifuge 10,000xg for 5 min at room temperature	125-250%	Le Doux et al., 2001
ULTRAFILTRATION					
Hollow fiber ultrafiltration	Oncoretrovector	13-40	Polysulfone 500 kDa MWCO, Filter 0.6-0.8 L in 3-6 h	54-86%	Paul et al., 1993
Tangential flow ultrafiltration	Oncoretrovector	16-25	Regenerated cellulose 300 kDa MWCO, Filter 8-10 L in 0.5 h	91-96%	Kotani et al., 1994
Hollow fiber ultrafiltration	HIV-1& MoMLV wild type	10-30	Regenerated cellulose (35 or 75 nm pore size), Filter 1L in 1h, EFA 300 cm²	~50%	Makino et al., 1994
Ultrafiltration in stirred cell tank	Oncoretrovector	50-75	Regenerated cellulose 100 KDa MWCO, Filter 0.1-0.15 L in 2.5 h, EFA 41.8cm²	20-55%	Miller et al., 1996
Centrifugal filtration	Lentivectors	61-69	Regenerated cellulose 100 kDa MWCO, Filter 0.06 L in 2-2.5 h, EFA 19 cm²	114-538%	Reiser 2000
Hollow fiber ultra/diafiltration	VSV-G lentivector	30-40	-	~70%	Slepushkin et al., 2003
Ultra/diafiltration in stirred cell tank	VSV-G oncoretrovector	20	Polyethersulfone 300 KDa MWCO, Filter 1L in 3 h, EFA 162 cm²	127± 10%	Segura et al., 2005

Table II Methods used for the concentration of virus stocks

(a) Concentration factors are based on volume change, (b) Recoveries are based on infectious particle quantitation

7.3 Purification

Concentrated viral stocks need to be further purified in order to obtain high quality vector preparations that meet regulatory requirements for clinical applications. The separation of retroviral particles from the remaining contaminating substances present in clarified concentrates is generally divided in two distinct operations: purification and polishing. The former is aimed at eliminating the bulk of impurities while the latter removes remaining low or trace amounts of contaminants and closely-related virus structures.

7.3.1 Density gradient ultracentrifugation

Density gradient ultracentrifugation is a powerful method for purifying retroviruses. More importantly, this is one of the few methods that offers potential to separate viral particles from closely-related species such as defective vector forms and/or cell membrane vesicles, all of which pose a serious challenge in downstream processing. Equilibrium density ultracentrifugation in sucrose gradients is the most widely used method for the preparation of highly purified retrovirus material for characterization of viral proteins and enzyme activities (Vogt, 1997). Using this technique, retrovirus particles are isolated from a band at a density of ~1.16 g/mL, corresponding to 35% (w/w) sucrose. Unfortunately, this technique often results in poor recovery of infective particles and is not reproducible. Therefore, this technique is best suited for studies whereby the preservation of viral activity is not required. Moreover, virus preparations obtained by this procedure are usually contaminated with variable amounts of cell membrane vesicles (microvesicles or exosomes) that have a density similar to that of the virus (Bess et al., 1997; Gluschankof et al., 1997). Since these vesicles show a wider range of size (50-500 nm), higher levels of purification can be achieved by rate zonal ultracentrifugation. In this case, separation of viral particles from contaminants is based on size and density, in contrast to the standard equilibrium ultracentrifugation procedure in which irrespective of the size, particles are separated according to their buoyant density alone. Rate zonal ultracentrifugation showed promising results in studies performed in our laboratory (Chapter III).

The most widely used gradient media for virus purification are sucrose and cesium chloride (CsCl). Both media are hyperosmotic at the densities used to band retrovirus particles. Sucrose solutions are very viscous and thus require longer sedimentation times for efficient separation of virus particles. Moreover, the high viscosity of sucrose has been associated with loss of surface structures and thus loss of infectivity upon purification (Moller-Larsen and Christensen, 1998). The use of iodixanol, a relatively new gradient medium, has also been described for the purification of retrovirus particles. This gradient medium can be diluted in iso-osmotic buffers to form iso-osmotic solutions that help preserve retrovirus particle integrity and functionality (Dettenhofer and Yu, 1999; Moller-Larsen and Christensen, 1998). In addition, it is less viscous than sucrose resulting in shorter processing times. Moreover, this medium, originally designed as an X-ray contrast solution, is non-toxic to cells.

Several practical disadvantages are associated with density gradient ultracentrifugation methods. The preparation of density gradients requires technical expertise, time and patience. The method is currently not being used at large scale since it would require the use of costly equipment that has not yet been tested for the purification of viruses. Additionally, separations usually require long processing times which may be detrimental to preserving retroviral infectivity. Therefore, the adoption of adsorptive chromatographic procedures has been strongly encouraged to move away from these conventional virus purification procedures (Andreadis et al., 1999; Braas et al., 1996; Lyddiatt and O'Sullivan, 1998).

7.3.2 Chromatography

Chromatography is the method of choice for selective fractionation of bioproducts in large-scale since it enables fast, efficient and reproducible separations (Lyddiatt, 2002). In chromatography, clarified and usually concentrated retroviral stocks are passed though a column containing beads coated with functional groups that capture the viral particles while the rest of the solution containing undesired impurities passes through. Captured particles are then displaced from the column using desorption agents and collected in purified fractions. This process is currently being employed for the purification of plasmid DNA

(Ferreira et al., 2000; Stadler et al., 2004) as well as viral gene therapy vectors including adenovirus and adeno-associated virus vectors (Arcand et al., 2003; Davidoff et al., 2004; Debelak et al., 2000; Smith et al., 2003; Zolotukhin et al., 2002). A number of adsorptive chromatography procedures have been described for the purification of retroviral particles (Table III). In contrast with the techniques mentioned above, that separate virus particles simply based on size and density, adsorptive chromatography can purify vectors based on chemical surface properties or the molecular composition of the viral envelope. Therefore, chromatographic purification greatly contributes to the manufacture of high-purity vector stocks for clinical applications. However, with the exemption of immunoaffinity chromatography, most chromatography methods are unlikely to remove a significant amount of closely-related species such as defective vector forms and/or cell membrane vesicles from viral preparations.

Ion-exchange chromatography

Anion exchange chromatography exploits the negatively charged surface of retroviruses for purification purposes. Retroviral particles bind strongly to anion exchangers carrying positively charged quaternary ammonium functional groups. Anion exchange chromatography, first used for the preparation of an inactivated HIV-1 vaccine (Prior et al., 1995), was more recently adapted to the purification of lentiviral vectors (Scherr et al., 2002; Yamada et al., 2003). Similarly, hydroxyapatite chromatography matrices, originally used to purify wild-type inactive MoMLV (Smith and Lee, 1978), were found to bind oncoretroviral vectors, although only moderate recoveries of infective particles (18-31%) were obtained (Kuiper et al., 2002). The mechanism of interaction of hydroxyapatite resins is not completely understood, but it appears to be a combined effect of anion exchange, cation exchange and calcium coordination (Gagnon, 1998). Ion exchange matrices show imperfect selectivity and high salt concentrations are required to elute retroviral particles. Consequently, in most cases further purification steps are required to eliminate similarly charged contaminants (i.e. DNA) and salt.

Affinity chromatography

In order to limit the number of purification steps, highly selective affinity chromatographic adsorbents would be ideal. Unfortunately, little is known about the composition of the viral membrane, which complicates the selection of suitable adsorbents. A possibility is to engineer vectors to contain affinity tags inserted on the surface of the virus to facilitate their purification. Hexahistidine affinity tags, often used for the purification of recombinant proteins by immobilized metal affinity chromatography (IMAC), have been inserted into the MoMLV ecotropic Env-protein. His_6-tagged retroviruses showed high affinity for immobilized nickel ions and were succesfully purified by one-step IMAC with good recovery of infective particles (Ye et al., 2004). Additionally, Williams and collaborators (Williams et al., 2005b) have demonstrated the feasibility of purifying MoMLV particles by exploiting the interaction between streptavidin and biotin. Chemically biotinylated oncoretroviral particles strongly bound streptavidin coated adsorbents in batch experiments. However, low recoveries of infective particles were obtained after elution with optimized concentrations of d-biotin (maximum recovery ~17%).

Engineering vectors by inserting tags or chemically modifying the envelope structure without reducing or eliminating the virus ability to transduce cells has proved to be a difficult task as demonstrated by unsuccessful efforts to alter the structure of envelope proteins for targeting purposes (Palù et al., 2000). Another possibility is to explore the natural ability of retroviruses to bind commercially available affinity ligands. Heparin affinity chromatography proved to be a powerful tool for the purification of viruses that use heparan sulfate as cell surface receptor, including herpes simplex virus, foot and mouth disease virus and adeno-associated viral vectors (Navarro del Cañizo et al., 1996; O'Keeffe et al., 1999; Zolotukhin et al., 1999). More recently, heparin affinity chromatography was found to be useful for the purification of oncoretroviral vectors giving excellent results in terms of recovery of active particles (61%), reproducibility and selectivity (Segura et al., 2005). Although the method was described for the purification of VSV-G pseudotyped particles, we have recently found that other vector pseudotypes also show affinity for heparin ligands (Chapter IV).

The most selective affinity chromatography technique is immunoaffinity chromatography which relies on the specific interaction between immobilized antibodies and surface viral antigens. This technique could separate retroviral particles from cell membrane vesicles that frequently contaminate retrovirus preparations provided that a surface protein is found to be exclusively incorporated into either the virions or the vesicles. For instance, taking advantage of the differential incorporation of CD45 into HIV-1 and cell membrane vesicles derived from lymphoid cells (Esser et al., 2001), Trubey et al. developed an immunoaffinity approach to selectively deplete these vesicles from density-purified retrovirus preparations (Trubey et al., 2003). However, non-hematopoietic cells (i.e. HEK 293) are not expected to express CD45, limiting the usefulness of this technique. Moreover, the high costs associated with antibody purification and immobilization and the low stability of these ligands towards sanitizing agents do not favour the use of this method for large-scale (Andreadis et al., 1999).

Size-exclusion chromatography

Size-exclusion chromatography (SEC) was successfully used for the purification of wild-type retroviruses and retroviral vectors (McGrath et al., 1978; Slepushkin et al., 2003; Transfiguracion et al., 2004). Using this chromatographic method, retroviruses are excluded from the internal pores of the gel due to their large size and elute in the void volume of the column while low molecular weight contaminants are retarded by the column. Unfortunately, SEC is a non-adsorptive method likely to be useful only as a final polishing step due to its limited resolution and inherent low capacity (<10% bed volume for best peak resolution). Moreover, SEC using conventional matrices tends to operate at low linear flow rates (~15 cm/h) and typically results in product dilution of 2 to 4 folds.

Most currently available chromatographic matrices were designed to maximize the adsorption of protein macromolecules (diameter <5nm) rather than viruses. Consideration of the pore dimensions of most commercially available chromatography adsorbents (typically 30-80 nm) suggests that adsorption of retroviruses will be restricted to the bead surface area while most contaminating proteins have access to the area inside the pores as

well (Lyddiatt and O'Sullivan, 1998). Therefore, using conventional matrices, both available binding capacity and purification efficiency are predicted to be poor.

In order to circumvent problems found with conventional chromatography supports, several new chromatography technologies have been proposed and tested. Tentacle supports offer the possibility of increased virus binding capacities. The advantage of using these supports is that they have sterically accessible ligands available for virus capture. The ligands are attached to an inert and flexible spacer arm that separates them from the bead. Therefore, tentacle ligands can access otherwise sterically hindered binding sites and compensate in part for the loss of surface area inside the pores. In addition, since they are no longer exclusively on the surface of the chromatographic bead, larger amounts of ligands are available for binding (Kaufmann, 1997). Tentacle matrices have been employed for the purification of inactivated HIV-1 for viral vaccines and oncoretroviral particles (Prior et al., 1995; Segura et al., 2005; Williams et al., 2005b). The superiority of these supports compared to non-tentacle ones was demonstrated in these reports. Membrane chromatography is another interesting alternative to traditional column chromatography since it combines the advantages of membrane technology (high flow rates) and liquid chromatography (high selectivity). Anion exchange membrane adsorbers have been tested for the purification of lentiviral vectors showing excellent results (Slepushkin et al., 2003). Another way to improve virus purification using chromatography is to use monolithic adsorbents instead of the traditional biopolymers (or so–called soft supports). The application of compact, macroporous monoliths as supports for the purification of retroviral vectors has been described (Williams et al., 2005a). Biotinylated oncoretroviral particles were recovered by elution from streptavidin coated monoliths with 8% recovery of infective particles.

Another key-point to consider for the successful recovery of active viral particles is to select adsorbents that do not require harsh conditions for virus adsorption and/or elution. Most methods developed so far maintain physiological pH throughout the whole purification process (Table III). Elution of retrovirus particles from heparin affinity columns is achieved under mild conditions (neutral pH and 0.35 M NaCl). In contrast,

elution of strongly bound viral particles from anion exchange chromatography adsorbents requires the addition of high salt concentrations (~1 M NaCl) which may compromise the activity of retroviral particles. The concentration of imidazole and d-biotin for the elution of retroviral particles from Ni-NTA and streptavidin affinity matrices respectively was adjusted to minimize the detrimental effect of these desorption agents on virus activity (Williams et al., 2005b; Ye et al., 2004). Immunoaffinity chromatography usually requires stringent elution conditions to break antibody-antigen interactions including low pH, high salt or the use of denaturing agents. Therefore, using this method for virus capture, low recoveries of active particles are predictable upon elution.

Table III Chromatography methods used for the purification of retroviral particles

CHROMATOGRAPHY	Retrovirus	Matrix	Desorption reagents	Buffer	pH	Recovery[a]	Reference
ION-EXCHANGE CHROMATOGRAPHY							
Anion-exchange chromatography	HIV-1 wild type (inactivated)	Fractogel® TMAE	1 M NaCl	Phosphate	6.5	N/A	Prior et al., 1995
	HIV-1 wild type (inactivated)	Q-Sepharose FF	1M NaCl	Phosphate	6.5	N/A	Prior et al., 1995
	VSV-G lentivector	HiTrap® Q HP	0.5 M and 1M NaCl	PBS	7.4	33% and 17% respectively[b]	Yamada et al., 2003
	VSV-G lentivector	Fractogel® TMAE	2M NaCl	PBS	7.4	45% ± 15% (PCR-based)	Scherr et al., 2002
Hydroxyapatite chromatography	MoMLV wild type	Hydroxyapatite	0.4 M sodium phosphate	Phosphate	7.2	1-5%	Smith et al., 1978
	Oncoretrovector	Ceramic Hydroxyapatite	0-0.5 M sodium phosphate	Phosphate	6.8	18-20%[c]	Kuiper et al., 2002
AFFINITY CHROMATOGRAPHY							
Heparin affinity chromatography	VSV-G oncoretrovector	Fractogel® Heparin	0.35 M NaCl	20 mM Tris/HCl	7.5	61 ± 2%	Segura et al., 2005
Immobilized metal affinity chromatography	His6-tagged oncoretrovector	Ni-NTA agarose	75 mM imidazole	PBS	7.4	56%	Ye et al., 2004
Biotin-streptavidin affinity chromatography	Biotinylated oncoretrovector	Fractogel® Streptavidin	0.6 mM d-biotin	PBS	7.4	16.7%	Williams et al., 2005a
SIZE-EXCLUSION CHROMATOGRAPHY							
	MoMLV wild type	Sepharose CL-4B	N/A	TEN	7.4	85 ± 15%	McGrath et al., 1978
	VSV-G oncoretrovector	Sepharose CL-4B	N/A	TEN	7.4	70 ± 1%	Transfiguration et al., 2003
	VSV-G lentivector	Sephacryl® S-500	N/A	-	-	~80 %	Slepushkin et al., 2003

(a) Recoveries are titer-based unless otherwise indicated, (b) The authors found two peaks of activity using a linear NaCl gradient, (c) Pool of fractions obtained throughout a gradient (0-0.5 M NaCl)

Abbreviation: N/A not applicable

7.4 Formulation and storage

Retrovirus supernatants are commonly aliquoted and frozen at −80°C to protect the virus from thermal inactivation. These stocks were shown to maintain their potency over months (McTaggart and Al-Rubeai, 2000; Wikstrom et al., 2004). However, there is a high cost and impracticality associated with long-term cryostorage of large volumes of supernatant which encourages the development of alternative storage strategies (Andreadis et al., 1999; McTaggart and Al-Rubeai, 2002). Retroviral particles can be preserved in a lyophilized form (Levy and Fieldsteel, 1982). The lyophilization of retroviral vector supernatants with and without additives was investigated by Kotani and collaborators (Kotani et al., 1994). The authors found that the recovery of active retroviral particles after lyophilization is more efficient in the presence of glucose or sorbitol with gelatin (64-83 %) than without these additives (21%). Conversely, Lee and collaborators observed a severe loss of virus activity during lyophilization (>60%) that could not be circumvented by addition of additives (Lee et al., 1996). The authors also reported that commonly used cryoprotectants, DMSO and glycerol, did not play an important role in preserving retroviral activity during long-term cryostorage. Little is known about the stability of purified vector stocks during storage. More research in this area will be required.

8 Conclusion

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. Significant advances in the downstream processing of retroviral vectors have been made in the past several years. Studies have shown that the potency of retroviral preparations can be significantly improved by concentration and removal of inhibitors of cell transduction. Various selective chromatography matrices have been identified and new chromatography technologies, better suited for virus purification purposes, are being tested with very promising results. However, most of these techniques have only been tested at laboratory scale. Very few reports show a complete purification scheme, from retrovirus supernatant to clinical grade virus, in which final overall recoveries are presented (Kuiper et al., 2002; Segura et al., 2005; Slepushkin et al., 2003). Yet, they indicate that overall recoveries of active retrovirus particles in the range of 30% should be considered satisfactory. Considering the instability of retroviral vectors and its susceptibility to several factors as discussed in this review, we would like to emphasize the importance of selecting gentle purification procedures. The yield in each step is critical in view of the fact that even small losses in each purification step will have a strong impact in the final overall recovery when several purification steps are required to reach the desired level of purity. On the other hand, in most reported cases the final purities achieved are poorly documented and the ability (or inability) of the different methods to remove closely related structures such as cell membrane vesicles or defective virus particles, or the possible implications of having these contaminants in clinical grade preparations, are rarely discussed. Moreover, studies concerning the proper conditions and procedures for formulation and long-term storage of purified material are often incomplete, or non-existent. Undoubtedly, downstream processing of retroviral vectors will continue to be an area of intensive research in the coming years.

9 Acknowledgements

10 References

Aboud M, Wolfson M, Hassan Y, Huleihel M. 1982. Rapid purification of extracellular and intracellular Moloney murine leukemia virus. Arch Virol 71(3):185-95.

Alain R. 1997. Quantitation of virus particles by negative stain electron microscopy. Microscopy Today 97(5):20.

Andreadis S, Lavery T, Davis HE, Le Doux JM, Yarmush ML, Morgan JR. 2000. Toward a more accurate quantitation of the activity of recombinant retroviruses: alternatives to titer and multiplicity of infection. J Virol 74(7):3431-9.

Andreadis ST, Brott D, Fuller AO, Palsson BO. 1997. Moloney murine leukemia virus-derived retroviral vectors decay intracellularly with a half-life in the range of 5.5 to 7.5 hours. J Virol 71(10):7541-8.

Andreadis ST, Roth CM, Le Doux JM, Morgan JR, Yarmush ML. 1999. Large-scale processing of recombinant retroviruses for gene therapy. Biotechnol Prog 15(1):1-11.

Arcand N, Bernier A, Transfiguracion J, Jacob D, Coelho H, Kamen A. 2003. Adenovirus type 5 (Ad5) chromatographic purification process at the 20L scale. Bioprocessing J 2:72-75.

Baekelandt V, Eggermont K, Michiels M, Nuttin B, Debyser Z. 2003. Optimized lentiviral vector production and purification procedure prevents immune response after transduction of mouse brain. Gene Ther 10(23):1933-40.

Bajaj B, Lei P, Andreadis ST. 2001. High efficiencies of gene transfer with immobilized recombinant retrovirus: kinetics and optimization. Biotechnol Prog 17(4):587-96.

Beer C, Meyer A, Muller K, Wirth M. 2003. The temperature stability of mouse retroviruses depends on the cholesterol levels of viral lipid shell and cellular plasma membrane. Virology 308(1):137-46.

Bess JW, Jr., Gorelick RJ, Bosche WJ, Henderson LE, Arthur LO. 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology 230(1):134-44.

Beyer WR, Westphal M, Ostertag W, von Laer D. 2002. Oncoretrovirus and lentivirus vectors pseudotyped with lymphocytic choriomeningitis virus glycoprotein: generation, concentration, and broad host range. J Virol 76(3):1488-95.

Bowles NE, Eisensmith RC, Mohuiddin R, Pyron M, Woo SL. 1996. A simple and efficient method for the concentration and purification of recombinant retrovirus for increased hepatocyte transduction in vivo. Hum Gene Ther 7(14):1735-42.

Braas G, Searle PF, Slater NK, Lyddiatt A. 1996. Strategies for the isolation and purification of retroviral vectors for gene therapy. Bioseparation 6(4):211-28.

Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci U S A 90(17):8033-7.

Carmo M, Peixoto C, Coroadinha AS, Alves PM, Cruz PE, Carrondo MJ. 2004. Quantitation of MLV-based retroviral vectors using real-time RT-PCR. J Virol Methods 119(2):115-9.

CBER. 2001. Supplemental guidance on testing for replication-competent retrovirus in retroviral vector-based gene therapy products and during follow-up of patients in clinical trials using retroviral vectors. Hum Gene Ther 12(3):315-20.

Chang LJ, Zaiss AK. 2002. Lentiviral vectors. Preparation and use. Methods Mol Med 69:303-18.

Chen J, Reeves L, Sanburn N, Croop J, Williams DA, Cornetta K. 2001. Packaging cell line DNA contamination of vector supernatants: implication for laboratory and clinical research. Virology 282(1):186-97.

Cruz PE, Goncalves D, Almeida J, Moreira JL, Carrondo MJ. 2000. Modeling retrovirus production for gene therapy. 2. Integrated optimization of bioreaction and downstream processing. Biotechnol Prog 16(3):350-7.

Davidoff AM, Ng CY, Sleep S, Gray J, Azam S, Zhao Y, McIntosh JH, Karimipoor M, Nathwani AC. 2004. Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock. J Virol Methods 121(2):209-15.

Debelak D, Fisher J, Iuliano S, Sesholtz D, Sloane DL, Atkinson EM. 2000. Cation-exchange high-performance liquid chromatography of recombinant adeno-associated virus type 2. J Chromatogr B Biomed Sci Appl 740(2):195-202.

Dettenhofer M, Yu XF. 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. J Virol 73(2):1460-7.

Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol 72(11):8463-71.

Esser MT, Graham DR, Coren LV, Trubey CM, Bess JW, Jr., Arthur LO, Ott DE, Lifson JD. 2001. Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation. J Virol 75(13):6173-82.

Ferreira GN, Monteiro GA, Prazeres DM, Cabral JM. 2000. Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications. Trends Biotechnol 18(9):380-8.

Flotte T, Burd P, Snyder R. 2002. Utility of a recombinant adeno-associated viral vector reference standard. Bioprocessing J:75-77.

Gagnon P. 1998. An Enigma Unmasked: How Hydroxyapatite Works and How to Make It Work for You Validated Biosystems Quarterly Web Newsletter.

Gatlin J, Melkus MW, Padgett A, Kelly PF, Garcia JV. 2001. Engraftment of NOD/SCID mice with human CD34(+) cells transduced by concentrated oncoretroviral vector particles pseudotyped with the feline endogenous retrovirus (RD114) envelope protein. J Virol 75(20):9995-9.

Gluschankof P, Mondor I, Gelderblom HR, Sattentau QJ. 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology 230(1):125-33.

Higashikawa F, Chang L. 2001. Kinetic analyses of stability of simple and complex retroviral vectors. Virology 280(1):124-31.

Kaufmann M. 1997. Unstable proteins: how to subject them to chromatographic separations for purification procedures. J Chromatogr B Biomed Sci Appl 699(1-2):347-69.

Kotani H, Newton PB, 3rd, Zhang S, Chiang YL, Otto E, Weaver L, Blaese RM, Anderson WF, McGarrity GJ. 1994. Improved methods of retroviral vector transduction and production for gene therapy. Hum Gene Ther 5(1):19-28.

Kuiper M, Sanches RM, Walford JA, Slater NK. 2002. Purification of a functional gene therapy vector derived from Moloney murine leukaemia virus using membrane filtration and ceramic hydroxyapatite chromatography. Biotechnol Bioeng 80(4):445-53.

Kwon YJ, Hung G, Anderson WF, Peng CA, Yu H. 2003. Determination of infectious retrovirus concentration from colony-forming assay with quantitative analysis. J Virol 77(10):5712-20.

Kwon YJ, Peng CA. 2002. Transduction rate constant as more reliable index quantifying efficiency of retroviral gene delivery. Biotechnol Bioeng 77(6):668-77.

Le Doux JM, Davis HE, Morgan JR, Yarmush ML. 1999. Kinetics of retrovirus production and decay. Biotechnol Bioeng 63(6):654-62.

Le Doux JM, Landazuri N, Yarmush ML, Morgan JR. 2001. Complexation of retrovirus with cationic and anionic polymers increases the efficiency of gene transfer. Hum Gene Ther 12(13):1611-21.

Le Doux JM, Morgan JR, Snow RG, Yarmush ML. 1996. Proteoglycans secreted by packaging cell lines inhibit retrovirus infection. J Virol 70(9):6468-73.

Le Doux JM, Morgan JR, Yarmush ML. 1998. Removal of proteoglycans increases efficiency of retroviral gene transfer. Biotechnol Bioeng 58(1):23-34.

Lee SG, Kim S, Robbins PD, Kim BG. 1996. Optimization of environmental factors for the production and handling of recombinant retrovirus. Appl Microbiol Biotechnol 45(4):477-83.

Levy JA, Fieldsteel AH. 1982. Freeze-drying is an effective method for preserving infectious type C retroviruses. J Virol Methods 5(3-4):165-71.

Lizee G, Aerts JL, Gonzales MI, Chinnasamy N, Morgan RA, Topalian SL. 2003. Real-time quantitative reverse transcriptase-polymerase chain reaction as a method for determining lentiviral vector titers and measuring transgene expression. Hum Gene Ther 14(6):497-507.

Logan AC, Nightingale SJ, Haas DL, Cho GJ, Pepper KA, Kohn DB. 2004. Factors influencing the titer and infectivity of lentiviral vectors. Hum Gene Ther 15(10):976-88.

Lyddiatt A. 2002. Process chromatography: current constraints and future options for the adsorptive recovery of bioproducts. Curr Opin Biotechnol 13(2):95-103.

Lyddiatt A, O'Sullivan DA. 1998. Biochemical recovery and purification of gene therapy vectors. Curr Opin Biotechnol 9(2):177-85.

Makino M, Ishikawa G, Yamaguchi K, Okada Y, Watanabe K, Sasaki-Iwaki Y, Manabe S, Honda M, Komuro K. 1994. Concentration of live retrovirus with a regenerated cellulose hollow fiber, BMM. Arch Virol 139(1-2):87-96.

McGrath M, Witte O, Pincus T, Weissman IL. 1978. Retrovirus purification: method that conserves envelope glycoprotein and maximizes infectivity. J Virol 25(3):923-7.

McTaggart S, Al-Rubeai M. 2000. Effects of culture parameters on the production of retroviral vectors by a human packaging cell line. Biotechnol Prog 16(5):859-65.

McTaggart S, Al-Rubeai M. 2002. Retroviral vectors for human gene delivery. Biotechnol Adv 20(1):1-31.

Merten OW. 2004. State-of-the-art of the production of retroviral vectors. J Gene Med 6 Suppl 1:S105-24.

Miller DL, Meikle PJ, Anson DS. 1996. A rapid and efficient method for concentration of small volumes of retroviral supernatant. Nucleic Acids Res 24(8):1576-7.

Moller-Larsen A, Christensen T. 1998. Isolation of a retrovirus from multiple sclerosis patients in self-generated Iodixanol gradients. J Virol Methods 73(2):151-61.

Morling FJ, Russell SJ. 1995. Enhanced transduction efficiency of retroviral vectors coprecipitated with calcium phosphate. Gene Ther 2(7):504-8.

Moy N, Dillingham M, McGowan S, Rotherham M, Clayton T. 2000. Large scale GMP production of retroviral gene therapy vector in very low serum medium. Bernard A, Griffiths B, Noé W, Wurm F, editors: Kluwer Academic publishers. 441-443 p.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272(5259):263-7.

Navarro del Cañizo AA, Mazza M, Bellinzoni R, Cascone O. 1996. Foot and mouth disease virus concentration and purification by affinity chromatography. Appl Biochem Biotechnol 61(3):399-409.

Neff T, Peterson LJ, Morris JC, Thompson J, Zhang X, Horn PA, Thomasson BM, Kiem HP. 2004. Efficient gene transfer to hematopoietic repopulating cells using concentrated RD114-pseudotype vectors produced by human packaging cells. Mol Ther 9(2):157-9.

O'Keeffe RS, Johnston MD, Slater NK. 1999. The affinity adsorptive recovery of an infectious herpes simplex virus vaccine. Biotechnol Bioeng 62(5):537-45.

Ott DE. 2002. Potential roles of cellular proteins in HIV-1. Rev Med Virol 12(6):359-74.

Palù G, Parolin C, Takeuchi Y, Pizzato M. 2000. Progress with retroviral gene vectors. Rev Med Virol 10:185-202.

Pan D, Gunther R, Duan W, Wendell S, Kaemmerer W, Kafri T, Verma IM, Whitley CB. 2002. Biodistribution and toxicity studies of VSVG-pseudotyped lentiviral vector after intravenous administration in mice with the observation of in vivo transduction of bone marrow. Mol Ther 6(1):19-29.

Paul RW, Morris D, Hess BW, Dunn J, Overell RW. 1993. Increased viral titer through concentration of viral harvests from retroviral packaging lines. Hum Gene Ther 4(5):609-15.

Pham L, Ye H, Cosset FL, Russell SJ, Peng KW. 2001. Concentration of viral vectors by co-precipitation with calcium phosphate. J Gene Med 3(2):188-94.

Pizzato M, Marlow SA, Blair ED, Takeuchi Y. 1999. Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction. J Virol 73(10):8599-611.

Prior C, Bay P, Ebert B, Gore R, Holt J, Irish T, Jensen F, Leone C, Mitschelen J, Stiglitz M and others. 1995. Process development for the manufacture of inactivated HIV-1. BioPharmacology 8:25-35.

Rainov NG, Ren H. 2003. Clinical trials with retrovirus mediated gene therapy--what have we learned? J Neurooncol 65(3):227-36.

Reeves L, Cornetta K. 2000. Clinical retroviral vector production: step filtration using clinically approved filters improves titers. Gene Ther 7(23):1993-8.

Reiser J. 2000. Production and concentration of pseudotyped HIV-1-based gene transfer vectors. Gene Ther 7(11):910-3.

Rigg RJ, Chen J, Dando JS, Forestell SP, Plavec I, Bohnlein E. 1996. A novel human amphotropic packaging cell line: high titer, complement resistance, and improved safety. Virology 218(1):290-5.

Sastry L, Johnson T, Hobson MJ, Smucker B, Cornetta K. 2002. Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods. Gene Ther 9(17):1155-62.

Sastry L, Xu Y, Cooper R, Pollok K, Cornetta K. 2004. Evaluation of plasmid DNA removal from lentiviral vectors by benzonase treatment. Hum Gene Ther 15(2):221-6.

Scherr M, Battmer K, Eder M, Schule S, Hohenberg H, Ganser A, Grez M, Blomer U. 2002. Efficient gene transfer into the CNS by lentiviral vectors purified by anion exchange chromatography. Gene Ther 9(24):1708-14.

Segura M, Kamen A, Trudel P, Garnier A. 2005. A novel purification strategy for retrovirus gene therapy vectors using heparin affinity chromatography. Biotechnol Bioeng 90(4):391-404.

Slepushkin V, Chang N, Cohen R, Gan Y, Jiang B, Deausen E, Berlinger D, Binder G, Andre K, Humeau L and others. 2003. Large-scale purification of a lentiviral vector by size exclusion chromatography or mustang Q ion exchange capsule. Bioprocessing J 2:89-95.

Slingsby JH, Baban D, Sutton J, Esapa M, Price T, Kingsman SM, Kingsman AJ, Slade A. 2000. Analysis of 4070A envelope levels in retroviral preparations and effect on target cell transduction efficiency. Hum Gene Ther 11(10):1439-51.

Smith KT, Shepherd AJ, Boyd JE, Lees GM. 1996. Gene delivery systems for use in gene therapy: an overview of quality assurance and safety issues. Gene Ther 3(3):190-200.

Smith RG, Lee SA. 1978. Large-scale isolation and partial purification of type C RNA viruses on hydroxyapatite. 1. Biochemical characterization. Anal Biochem 86(1):252-63.

Smith RH, Ding C, Kotin RM. 2003. Serum-free production and column purification of adeno-associated virus type 5. J Virol Methods 114(2):115-24.

Srinivasakumar N. 2002. Packaging cell system for lentivirus vectors. Preparation and use. Methods Mol Med 69:275-302.

Stadler J, Lemmens R, Nyhammar T. 2004. Plasmid DNA purification. J Gene Med 6 Suppl 1:S54-66.

Transfiguracion J, Coelho H, Kamen A. 2004. High-performance liquid chromatographic total particles quantification of retroviral vectors pseudotyped with vesicular stomatitis virus-G glycoprotein. J Chromatogr B Analyt Technol Biomed Life Sci 813(1-2):167-73.

Transfiguracion J, Jaalouk DE, Ghani K, Galipeau J, Kamen A. 2003. Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications. Hum Gene Ther 14(12):1139-53.

Trubey CM, Chertova E, Coren LV, Hilburn JM, Hixson CV, Nagashima K, Lifson JD, Ott DE. 2003. Quantitation of HLA class II protein incorporated into human immunodeficiency type 1 virions purified by anti-CD45 immunoaffinity depletion of microvesicles. J Virol 77(23):12699-709.

Vogt VM. 1997. Retroviral virions and genomes. In: Coffin JM HS, Varmus HE., editor. Retroviruses. NY: Cold Spring Harbor Laboratory Press. p 27-69.

Vogt VM, Simon MN. 1999. Mass determination of rous sarcoma virus virions by scanning transmission electron microscopy. J Virol 73(8):7050-5.

White SM, Renda M, Nam NY, Klimatcheva E, Zhu Y, Fisk J, Halterman M, Rimel BJ, Federoff H, Pandya S and others. 1999. Lentivirus vectors using human and simian immunodeficiency virus elements. J Virol 73(4):2832-40.

Wikstrom K, Blomberg P, Islam KB. 2004. Clinical grade vector production: analysis of yield, stability, and storage of gmp-produced retroviral vectors for gene therapy. Biotechnol Prog 20(4):1198-203.

Williams SL, Eccleston ME, Slater NK. 2005a. Affinity capture of a biotinylated retrovirus on macroporous monolithic adsorbents: towards a rapid single-step purification process. Biotechnol Bioeng 89(7):783-7.

Williams SL, Nesbeth D, Darling DC, Farzaneh F, Slater NK. 2005b. Affinity recovery of Moloney Murine Leukaemia Virus. J Chromatogr B Analyt Technol Biomed Life Sci 820(1):111-9.

Yamada K, McCarty DM, Madden VJ, Walsh CE. 2003. Lentivirus vector purification using anion exchange HPLC leads to improved gene transfer. Biotechniques 34(5):1074-8, 1080.

Ye K, Dhiman HK, Suhan J, Schultz JS. 2003. Effect of pH on infectivity and morphology of ecotropic moloney murine leukemia virus. Biotechnol Prog 19(2):538-43.

Ye K, Jin S, Ataai MM, Schultz JS, Ibeh J. 2004. Tagging retrovirus vectors with a metal binding peptide and one-step purification by immobilized metal affinity chromatography. J Virol 78(18):9820-7.

Zhang B, Xia HQ, Cleghorn G, Gobe G, West M, Wei MQ. 2001. A highly efficient and consistent method for harvesting large volumes of high-titre lentiviral vectors. Gene Ther 8(22):1745-51.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6(6):973-85.

Zolotukhin S, Potter M, Zolotukhin I, Sakai Y, Loiler S, Fraites TJ, Jr., Chiodo VA, Phillipsberg T, Muzyczka N, Hauswirth WW and others. 2002. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28(2):158-67.

Zufferey R. 2002. Production of lentiviral vectors. Curr Top Microbiol Immunol 261:107-21.

Chapter II

A novel purification strategy for retrovirus gene therapy vectors using heparin affinity chromatography

María de las Mercedes Segura [1,2], Amine Kamen [2], Pierre Trudel [1,2] and Alain Garnier [1*]

1- Department of Chemical Engineering, Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines, Université Laval, Québec, Canada, G1K 7P4; phone : 418-656-3106; fax: 418-656-5993; e-mail: alain.garnier@gch.ulaval.ca

2- Biotechnology Research Institute, NRC, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

1 Résumé

Les technologies chromatographiques et la séparation par membranes sont vues comme une alternative attrayante aux procédés conventionnels d'ultracentrifugation à petite échelle utilisées pour la purification des rétrovirus. Cependant, en dépit des demandes de plus en plus élevées pour des préparations de vecteurs rétroviraux purifiés, de nouvelles résines chromatographiques, ayant une spécificité particulière pour les virus, n'ont pas été rapportées. La chromatographie d'affinité sur colonne d'héparine est présentée ici comme un nouvel outil pratique pour la purification des rétrovirus. La capacité des particules rétrovirales bioactives de se lier spécifiquement au ligand héparine immobilisé sur la matrice chromatographique est démontrée. Un facteur de purification de 63 avec une récupération de 61% des rétroparticules fonctionnelles a été obtenu en une seule étape. Le support tentaculaire testé a permis la capture des particules rétrovirales plus efficacement que le support chromatographique héparine conventionnel (sans tentacules) qui donnait une récupération plus faible (18%). Les particules rétrovirales intactes et infectieuses ont été obtenues par une élution à faible concentration de NaCl (350 mM). Ces conditions d'élution douces sont requises pour préserver l'infectivité virale. Les rétrovirus pseudo-typés VSV-G, ont démontrés une grande fragilité aux forces ioniques élevées, perdant 50% de leur activité et montrant un dommage membranaire après une exposition de 1 hr à 1 M NaCl. Nous rapportons également un schéma complet de procédé de purification (pouvant être mis à l'échelle) pour les vecteurs dérivés de MoMLV qui comprend des étapes séquentielles de microfiltration et d'ultra/diafiltration pour la clarification et la concentration respectives des virus, suivi d'une purification par chromatographie d'affinité sur colonne d'héparine et d'une étape finale de purification sur tamis moléculaire. Grâce à cette stratégie, une récupération finale de 38% de particules infectieuses a été obtenue avec un facteur de purification de 2000.

2 Abstract

Membrane separation and chromatographic technologies are regarded as an attractive alternative to conventional academic small-scale ultracentrifugation procedures used for retrovirus purification. However, in spite of the increasing demands for purified retroviral vector preparations, new chromatography adsorbents with high specificity for the virus have not been reported. Heparin affinity chromatography is presented here as a novel convenient tool for retrovirus purification. The ability of bioactive retroviral particles to specifically bind to heparin ligands immobilized on a chromatographic gel is shown. A purification factor of 63 with a recovery of 61% of functional retroparticles was achieved using this single step. Tentacle heparin affinity supports captured retroviral particles more efficiently than conventional heparin affinity chromatography supports with which a lower recovery was obtained (18%). Intact, infective retroviral particles were recovered by elution with low salt concentrations (350 mM NaCl). Mild conditions for retrovirus elution from chromatographic columns are required to preserve virus infectivity. VSV-G pseudotyped retroviruses have shown to be very sensitive to high ionic strength, losing 50% of their activity and showing membrane damage after a 1 h exposure to 1 M NaCl. We also report a complete scaleable downstream processing scheme for the purification of MoMLV-derived vectors that involves sequential microfiltration and ultra/diafiltration steps for virus clarification and concentration respectively, followed by fractionation by heparin affinity chromatography and final polishing by size-exclusion chromatography. Overall, by using this strategy, a 38% yield of infective particles can be achieved with a final purification factor of 2,000.

3 Introduction

Being the most popular viral vectors used in both clinical trials and research studies, retrovectors derived from the oncovirus Moloney murine leukemia virus (MoMLV) have demonstrated a great potential as gene delivery vehicles. Although originally envisioned as a tool for the treatment of genetic disorders *ex vivo* (Grossman et al., 1994; Hacein-Bey-Abina et al., 2002; Parkman et al., 2000), retroviral vectors were found to be well suited for the treatment of solid malignant tumours *in vivo* (Gomez-Navarro et al., 1999). Retrovirus vectors constitute an efficient means to transfer genes to dividing cells allowing permanent integration of the transgene into the host cell genome and therefore long-term gene expression. Additionally, retroviral vectors are relatively non-immunogenic and non-toxic. Despite the fact that retrovirus-mediated gene transfer presents a potential risk of insertional mutagenesis, ways to prevent this possibility have been proposed and are under investigation (Williams and Baum, 2003). Major obstacles associated with retrovirus vectors include the low-titer viral stocks generated by currently available packaging systems and the instability of the viral particles, which taken together reduce their potential for efficient gene delivery. Between 10^6 to 10^7 infective viral particles per mL of cell culture supernatant are produced by commonly used retrovirus packaging cell lines (Andreadis et al., 1999). These concentrations may be high enough for *in vitro* but not for *in vivo* applications and viral stocks still contain serum supplement and cell secreted contaminants that need to be inactivated or removed in order to increase the potency and safety of the final product (Andreadis et al., 1999). In this sense, the development of mild downstream processing strategies that render high-titer clinical-grade retrovirus preparations is critical to advances in gene therapy applications.

The separation of unstable proteins is a challenge to the scientist working in the field of downstream processing. The challenge is even greater when considering that the unstable product to be separated is not a protein, for which most commercially available purification supports were originally designed, but a large ill-defined virus particle. Ultracentrifugation has traditionally been used as a method of choice for isolating retroviruses in small quantities. However, density gradient ultracentrifugation is difficult to

scale-up, is labour-intensive and requires long processing times which may be detrimental to preserving retroviral infectivity (Andreadis et al., 1999; Brass et al., 1996). Moreover, recovery of infective particles depends on the susceptibility of the particular pseudotyped retroviral vectors to hydrodynamic shear (Burns et al., 1993) and cellular debris, host nucleic acid and serum proteins that co-purify with viral particles still need to be removed in subsequent purification steps (Transfiguracion et al., 2003). The adoption of selective chromatographic procedures was strongly encouraged to move away from conventional virus purification techniques (Andreadis et al., 1999; Braas et al., 1996; Lyddiatt and O'Sullivan, 1998). Hydroxyapatite adsorptive chromatography was found to purify wild type inactive MoMLV (Smith and Lee, 1978) as well as recombinant retroviral vectors (Kuiper et al., 2002), with moderate recoveries of infective particles (18-31%). Size-exclusion chromatography employing cross-linked agarose media (Sepharose CL-4B) was successfully used in combination with concentration and partial purification procedures, such as ultracentrifugation, ultrafiltration and polyethylene glycol-6000 precipitation for the purification of infectious MoMLV and derived vectors (Aboud et al., 1982; McGrath et al., 1978; Transfiguracion et al., 2003). However, this method is not adsorptive and likely to be useful only as a final polishing step due to its limited resolution and inherent low capacity. Unfortunately, little is known about the physicochemical characteristics of retroviral particles and molecular structure of the viral membrane, which complicates the selection of a suitable selective chromatographic adsorbent (Braas et al., 1996). To the best of our knowledge, no affinity chromatography procedure for the purification of retroviral vectors was reported to date.

Heparin and the structurally related heparan sulfate belong to the family of glycosaminoglycans (GAGs) and are linear anionic polymers made up of repeating disaccharides units of glucosamine and uronic acid. The ubiquitously distributed cell surface heparan sulfate serves as receptor for several viruses to bind to and gain access to susceptible cells (Flint et al., 2000). Recently, a number of research groups found that soluble heparin and other GAGs inhibit retrovirus vector transduction, indicating a possible interaction between retroviruses with the heparin molecule (Arai et al., 1998; Guibinga et al., 2002; Jinno-Que et al., 2001; Le Doux et al., 1999; Walker et al., 2002). This

observation strongly motivated the selection of heparin ligand to attempt the capture of retroviral particles by heparin affinity media for purification purposes. Although heparin affinity chromatography proved to be a powerful tool for the purification of viruses that use heparan sulfate as cell surface receptor, including herpes simplex virus, foot and mouth disease virus and adeno-associated viral vectors (Navarro del Cañizo et al., 1996; O'Keeffe et al., 1999; Zolotukhin et al., 1999), the purification of retroviruses by this strategy was not reported to date. It is interesting to note that no new chromatographic methods to purify MoMLV-derived vectors have been reported in the literature since the development of characterization protocols for wild-type MoMLV particles more than twenty years ago (Aboud et al., 1982; McGrath et al., 1978; Smith and Lee, 1978), despite the increasing demand for large quantities of active viral vectors. Thus, the development of high recovery new selective chromatography procedures that provide highly purified active vector preparations is needed to overcome the limitations found with the existing chromatography techniques.

We developed a novel, scaleable method for the purification of bioactive VSV-G pseudotyped retrovirus particles that employs membrane filtration and chromatography. First, primary recovery of infective retroviral particles from crude supernatants was achieved by the use of membrane technologies. Concentrated viral particles suspended in the appropriate binding buffer were purified by heparin affinity chromatography. Finally, a highly purified vector preparation was obtained by loading this semi-purified concentrated material onto a size-exclusion chromatography column.

4 Materials and methods

Retrovirus vector, cell lines and virus stock preparation

The retrovirus model used in this study is a MoMLV-derived VSV-G pseudotyped retrovector produced by 293-GPG packaging cells (Ory et al., 1996) which were 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) (Paquin et al., 2001). This cell line, a generous gift from Dr. J. Galipeau (Lady Davis Institute for Medical Research, Montreal, QC, Canada), was adapted to suspension culture (Ghani et al., manuscript in preparation). Tissue culture flasks were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS; HyClone, Logan, UT) and tetracycline (1 µg/mL; Fisher Scientific, Nepean, ON, Canada) at 37°C, 100% humidity and a 5% CO_2 atmosphere. Vector production was carried out by 293-GPG suspension-adapted cultures in a 1-L shake flask (200 mL working volume) inoculated at 1×10^5 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 virus production phase was initiated by entirely removing the tetracycline-containing medium by centrifugation of the culture (420×g, 10 min) and washing the cells with phosphate-buffered saline pH 7.4 (PBS). The cell pellet was resuspended in tetracycline-free fresh medium, re-introduced into the shake flask and incubated at 37°C. The culture supernatant was harvested every 24 h, centrifuged at 420×g during 10 min, aliquoted in 50 mL sterile tubes and kept at –80°C until use. Target 143B cells were graciously provided by M. Caruso (Centre de recherche en cancérologie de l'Université Laval, Québec, QC, Canada). These cells were maintained in DMEM (GIBCO-BRL, Gaithersburg, MD) medium supplemented with 10% FBS at 37°C, 100% humidity and a 5% CO_2 atmosphere.

Clarification and concentration

Sequential microfiltration and ultra/diafiltration processes were chosen for clarification, concentration and partial purification of retroviral particles from crude supernatants. Supernatants were filtered at room temperature using a dual HT Tuffryn® polysulfone membrane (0.45/0.2 μm) capsule filtration device with an effective filtration area of 500 cm^2 (Pall Gelman Sciences, Montreal, PQ, Canada) to remove any cells and cell debris. Clarification was carried out at 50 mL/min in 1 or 2 L scale with low backpressure (below 0.01 MPa). Clarified permeates were collected into sterile containers and subjected to ultra/diafiltration processes. Retroviral particles were concentrated using a 150 mm diameter Omega^{TM} polyethersulfone membrane disc filter (effective filtration area = 162 cm^2) with a molecular weight cut-off of 300,000 (Pall Gelman Sciences) in a 2 L stirred cell ultrafiltration unit (Amicon 2000; Millipore, Etobicoke, ON, Canada). The membrane was prewashed with 1 L of Milli-Q water and prefouled with 40 mL of DMEM medium supplemented with 10% FBS. Retrovirus supernatants (1 L) were concentrated 20-fold under constant nitrogen pressure (30 psi) and tip speed (33.5 cm/sec). Retrovirus-enriched retentate was diafiltered against cold heparin affinity adsorption buffer (150 mM NaCl in 20 mM Tris-HCl buffer, pH 7.5). The diafiltration step was repeated 3 times with 100 mL buffer in discontinuous mode. Virus was aliquoted and stored at -80ºC. Smaller samples for stability studies and matrix screening experiments were clarified using 0.45 μm pore size Acrodisc syringe-mounted filters with HT Tuffryn® polysulfone membrane (Pall Gelman Sciences) and concentrated 15-fold using a smaller stirred cell unit (Amicon 8050) with a 43 mm diameter Omega^{TM} membrane disc filter (effective filtration area = 13.4 cm^2), respecting the same operational parameters, but diafiltering against either 20 mM HEPES buffer, pH 7.5 (cation exchange matrix screening studies) or 20 mM Tris-HCl buffer, pH 7.5 (anion exchange or heparin affinity and stability studies) without salt. Virus was aliquoted and stored at -80ºC.

Retrovirus vector titer determination

In addition to the therapeutic gene (tyrosine kinase), the model vector expresses the GFP reporter gene, which allows the quantification of infective particles using 143B target cells

and flow cytometric analysis. For this purpose, $2\text{-}3\times10^5$ target cells per well were seeded in 6-well plates 1 day before transduction and exposed to 1 mL aliquots of serial dilutions of virus in DMEM containing 8 µg/mL of polybrene during 3 h at 37ºC. After the addition of 1 mL of DMEM containing 20% FBS, cells were further incubated for 48 h at 37°C and a 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:

Titer (IVP/mL) = (% GFP+ cells) × (number of cells at time of exposure) × (dilution factor)/ (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 to minimize intra-assay variability (maximum observed RSD was 9%). Virus dilutions that resulted in %GFP+ cell values ranging from 3 to 20% were selected for titer determination.

Retrovirus vector stability studies

The degradation constant of the virus at different temperatures was determined by placing the concentrated virus stock in the incubator (37ºC), in the fridge (4ºC) and on the bench (22ºC). The samples were incubated during 24 h and titers of treated samples and a freshly thawed negative control were determined as previously described. Every experiment was performed in triplicate. To study the effect of different salt concentrations on retrovirus vector stability, a 15-fold concentrated virus stock was rapidly thawed at 37°C, aliquoted and incubated in the presence of 0.2, 0.4, 0.5, 0.75 and 1 M NaCl concentrations for 1 h at room temperature. All buffers used were 0.2 µm filter-sterilized solutions containing varying concentrations of NaCl in 20 mM Tris-HCl buffer, pH 7.5. The treatment was stopped by sample dilution in 20 mM Tris-HCl buffer, pH 7.5, to a final concentration of 0.1 M NaCl. Viral titers of treated samples and negative controls incubated without salt were determined as previously described. The morphology of non-treated versus treated viral particles with 1 M NaCl during 3 h at room temperature was analyzed by negative

stain electron microscopy (NSEM). For this purpose, 100 µL aliquots of concentrated virus stock were diluted ½ in filter-sterilized 20 mM Tris-HCl buffer, pH 7.5 (negative control sample) or in the same buffer containing 2 M NaCl (1 M treated sample). Both samples were incubated for 3 h on the bench and then diluted with 800 µL of 20 mM Tris-HCl buffer, pH 7.5, to stop the treatment and reach a low final salt concentration (below 0.2 M NaCl) for NSEM analysis.

Liquid chromatography

Virus purification experiments were performed at room temperature using a low-pressure liquid chromatography system (GradiFrac; GE Healthcare, Uppsala, Sweden) and monitoring protein elution by UV absorbance at 280 nm. Samples concentrated by ultra/diafiltration in the appropriate binding buffer were rapidly thawed at 37ºC and filtered with a 0.45 µm GHP Acrodisc filter membrane (Pall Gelman Sciences) before chromatography. For matrix screening experiments, tentacle-type Fractogel® EMD Heparin (S), Fractogel® EMD DEAE (M) and Fractogel® EMD SO3- (M) gels (Merck, Darmstadt, Germany) were packed into an HR 5/5 column to a final volume of 1 mL and equilibrated in the appropriate buffers: 20 mM HEPES buffer, pH 7.5 (cation exchange matrix screening studies) or 20 mM Tris-HCl buffer, pH 7.5 (anion exchange or heparin affinity and stability studies) without salt. Concentrated samples (0.5 mL) were loaded onto Fractogel® packed columns. A linear gradient of NaCl (from 0-1000 mM at pH 7.5, 33 mM NaCl/min) at a linear flow rate of 153 cm/h was applied. Collected fractions (1 mL) and feed were stored at -80ºC. Duplicate runs per column type were carried out. Optimization of the adsorption and desorption conditions was performed with Fractogel® EMD Heparin (S) 1 mL column. Optimal salt concentrations for virus binding and elution were defined. The final stepwise elution strategy included a virus capture and wash step at 150 mM NaCl [19.5 column volumes (CV)], followed by virus elution at 350 mM NaCl (13 CV) and a final high-stringency wash step at 1200 mM NaCl (7.5 CV). The running linear flow rate was 153 cm/h. The column (1 mL) was pre-equilibrated with 150 mM NaCl in Tris-HCl buffer, pH 7.5, and typically 3 mL of a 20-fold concentrated feed were injected. Three peaks were collected (one at each step) and fractions were immediately titrated along with the feed or

kept at -80ºC for further analyses. The ability of pre-packed Hi-Trap® Heparin columns (1 mL, GE Healthcare, Piscataway, NJ), where heparin is fixed directly on a sepharose matrix, to capture viral particles was evaluated using the same purification strategy. The dynamic binding capacity of Fractogel® EMD Heparin (S) matrix was determined by pumping 10 mL of feed at a linear flow rate of 153 cm/h in a pre-equilibrated column with 150 mM NaCl in Tris-HCl buffer, pH 7.5. Eluted fractions (1 mL) during virus loading were collected and stored at -80ºC for titration. Sepharose CL-4B gel (GE Healthcare) packed into an XK 16/70 glass column was used for further purification at room temperature. Immediately after tentacle-type heparin affinity chromatography, a pool of eluted virus fractions (7.5 mL) was loaded onto a 52.5 cm bed height (CV = 105 mL) size-exclusion column equilibrated with PBS. Elution was performed with PBS at a linear flow rate of 15 cm/h and the flowthrough peak fractions were collected, pooled and immediately titrated or stored at –80ºC.

Electron microscopy

Retrovirus morphology and total virus particle counts were determined by negative stain electron microscopy (NSEM). Particles in suspension were mixed with a known concentration of latex beads, mounted onto grids, negatively stained with phosphotungstic acid and examined in the transmission electron microscope at the Institut Armand-Frappier (Laval, PQ, Canada) as previously described (Alain et al., 1987; Alain, 1997).

Protein and DNA analysis

Total protein concentration was determined by the Bradford Protein Assay (BioRad, Hercules, CA) according to the manufacturer's instructions using Bovine Serum Albumin (BSA) as the standard. Cellular double-stranded DNA was detected and quantified using the Picogreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). The microassay was performed according to the manufacturer's instructions using lambda DNA as the standard. Proteins were fractionated by electrophoresis in 4-20% gradient polyacrylamide gels (BioRad) ran under reducing conditions (SDS-PAGE). Protein bands were visualized by silver staining (BioRad Silver Stain Plus Kit) or transferred to a Hybond ECL

nitrocellulose membrane (GE Healthcare) using a Trans-Blot SD (semidry) transfer cell (BioRad). Viral proteins bound to the membrane were identified by indirect immunostaining using a monoclonal antibody against the envelope protein (mouse Mab anti-VSV-G; Roche Diagnostics, Indianapolis, IN) or a polyclonal antibody raised against wild type MoMLV (goat antiserum to MoMLV; ATCC VR-1522AS-Gt). Primary antibodies were incubated overnight at room temperature. Appropriate secondary antibodies coupled to horseradish peroxidase (Jackson Immunochemicals, Mississauga, ON, Canada) allowed visualization by enhanced chemiluminescence using the ECLTM system (GE Healthcare) and X-ray film detection. Images were scanned using the Kodak Digital Sciences Image Station (Eastman Kodak, New Haven, CT).

5 Results

Harvested supernatant variability

The retrovirus model vector used in our experiments is a MoMLV-derived recombinant retrovirus encoding the green fluorescent protein-herpes simplex thymidine kinase fusion protein. The virus was generated by a suspension-adapted 293GPG producer cell line. The vector is pseudotyped with the envelope glycoprotein of the Vesicular Stomatis Virus (VSV). The VSV glycoprotein (VSV-G) is cytotoxic and therefore its expression has to be limited to the virus production stage. To this end, a tetracycline inducible system is used to control the expression of VSV-G by this packaging cell line (Ory et al., 1996). Upon removal of the tetracycline, VSV-G expression is induced and infective retroviral particles are harvested daily up to 7 days post-induction. The quality of harvested supernatants during a 200 mL production was evaluated. Infective particle concentration, total protein and cellular double stranded DNA content of harvested supernatants on different days post-induction were measured (Figure 4). Infective particles were detectable at day 3 post-induction and the maximum peak of virus production was detected on day 5 post-induction (1×10^7 IVP/mL). The total protein content of the supernatant remained almost unchanged during the production procedure (~3 mg/mL). The main source of contaminating proteins is the serum supplement added to the packaging cell line culture media, BSA being the major contaminant. Cellular DNA concentrations in the supernatant continuously increased during the production procedure reaching a maximum of 11 μg/mL at day 6 post-induction. During viral vector production the viability of 293-GPG packaging cells typically decreases mainly due to VSV-G toxicity. Cellular lysis results in increasing cell debris and cellular DNA in harvested supernatants, which adversely affects the quality of harvested supernatants with time. Since the purification performance is affected by DNA viscosity and the presence of cell debris, we decided to use a pool of equal volumes of supernatants harvested from day 4 to day 7 post-induction as a feed for our purification experiments in order to avoid day-by-day variability.
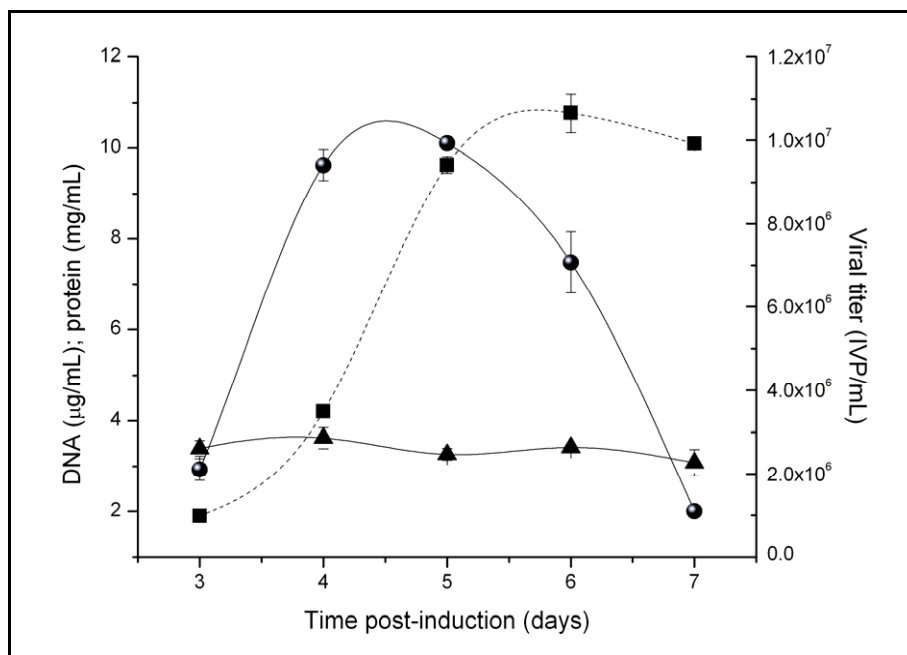
Figure 4 Analysis of the harvested supernatants during the retrovirus production phase. -■-Double stranded cellular DNA (µg/mL), -▲- total protein (mg/mL) and -●- viral titer (10^6 IVP/mL) measured in daily harvested supernatants. Values presented are the mean ± standard deviation of duplicate (titer) or triplicate (protein and DNA content) samples.

Membrane microfiltration and ultra/diafiltration

Microfiltration with 0.45-0.2 µm dual membranes resulted in clarified supernatants with minimal losses of infective particles. Infective retrovirus recovery was 93%. The amount of total protein (~3 mg/mL) and cellular DNA (~4 µg/mL) remained constant during clarification. Clarified supernatants were successfully concentrated, partially purified and conditioned by ultra/diafiltration at room temperature (Table IV). The virus (~100 nm) was recovered in an active form in the retentate fraction and in the appropriate adsorption buffer for subsequent fractionation. The process time was 5 h 30 min (3 h for concentration and 2.5 h for diafiltration). A minute amount of infective particles was found in the permeates separately collected during concentration and diafiltration (less than 1%) indicating that the virus was efficiently retained by the 300 kDa MWCO membrane employed. Additionally, a removal of 30% of the total proteins and 65% of small molecular weight DNA fragments

was achieved during this step. The final protein and DNA concentrations were 42.5 mg/mL and 28.4 µg/mL respectively. The apparent recovery of infective viral particles exceeded 100% (127 ± 10%). Although not demonstrated in this work, this effect could be attributed to the removal of small molecular weight species that interfere with retroviral transduction (Le Doux, 1996; Le Doux 1999). A possibility is that small DNA fragments or proteoglycans were neutralizing polybrene molecules by electrostatic interaction and therefore interfering with its enhancing effect. This would result in underestimation of viral titers in crude supernatants. A further explanation could be that vector preparations contained free-envelope VSV glycoprotein, which can interfere with transduction by competition with cell surface ligands. These results may reflect the limitations of biological assays for the quantitation of viral particles.

Table IV Ultra/diafiltration performance using a 300,000 MWCO membrane at 1 L scale

	Before concentration	After concentration
Viral titer (IVP/mL)	$1.4 \times 10^6 \pm 0.1 \times 10^6$	$3.6 \times 10^7 \pm 0.4 \times 10^7$
Protein concentration (mg/mL)	3.1 ± 0.1	42.5 ± 0.4
DNA concentration (µg/mL)	4.0 ± 0.1	28.4 ± 1.9
Concentration factor	1	19.9 ± 0.9
Purification factor	1	1.8 ± 0.2

The virus was concentrated 20-fold and diafiltered against 100 mL of 150 mM NaCl in 20 mM Tris-HCl buffer, pH 7.5, 3 times in discontinuous mode. Viral titer, total protein and DNA content of the retentate and the starting material are presented. All values presented are mean ± standard error of duplicate experiments.

Retrovirus stability

Although instability of retroviral particles to several physical factors (i.e. temperature, pH) to which the virus is exposed during the production phase is well documented, there are only few reported studies that allow us to predict virus behaviour during the purification procedure. Additionally, retrovirus stability to some factors was found to be dependent on the particular vector envelope protein and producer cell line type from which the viral envelope derives (Beer et al., 2003; Burns et al., 1993). Temperature stability studies of this model vector were required in order to reject a temperature-related degradation mechanism during virus purification (Figure 5). Virus half-lives were derived mathematically from a simple and fast 2-point kinetic experiment performed in triplicate. We found no significant loss of viral titer when the virus was stored at 4ºC in 24 h experiments. The virus half-life at 37ºC was calculated as 4.8 h. At room temperature the virus proved to be more stable showing a half-life of 40.7 h. These results are consistent with previous results published in the literature for retroviral vectors (Higashikawa and Chang 2001; Pizzato et al., 2001).
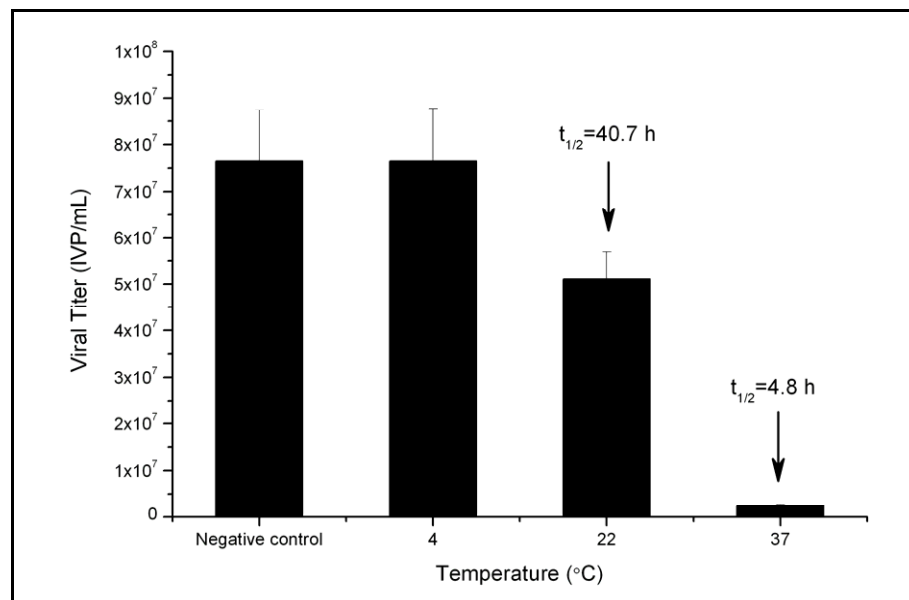
Figure 5 Effect of temperature on VSV-G pseudotyped retrovirus stability.
Values presented are the mean ± standard deviation of triplicate samples.

During chromatography, the virus could be exposed to high salt concentrations that are commonly used to elute the bound proteins from chromatographic columns. We further investigated the effect of salt on retrovirus stability and observed that the virus was progressively inactivated in the presence of increasing NaCl concentrations (Figure 6). The biological inactivation of the virus in the presence of salt was irreversible and happened very rapidly. Just 1 h of exposure to 1 M NaCl at room temperature was enough to inactivate 50% of the virus.
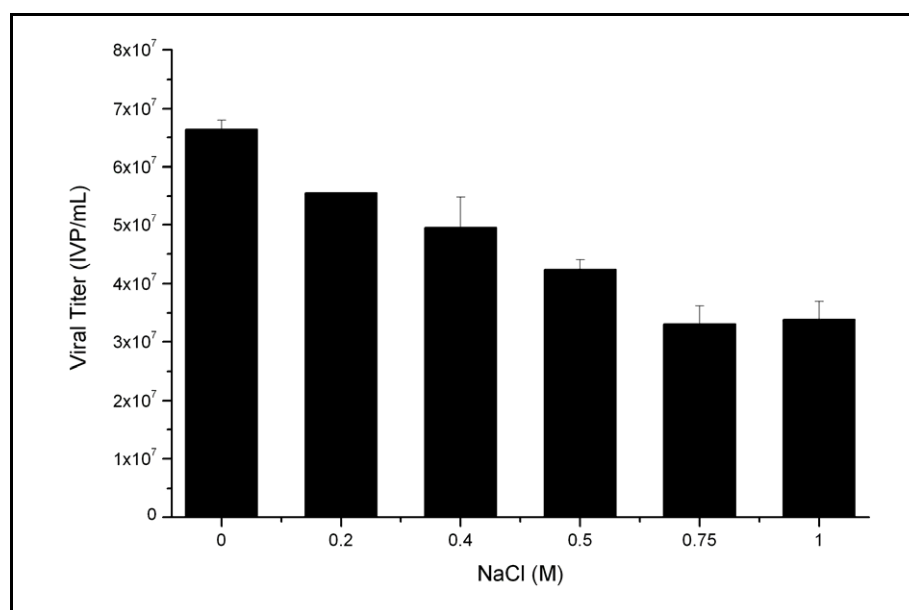
Figure 6 Effect of NaCl concentration on VSV-G pseudotyped retrovirus infectivity. Values presented are the mean ± standard deviation of triplicate samples.

The mechanism of infectivity loss due to virus exposure to salt remains unknown. In an effort to understand what causes virus inactivation, we investigated the effect of salt on virus morphology. Transmission electron micrographs of treated and untreated samples negatively stained with phosphotungstic acid are shown in Figure 7. Electron microscopy revealed that after a 3 h exposure to 1 M NaCl, retrovirus particles were broken or showed morphological changes. Retrovirus type C particles typically show a tailed-shape

appearance when negatively stained using phosphotungstic acid (PTA) (Schidlovsky, 1977). Although this "tail" is an artifact of the staining technique, it is claimed to be a very consistent and convenient artifact, which is used as a distinguishing feature for some RNA tumor viruses (i.e. MoMLV). In contrast to untreated samples, of which 96% were in excellent condition showing the typical tailed shape, 36% of the treated viral particles were broken and others showed predominantly a roughly round shape. It is interesting to note that particles with damaged envelopes will allow penetration of the PTA stain and will exhibit small or no tails (Schidlovsky, 1977). The roughly round shape of the virus in treated samples made it difficult to distinguish between cellular debris and round virus particles. However, since very few cellular debris was found in the original non-treated sample, it was assumed approximately the same amount of debris should be present in the sample after 1 M NaCl treatment and that the round particles observed were correctly identified as atypical viral particles. Moreover, the total virus particle count before and after treatment (3.3×10^8 and 3.6×10^8 respectively) has remained constant which indicates that the virus was not disintegrated but rather damaged after short exposure to 1 M NaCl. These observations strongly suggest that retrovirus infectivity loss following high salt concentration exposure is a consequence of membrane damage and is probably related to NaCl-associated increase in osmotic pressure.

Matrix screening

Fractogel® Heparin (S), Fractogel® DEAE (M), Fractogel® SO3- (M) and Heparin-Sepharose™ HP were evaluated for packed bed adsorption. Preliminary matrix screening studies demonstrated the ability of retroviral particles to bind heparin affinity matrices and also to strongly bind anion exchangers. Linear salt (NaCl) gradients were used to elute bound viral particles from the chromatographic columns. Viral particles were eluted at moderate (350 mM) or high salt concentrations (900 mM NaCl); from heparin and anion exchange columns respectively. On the other hand, cation exchangers were unable to capture viral particles, which were found in the flowthrough. Poor infectivity recoveries when using anion exchange chromatography (less than 5%) were attributed to virus sensitivity to the high salt concentrations required for elution from the DEAE tentacle

adsorbent. Heparin affinity chromatography resulted in poorer separation efficiency when a shallow linear gradient was applied but good recovery of infective viral particles. Thus, heparin affinity chromatography was chosen for further optimization.
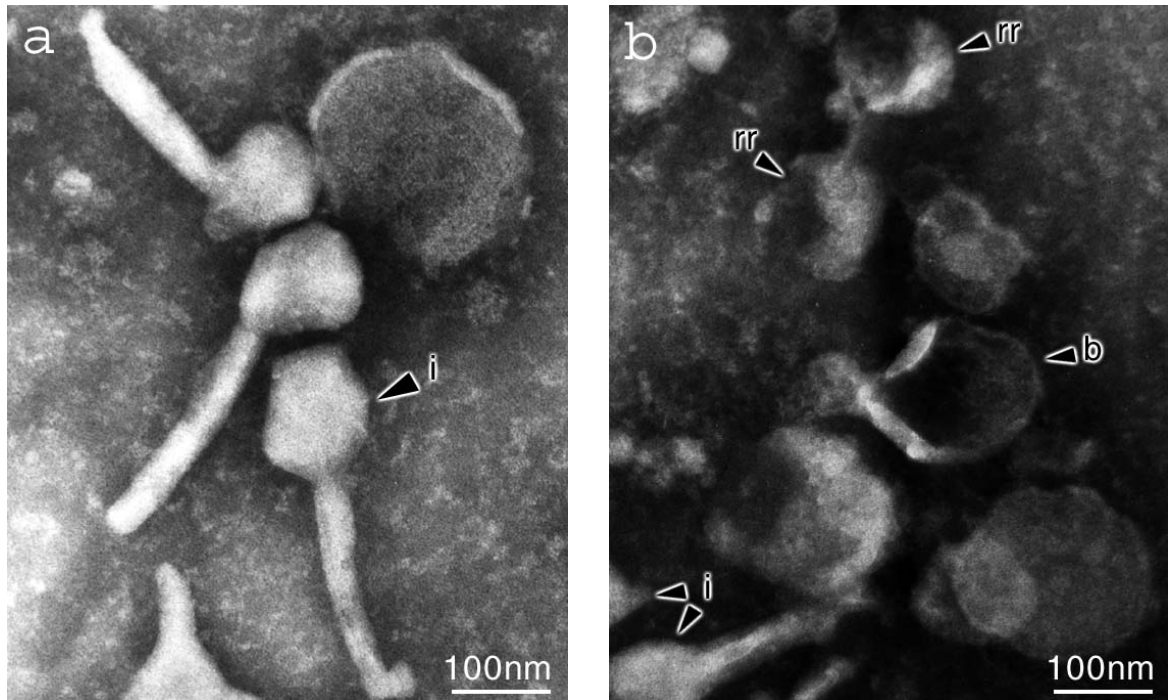
Figure 7 Effect of ionic strength on virus morphology by NSEM.
(a) Transmission electron micrograph of negatively stained MoMLV-derived vectors. Intact retroviral particles exhibit a typical pronounced rigid tail when stained with phosphotungstic acid (PTA). The total particle concentration is 3.3×10^8 VP/mL. 96% of the retroparticles are in excellent conditions. Few cellular debris are observed. (b) Transmission electron micrograph of negatively stained MoMLV-derived vectors after a 3 hr treatment with 1 M NaCl. Broken particles (37%) and atypical retrovirus particles exhibiting a roughly round shape are observed. The number of total particles was maintained (3.7×10^8 VP/mL). The bar represents 100 nm. Virus particle morphology: i, intact tailed shape retrovirus particle; b, broken retrovirus particle; rr, atypical roughly round retrovirus particle.

Heparin affinity chromatography purification

Since shallow linear gradients used in matrix screening experiments did not allow the separation of the virus from the bulk of serum-contaminating proteins eluting in the flowthrough, a step-wise elution strategy was developed (Figure 9). For the first step (virus adsorption), three different salt concentrations in the binding buffer were tested (0, 150 and 350 mM NaCl). The minimum loss of infective retroparticles in the flowthrough (19.4%) was observed when loading the concentrated feed at physiological ionic strength (150 mM NaCl) and this ionic strength was selected for the virus adsorption and wash step (data not shown). Most serum contaminating proteins were removed in this step (99% according to total protein determination). Viral particles were then eluted in a second step at 350 mM NaCl. The mean of five experiments indicated that $61.1 \pm 2.1\%$ of the infective viral particles were successfully recovered in a defined peak during this step. In a third step we were able to elute strongly bounded proteins along with 5.2% of infective viral particles by adding a higher salt concentration into the mobile phase (1200 mM). Chromatography was performed at room temperature and completed within 1.5 hours. Western blot analyses of fractions collected throughout the process confirmed the presence of viral particles in different fractions (Figure 11). Electron microscopy analysis of fractions eluted at 350 mM NaCl from the heparin column showed that 82% of retrovirus particles appeared in excellent condition and maintained their typical tailed shape (Figure 13). The infective: non infective particle ratio in the heparin eluant is 1:220. The purification factor for this single-chromatographic step was 62.8 ± 14.8. In addition, $86.8 \pm 7.1\%$ of the remaining DNA was removed (Table VI). Heparin-Sepharose, a non-tentacle media (Hi-Trap Heparin HP pre-packed columns), also showed potential in binding retrovirus particles using the described strategy; However, the binding efficiency was lower and only 18.6% of the infective retroviral particles were recovered by elution at 350 mM NaCl (Table V). Most retroviral particles failed to bind this chromatographic media and were recovered in the flowthrough (76.3%). Nevertheless, it must be pointed out that conditions for virus adsorption and desorption were only optimized for tentacle adsorbents in this work and the selected conditions tested with the non-tentacle media. Optimization of these conditions for the non-tentacle matrix, particularly flow rate, might result in increased virus recoveries.

Table V Recovery of virus using a tentacle vs. a conventional heparin affinity adsorbent

Fraction	Infective retroviral particles eluted (%)	
	Tentacle gel	Conventional gel
Load	100	100
Flowthrough (0.15 M NaCl)	19.4	76.3
Elution (0.35 M NaCl)	61.1	18.6
Wash (1.20 M NaCl)	5.2	4.5

1 mL packed bed column; step-wise elution strategy

Heparin affinity chromatography binding capacity

The dynamic binding capacity of Fractogel$^{®}$ EMD Heparin (S) gel was studied. Binding of the viral particles to heparin-immobilized ligands was achieved under physiological ionic strength and pH. The 20-fold concentrated feed contained 2.5×10^7 IVP/mL, which corresponds to 3.3×10^9 VP/mL according to the electron microscopy particle count (infective: non-infective particle ratio of 1:132) and a total protein concentration of 42 mg/mL. A breakthrough curve was generated using a 1 mL packed bed column (Figure 8). Breakthrough was expressed as a fraction of loaded infective virus particles in the column effluent or C/Co where C is the viral titer in the column effluent and Co is the viral titer in the feed. The C/Co values during breakthrough vary from 0 (when all loaded virus is bound to the column) to 1 (when the viral titer in the effluent equals that in the feed). The breakthrough curve was plotted as C/Co versus infective viral particles loaded calculated as Co $\times$ flow rate $\times$ time which allows dynamic capacity to be measured directly from the graph at C/Co of 0.1. The dynamic binding capacity was 1×10^8 IVP/mL of gel, which corresponds to 1.32×10^{10} VP/mL of gel. Since retroviral particles were found to optimally bind the chromatographic adsorbent under physiological conditions (150 mM NaCl and pH

7.5) that are also encountered in crude supernatants, the possibility of loading this material directly onto the column became very attractive. Direct loading of retrovirus clarified supernatants into heparin affinity packed bed columns would obviate the need for the initial concentration step of the material and thus reduce time and cost of downstream processing. Even more attractive was the idea of directly loading crude supernatants onto expanded bed adsorption (EBA) columns abrogating the need for both preliminary clarification and concentration, provided tentacle heparin affinity chromatography adsorbents suitable for EBA were available. We tested the ability of viral particles in clarified supernatants (2×10^6 IVP/mL, no ultrafiltration) to bind the heparin ligand. Unfortunately, the viral particles were not as efficiently retained (data not shown). This could be due to the partial purification accomplished during the ultrafiltration step, which could significantly reduce the amount of contaminating proteins able to compete for heparin binding sites on the support.
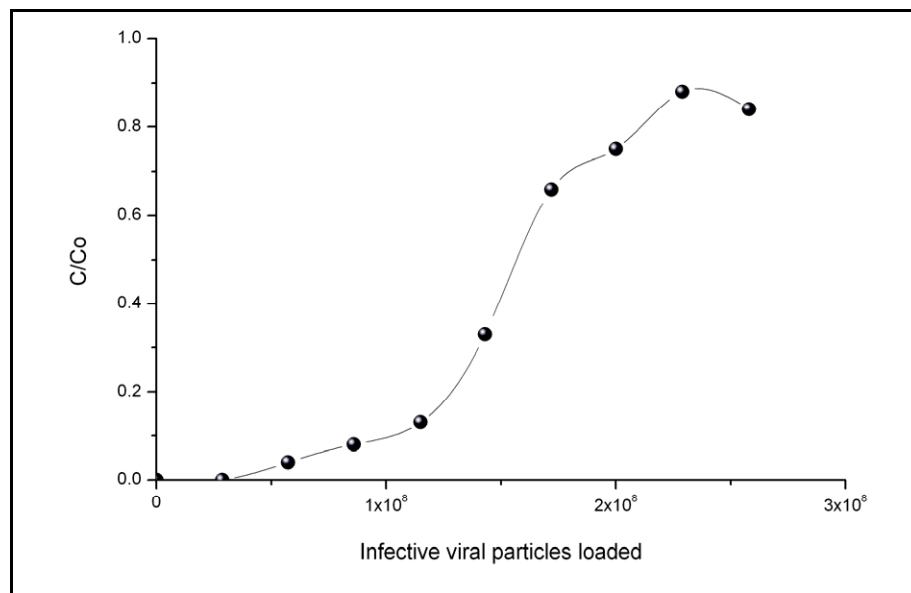
Figure 8 Breakthrough curve for retroviral particles in Fractogel$^®$ EMD Heparin (S) gel. The horizontal axis represents the infective virus particles loaded (Co × flow rate × time) onto a 1-mL column and the vertical axis is the fraction of loaded infective virus particles in the column effluent (C/Co). Dynamic capacity at C/Co=0.1 on the breakthrough curve is measured as ~$1x10^8$ IVP/mL of gel. This dynamic binding capacity corresponds to ~$1.3x10^{10}$ VP/mL of gel.

Size-exclusion chromatography polishing step

Heparin is a group-specific affinity ligand that binds proteins containing a heparin-binding domain. However, cationic proteins also have the ability to bind in a non-specific manner the strong negatively charged heparin molecule. A subsequent purification step is required to remove such impurities. Size-exclusion chromatography (SEC) was introduced to further purify the virus and also allowed us to desalt the preparation. Since the virus is large ($\sim$100 nm) it was recovered in the void volume of the size-exclusion gel (pore size 42 nm) whereas the contaminants that penetrate into the internal pores of the beads eluted later (column separation range for globular proteins is 70×10^3-20×10^6 Da). The heparin-binding contaminants showed a broad range of molecular weights with a maximum at $\sim$700 kDa as indicated in the chromatographic profile (Figure 10). A recovery of 53% of infective retroviral particles was achieved. This procedure required 4 hours for completion. The virus was further purified 19-fold in this step. Even though conditions were maximized to obtain a neat separation of the virus in the void volume, applying a very low flow rate (15 cm/h) and long SEC column (~60 cm), baseline resolution was not achieved. This indicates the presence of large molecular aggregates or macromolecular structures contaminating the final preparation as will be discussed in the next section.

Viral vector purity

The purity of viral preparations was evaluated by examining SDS-PAGE silver-stained protein electrophoretic profiles (Figure 12). After size-exclusion chromatography the major viral protein bands are clearly visible. The gel shows the presence of the VSV-G envelope protein ($\sim$68 kDa), capsid protein (p30), matrix protein (p15) and a polypeptide ~10 kDa (p10 or p12 viral protein) in the eluted fraction. A little background protein contamination was observed in the high-molecular-weight area (above 68 kDa). Purity was also evaluated in terms of specific virus activity. The specific virus activity, calculated as infective viral particles per mg of total protein, increased 3.3 logs after purification using the described strategy. This corresponds to a purification fold of $\sim$2000 (Table VI). The increase in

specific virus activity in terms of total viral particles per mg of total protein should be even higher if one considers that some viral particles that lost infectivity during the purification process and co-purify with infective particles will be part of the total contaminating protein content. The purification progress was also monitored by transmission electron microscopy. The electron micrograph of the starting material shows a significant amount of aggregated proteins (Figure 13). The background becomes clearer after each chromatographic step, thus indicating an efficient removal of contaminating proteins. Co-purification of $\sim$17-27 nm particles with retroviruses ($\sim$100 nm) was revealed by electron microscopy. These particles are clearly observed in the final SEC purified preparation. The identity of these contaminating particles was not established in this work. However, their known affinity to heparin ligand, their morphology and size suggest they may be low density lipoproteins (LDL) that are present in the 10% serum supplemented media at high concentrations ($\sim$5$\times$10^{12} particles/mL). Moreover, the LDL unique apoprotein (Apo B-100), one of the largest proteins known (550 kDa), is unable to migrate into 4-20% polyacrylamide gels, which complicates LDL detection by SDS-PAGE.
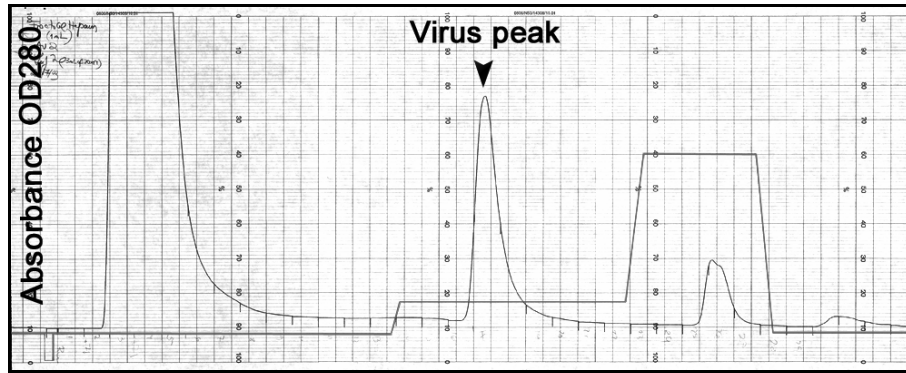
Figure 9 Heparin affinity chromatography step gradient elution profile.
3 mL of a 20-fold concentrated virus containing 2.4×10^7 IVP/mL were loaded onto a 1 mL Fractogel® EMD Heparin (S) column. The virus was eluted by addition of 350 mM NaCl into the mobile phase. Retroviral particles were recovered in a defined peak (6 mL) containing 7.6×10^6 IVP/mL.
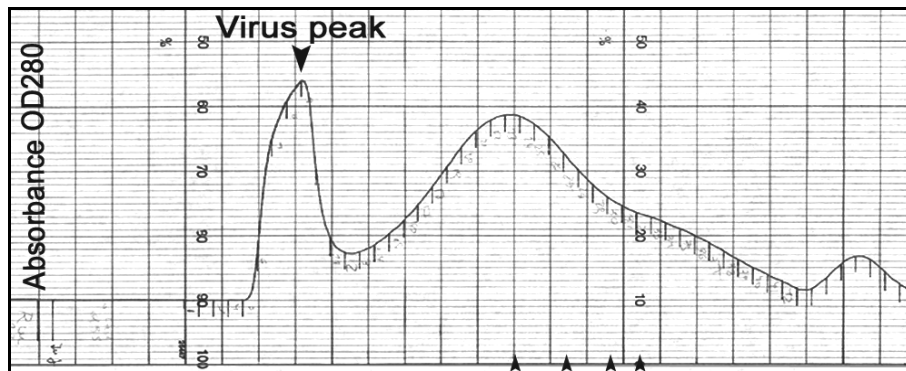
Figure 10 Size exclusion chromatography elution profile.
Elution pattern of heparin semi-purified retrovirus by size exclusion chromatography. 7.5 mL of semi-purified heparin fractions containing 3.9×10^6 IVP/mL were loaded onto a Sepharose CL-4B column (52.5 cm bed height). The virus was recovered in the flowthrough peak (12 mL) containing 1.3×10^6 IVP/mL. Arrows at the bottom of the chromatogram (from left to right) indicate the retention volumes of calibration weight standards previously ran: Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kDa) and BSA (67 kDa) respectively.
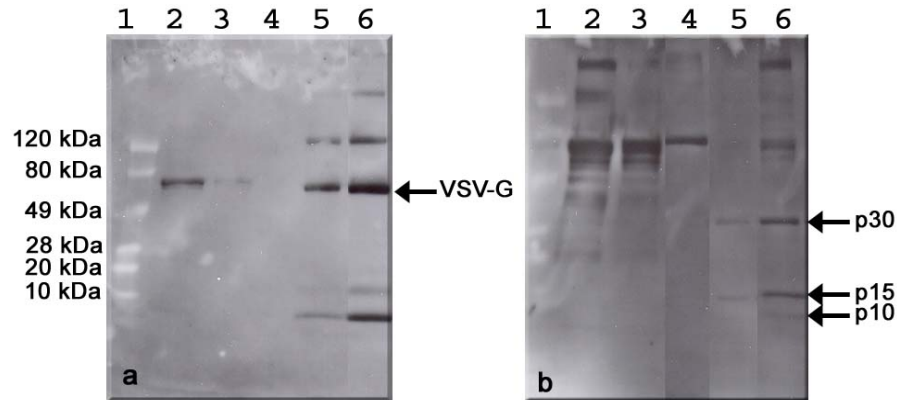
Figure 11 Western Blot analyses of heparin affinity chromatography fractions.
Western blot analyses for VSV-G (a) and Gag-proteins (b). The presence of retroviral particles in the three fractions collected throughout heparin affinity chromatography is shown. VSV envelope glycoprotein (68 kDa) was detected in the heparin affinity eluant fraction. VSV-G is also visible in the 1.2 M NaCl stringent wash, indicating that some viral particles were more strongly retained by the column. A faint band is also observed in the flowthrough fraction. Retroviral structural proteins (Gag-proteins including p30, p15 and a ∼10 kDa polypeptide) were detected in the heparin affinity eluant fraction using a polyclonal antibody against the virus. Gag proteins are also visible in the 1.2 M NaCl stringent wash. BSA cross-reacted with the polyclonal antibody as observed. Lane 1: Broad molecular weight markers; 2: 20-fold concentrated retentate; 3: Heparin affinity chromatography flowthrough; 4: BSA; 5: Heparin affinity chromatography 1.2 M wash; 6: Heparin affinity chromatography semi-purified virus fractions.
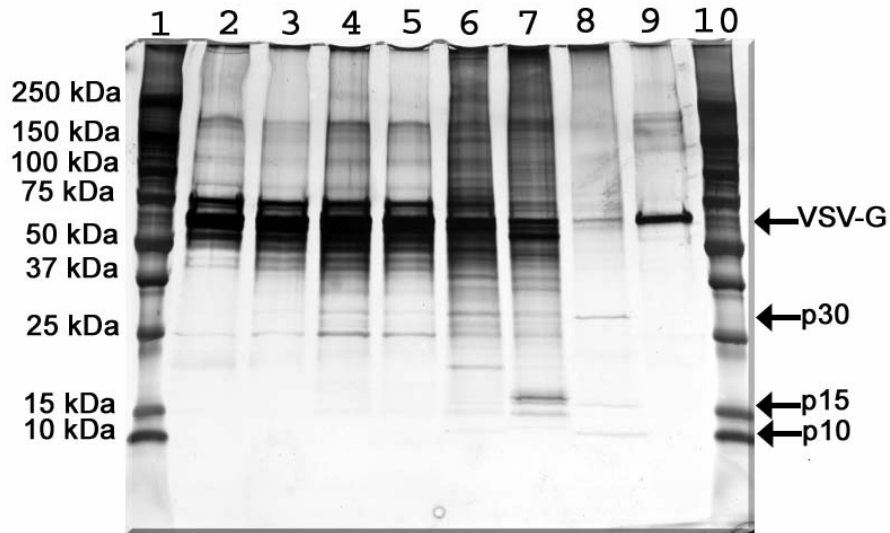
Figure 12 Purification performance analysis by SDS-PAGE silver stained.
Protein profiles of retrovirus samples obtained throughout the purification process. In the final purified preparation (lane 8) all major viral proteins are visualized including VSV-G (68 kDa), capsid protein (30 kDa), matrix protein (15 kDa) and a 10 kDa polypeptide (the nucleocapsid protein or p12 MoMLV protein). Little background contamination is observed. Lane 1: Broad molecular weight markers; 2: DMEM + 10% FBS; 3: Clarified supernatant; 4: 20-fold concentrated retentate; 5: Heparin affinity chromatography flowthrough; 6: Heparin affinity chromatography semi-purified virus fractions; 7: Heparin affinity chromatography 1.2 M wash; 8: Size-exclusion chromatography purified virus; 9: BSA; 10: Broad molecular weight markers. Protein loaded: 0.6 μg/lane; lane 8 loaded undiluted.
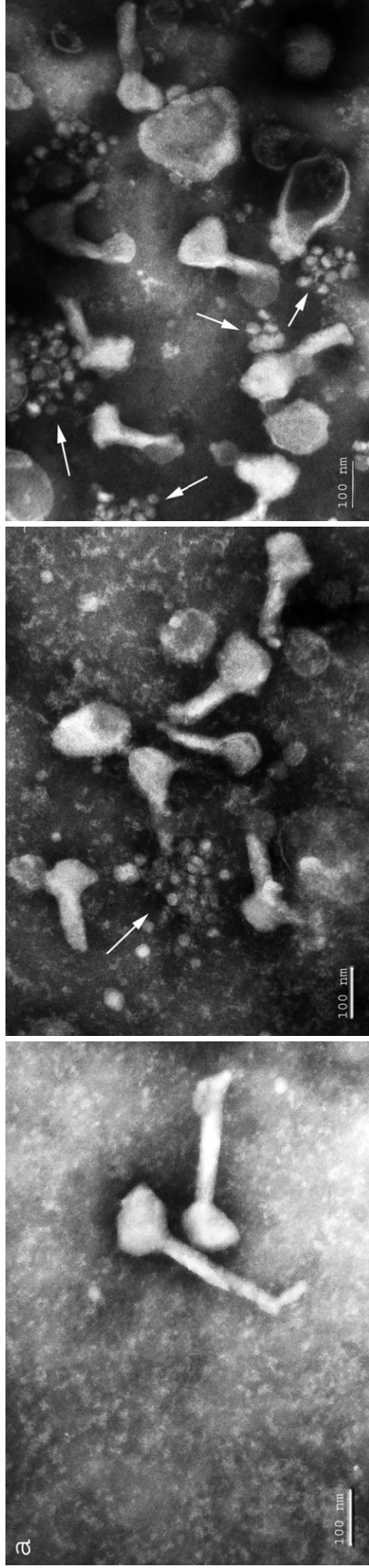
Figure 13 Monitoring purity by NSEM in sequential purification steps.
Transmission electron micrographs of negatively stained viral particles throughout the purification process using phosphotungstic acid and a final magnification of 182,000 X. The bar represents 100 nm. (a) The 20-fold concentrated sample used as feed for heparin affinity chromatography contained intact tailed-shape retrovirus particles, 96% of which were in excellent conditions. A significant amount of contaminating protein aggregates is visualized in the gray background but only few cellular debris. (b) Heparin affinity chromatography semi-purified preparations contained intact tailed-shape retrovirus particles, 82% of which were in excellent conditions. The sample contained few cellular debris and background contamination was significantly reduced. (c) In size exclusion chromatography purified preparations most retrovirus particles appeared in excellent conditions (62 %) but others were lightly broken. The background appeared extremely dark indicating the removal of contaminating proteins. Electron microscopy examination revealed the presence of small contaminating particles ranging from 17-27 nm (pointed with arrows). The diameter of the virion heads ranged from 80-100 nm.

6 Discussion

Retroviral vectors are currently the best clinically characterized gene therapy vehicles since they have been employed in the wide majority of clinical trials to date. Not surprisingly, there is an increasing demand for large volumes of high-quality retroviral vector preparations and it becomes important to establish vector concentration and purification schemes that are amenable to large-scale production (Andreadis et al., 1999; Brass et al., 1996). Adsorptive chromatography is the method of choice for selective fractionation of bioproducts in large-scale (Lyddiatt, 2002). However, suitable adsorbents for retrovirus purification are scarce. The aim of this work was to find a selective chromatographic adsorbent that bound retroviral particles and allowed its elution using mild conditions in order to maximize infective particle recoveries. Heparin affinity chromatography was found to meet these requirements. A multi-step strategy that included this highly selective purification step in combination with other developed tools for retrovirus purification was tested. Mean step values obtained from these studies were integrated together to construct a purification table where overall process recovery and purification factor were estimated (Table VI). Given the instability of retroviral particles to several factors including pH, temperature, shear stress, osmotic pressure and ionic strength, a key-point was to select the appropriate methods to obtain a final efficient recovery of infectious retrovirus particles. Particularly, the sensitivity of the viral particles to osmotic pressure may limit the adoption of many common bioseparation methods where suspension of the viral particles in high osmotic strength solutions is involved. Among these methods are sucrose density ultracentrifugation, aqueous two-phase extraction, precipitation with salts or nonionic polymers and also adsorptive chromatography procedures that require the use of high NaCl concentrations as discussed in this work. The choice of downstream processing steps was therefore focused on those methods that did not involve an abrupt change in the osmotic strength of the suspending virus fluid. Maintaining physiological pH and low ionic strength throughout the whole process contributed to preserving retrovirus functionality as revealed by the high overall recovery achieved (38%). Scalability of the concentration and purification steps was also taken into account when selecting the appropriate methods. In this sense, membrane and chromatographic technologies, regarded

as promising candidates for use at industrial scale (Andreadis et al., 1999; Brass et al., 1996), were employed in this work.

Membrane processes contributed to the clarification, concentration, buffer exchange and partial purification of retroviral particles from crude supernatants. Stability of retroviruses was found to be strongly dependent on the ultrafiltration operational parameters including pressure, stirring rate and process time (Cruz et al., 2000). These variables were kept low to ensure a maximum recovery of infective retroparticles. At the selected pressure and stirring rate, ultra/diafiltration process duration was directly dependent on volume reduction. Therefore, to keep process times within reasonable limits it was necessary to restrict the concentration factor to 20-fold. Under these mild conditions we were able to concentrate the virus without important infectivity loss. Additionally, levels of degraded DNA and protein contaminants were significantly reduced (65 and 30% respectively) during this filtration step. Progressive membrane fouling limited the volumes of supernatant to be processed and thus further scalability of this method. It is clear that this initial concentration step could greatly benefit from the development of serum-free adapted packaging cell lines.

The ability or inability of VSV-G pseudotyped MoMLV particles to bind different chromatographic supports, investigated in this work, led to interesting observations. First, at physiological pH, VSV-G pseudotyped retroviral particles are negatively charged and therefore strongly bind anion exchangers. The exploitation of the negatively charged surface of HIV for the purification of inactivated HIV-1 particles for viral vaccine purposes was previously reported (Prior et al., 1995). Anion exchange chromatography was also employed for the purification of lentiviral vectors (Scherr et al., 2002, Yamada et al., 2003). Lentivirus desorption from anion exchange matrices is accomplished using high salt concentrations steps (1-2M NaCl). The fact that high salt concentrations jeopardize the functional and structural integrity of the model vector used in this study and that low recoveries were obtained in preliminary experiments using anion exchange chromatography did not encourage the adoption of this method for further optimization. Second, the virus was recovered in the flowthrough when tentacle cation exchangers were tested. This

observation confirmed the anionic properties of the virus particles at neutral pH and allowed us to discard the possibility of nonspecific virus adsorption to Fractogel® chromatography supports. The structure and chemistry of all tentacle chromatography supports employed in the matrix screening experiments is basically the same. Fractogel® chromatography beads are made of a synthetic methacrylate based polymeric resin (matrix) to which long linear polymer chains (tentacles) are attached, carrying the functional ligands. The difference between the 3 Fractogel® supports used resides in the type of ligand coupled to the tentacles: diethylaminoethyl (DEAE), sulphoisobutyl (SO^{3-}) or heparin. Considering that the virus was unable to bind the tentacle sulfated cation exchange support, but bind the heparin ligand (also a strongly negatively charged sulfated molecule) we hypothesize that a specific interaction takes place between the retrovirus and the heparin molecule.

Heparin affinity chromatography is presented in this work as a novel valuable tool for retrovector purification that can be included in this or other purification schemes. Compared to other chromatographic adsorbents, such as hydroxyapatite and anion exchangers, where vector elution requires the addition of high salt concentrations into the mobile phase (Kuiper et al., 2002; Scherr et al., 2002, Yamada et al., 2003), elution of retrovirus particles from the heparin column was accomplished under mild conditions (0.35 M NaCl) and as a result high recoveries of intact functional retroviral particles were reproducibly obtained (61.1 ± 2.1%; n=5). By using this single-chromatographic step an increase of 62.8 ± 14.8 in purification factor was accomplished due to the high selectivity of the heparin media. Moreover, at this stage host cell DNA removal reached 95.8%.

Consideration of the pore dimensions (80 nm) of the heparin affinity chromatography adsorbent employed, suggests that adsorption of retroviruses (~100 nm) is restricted to the external bead area while most heparin-binding contaminating proteins have access to the area inside the pores as well, predicting both poor binding capacities and purification performance. The same is true for most available chromatography adsorbents since they were designed for protein purification rather than virus purification purposes (Lyddiatt and O'Sullivan, 1998). The advantage of using tentacle supports is the presence

of large amounts of sterically accessible ligands available for virus capture. In contrast to conventional supports, the ligands in tentacle affinity supports are attached to an inert and flexible spacer arm that separates them from the bead. Therefore, tentacle ligands can access binding sites located deeply inside the virus particle. In addition, since they are no longer exclusively on the surface area of the chromatographic bead larger amounts of ligands are available for binding (Kaufmann, 1997). This was reflected in our work by the good dynamic binding capacity achieved with Heparin Fractogel® matrix (1.3×10^{10} total viral particles/mL of gel) compared to other binding capacities of viral vectors reported in the literature (Lyddiatt and O'Sullivan, 1998).

The overall purification of the virus was 2,000-fold. The final purified product showed the presence of the major viral proteins on SDS-PAGE. There were no other major bands, although a few faint non-viral encoded high molecular weight bands were visible. The origin of these bands is unknown. While some of these proteins could be present in the SEC fractions as a consequence of sub-optimal chromatography resolution, we do not exclude the possibility that some of them may in fact be integral parts of the virus. Pure HIV-1 and lentiviral preparations were found to contain large amounts of cellular proteins (Richieri et al., 1998). Because retroviruses bud through the plasma membrane, they acquire a number of host cell proteins that are embedded in the membrane during this process (Weiss, 1993). Therefore, the estimation of virus purity by SDS-PAGE analysis might be complex, if not inappropriate, in the absence of a purified virus standard. Moreover, we would expect that MoMLV host cell content is dependent to some extent on the packaging cell line used to generate the retrovector particles, and therefore an appropriate virus standard should derive from the same cells as the vector under investigation. Unfortunately, highly purified retrovirus vector standards are very rarely available. In the absence of such a virus standard, electron microscopy is an essential tool to monitor sequential steps leading to virus purification. The dramatic consequences of omitting this control have been demonstrated in the past by HIV researchers (Papadopulos-Eleopulos et al., 1993). In this case, "pure" HIV-1 preparations were used to generate antibodies for ELISA and Western Blot diagnostic kits that incidentally lacked specificity for the virus. Massive amounts of cell debris and microvesicles were found in these

presumably pure retrovirus preparations isolated from 1.16 g/mL sucrose bands using electron microscopy (Bess et al., 1997; Gluschankof et al., 1997). Similarly, we found large amounts of 17-27 nm contaminating particles, presumably LDL particles, in the final vector preparations as revealed by electron microscopy. Although, this should not be a concern for *ex vivo* gene therapy applications, a complete removal of these contaminating particles as well as the 5% residual DNA should be required for safer *in vivo* gene therapy.

The ability of heparin chromatographic media to specifically bind MoMLV particles was exploited in this work for vector purification purposes. The selection of heparin affinity ligand was motivated by the recent observation that soluble heparin inhibits retrovirus infection (Arai et al., 1998; Guibinga et al., 2002; Jinno-Que et al., 2001; Le Doux et al., 1999; Walker et al., 2002). These observations were confirmed in our laboratory for the model vector used. Our results showed that concentrations of 1.5 U/mL of heparin or greater in the culture supernatant significantly inhibited retrovirus vector transduction to 143B target cells (data not shown). Soluble heparin also inhibits the attachment of Env-protein deficient retrovirus-like particles that have the ability to attach to target cells as efficiently as complete virus particles (Guibinga et al., 2002; Pizzato et al., 1999). Guibinga et al. (2002) proposed that heparan sulfate on the cell surface would promote this initial virus-cell attachment step in a similar way they do for a number of other viruses, but independently of the virus Env-protein. However, since polybrene as well as other polycations are well known enhancers of retrovirus transduction and heparin is a polyanion, it could be argued that the effect heparin has on transduction and attachment results from purely electrostatic interference rather than competition with cell surface heparan sulfate receptors. In this case, one would expect that the polyanionic nature of heparin repels the negatively charged virus rather than bind to it. By reporting the ability of 293-GPG-derived VSV-G pseudotyped retroparticles to directly and specifically bind immobilized heparin ligands, we provide further evidence to support the proposed mechanism for initial retrovirus binding to cells. Since VSV-G would not be responsible for the virus binding to heparin, we assume that virus binding to heparin would be mediated by an unknown envelope component derived from the 293-GPG packaging cell line membrane. Cellular adhesion molecules taken by the virus during budding may confer

heparin-binding properties to the viral particles. Whether these heparin-binding components are shared by retroviruses produced by different cell types is currently unknown. Studies with MoMLV Gag particles have shown that these particles randomly include various cellular membrane proteins with minimal exclusion (Hammarstedt et al., 2000). The extension of heparin affinity chromatography to the purification of retroviruses produced by different packaging cell lines may ultimately depend on the distribution of the heparin-binding components on the different cell types. The fact that the studies on the Env-protein independent retrovirus binding and binding inhibition by heparin were performed with retroviral particles derived from different cell types suggests that the virus surface heparin-binding component could be ubiquitous. Only a thorough investigation of the heparin-binding host cell derived proteins on retrovirus surface could bring a definitive answer to this question, contribute to the full understanding of the mechanism of retrovirus attachment to target cells as well as help design better targeted retroviral vectors for *in vivo* applications. We are currently working along this line.

In conclusion, we developed a novel affinity chromatography method for the purification of retroviral vectors that exploits the selective interaction of retroviruses with immobilized heparin ligands. This purification method was reproducible as determined in five different experiments. The method could be generic since the binding of retrovirus particles to heparin columns would be independent of the Env-protein and therapeutic gene. The method constitutes a valuable tool for researchers looking for a convenient way to purify retroviral vectors without significantly losing viral infectivity. A complete multi-step purification scheme that employs scaleable membrane filtration and chromatographic technologies is described. The final purified preparation containing functional and intact viral particles is characterized by minimal contamination with nucleic acids and proteins. Aside from the development of this original method, our findings provide further evidence to support the proposed mechanism of retrovirus initial binding to cell surface through an interaction with heparan sulfate.

Table VI Overall purification process results

Step	Volume (mL)	Viral titer (IVP/mL)	Protein (mg/mL)	DNA (µg/mL)	SVA (IVP/mg)	Recovery (%)	Concentration factor	Purification factor	DNA removal (%)
Crude supernatant	1000	2.50E+06	3.000	4.0	8.33E+05	100.0	1.0	1.0	0
Clarification	1000	2.34E+06	2.981	3.7	7.83E+05	93.4	1.0	0.9	6.9
Ultra/diafiltration	50	5.89E+07	41.759	26.2	1.41E+06	126.7	19.9	1.8	64.6
Heparin affinity	101	1.80E+07	0.203	1.7	8.85E+07	61.1	0.5	62.8	86.8
SEC	168	5.71E+06	0.003	1.0	1.65E+09	52.9	0.6	18.6	0
Overall values						**38.3**	**6.0**	**1976.4**	**95.8**

Mean values from five independent runs. Abbreviation: SVA specific virus activity

7 Acknowledgements

8 References

Aboud M, Wolfson M, Hassan Y, Huleihel M. 1982. Rapid purification of extracellular and intracellular Moloney murine leukemia virus. Arch Virol 71(3):185-95.

Alain R. 1997. Quantitation of virus particles by negative stain electron microscopy. Microsc. Today 97:20.

Alain R, Nadon F, Seguin C, Payment P, Trudel M. 1987. Rapid virus subunit visualization by direct sedimentation of samples on electron microscope grids. J Virol Methods 16(3):209-16.

Andreadis ST, Roth CM, Le Doux JM, Morgan JR, Yarmush ML. 1999. Large-scale processing of recombinant retroviruses for gene therapy. Biotechnol Prog 15(1):1-11.

Arai T, Matsumoto K, Saitoh K, Ui M, Ito T, Murakami M, Kanegae Y, Saito I, Cosset FL, Takeuchi Y and others. 1998. A new system for stringent, high-titer vesicular stomatitis virus G protein-pseudotyped retrovirus vector induction by introduction of Cre recombinase into stable prepackaging cell lines. J Virol 72(2):1115-21.

Beer C, Meyer A, Muller K, Wirth M. 2003. The temperature stability of mouse retroviruses depends on the cholesterol levels of viral lipid shell and cellular plasma membrane. Virology 308(1):137-46.

Bess JW, Jr., Gorelick RJ, Bosche WJ, Henderson LE, Arthur LO. 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology 230(1):134-44.

Braas G, Searle PF, Slater NK, Lyddiatt A. 1996. Strategies for the isolation and purification of retroviral vectors for gene therapy. Bioseparation 6(4):211-28.

Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. 1993. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and

efficient gene transfer into mammalian and nonmammalian cells. Proc Natl Acad Sci USA 90(17):8033-7.

Cruz PE, Goncalves D, Almeida J, Moreira JL, Carrondo MJ. 2000. Modeling retrovirus production for gene therapy. 2. Integrated optimization of bioreaction and downstream processing. Biotechnol Prog 16(3):350-7.

Davis HE, Morgan JR, Yarmush ML. 2002. Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophys Chem 97(2-3):159-72.

Flint SJ, Enquist LW, Krug RM, Racaniello VR, Skalka AM. 2000. Virus attachment to host cells. In: Principles of virology: molecular biology, pathogenesis and control. Washington: ASM Press, p 100-131.

Galipeau J, Li H, Paquin A, Sicilia F, Karpati G, Nalbantoglu J. 1999. Vesicular stomatitis virus G pseudotyped retrovector mediates effective *in vivo* suicide gene delivery in experimental brain cancer. Cancer Res 59(10):2384-94.

Gluschankof P, Mondor I, Gelderblom HR, Sattentau QJ. 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology 230(1):125-33.

Gomez-Navarro J, Curiel DT, Douglas JT. 1999. Gene therapy for cancer. Eur J Cancer 35(14):2039-57.

Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, Lupien PJ, Wilson JM. 1994. Successful *ex vivo* gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nat Genet 6(4):335-41.

Guibinga GH, Miyanohara A, Esko JD, Friedmann T. 2002. Cell surface heparan sulfate is a receptor for attachment of envelope protein-free retrovirus-like particles and VSV-G pseudotyped MLV-derived retrovirus vectors to target cells. Mol Ther 5(5 Pt 1):538-46.

Hammarstedt M, Wallengren K, Pedersen KW, Roos N, Garoff H.2000. Minimal exclusion of plasma membrane proteins during retroviral envelope formation. Proc Natl Acad Sci U S A. Jun 20;97(13):7527-32.

Hacein-Bey-Abina S, Le Deist F, Carlier F, Bouneaud C, Hue C, De Villartay JP, Thrasher AJ, Wulffraat N, Sorensen R, Dupuis-Girod S, Fischer A, Davies EG, Kuis W, Leiva L, Cavazzana-Calvo M. 2002. Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. N Engl J Med Apr 18, 346(16):1185-93.

Higashikawa F, Chang L. 2001. Kinetic analyses of stability of simple and complex retroviral vectors. Virol 280 (1):124-31.

Jinno-Oue A, Oue M, Ruscetti SK. 2001. A unique heparin-binding domain in the envelope protein of the neuropathogenic PVC-211 murine leukemia virus may contribute to its brain capillary endothelial cell tropism. J Virol 75(24):12439-45.

Kaufmann M. 1997. Unstable proteins: how to subject them to chromatographic separations for purification procedures. J Chromatogr B Biomed Sci Appl 699(1-2):347-69.

Kuiper M, Sanches RM, Walford JA, Slater NK. 2002. Purification of a functional gene therapy vector derived from Moloney murine leukaemia virus using membrane filtration and ceramic hydroxyapatite chromatography. Biotechnol Bioeng 80(4):445-53.

Le Doux JM, Morgan JR, Snow RG, Yarmush ML. 1996. Proteoglycans secreted by packaging cell lines inhibit retrovirus infection. J Virol 70(9):6468-73.

Le Doux JM, Morgan JR, Yarmush ML. 1999. Differential inhibition of retrovirus transduction by proteoglycans and free glycosaminoglycans. Biotechnol Prog 15(3):397-406.

Lyddiatt A. 2002. Process chromatography: current constraints and future options for the adsorptive recovery of bioproducts. Curr Opin Biotechnol 13(2):95-103.

Lyddiatt A, O'Sullivan DA. 1998. Biochemical recovery and purification of gene therapy vectors. Curr Opin Biotechnol 9(2):177-85.

McGrath M, Witte O, Pincus T, Weissman IL. 1978. Retrovirus purification: method that conserves envelope glycoprotein and maximizes infectivity. J Virol 25(3):923-7.

Navarro del Cañizo AA, Mazza M, Bellinzoni R, Cascone O. 1996. Foot and mouth disease virus concentration and purification by affinity chromatography. Appl Biochem Biotechnol 61(3):399-409.

Ory DS, Neugeboren BA, Mulligan RC. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A 93(21):11400-6.

O'Keeffe RS, Johnston MD, Slater NK. 1999. The affinity adsorptive recovery of an infectious herpes simplex virus vaccine. Biotechnol Bioeng 62(5):537-45.

Palu G, Parolin C, Takeuchi Y, Pizzato M. 2000. Progress with retroviral gene vectors. Rev Med Virol 10(3):185-202.

Papadopulos-Eleopulos E, Turner VF, Papadimitriou JM. 1993. Is a positive western blot proof of HIV infection? Biotechnology (N Y) 11(6):696-707.

Parkman R, Weinberg K, Crooks G, Nolta J, Kapoor N, Kohn D. 2000. Gene therapy for adenosine deaminase deficiency. Annu Rev Med 51:33-47.

Pizzato M, Marlow SA, Blair ED, Takeuchi Y. 1999. Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction. J Virol 73(10):8599-611.

Pizzato M, Merten OW, Blair ED, Takeuchi Y. 2001. Development of a suspension packaging cell line for production of high titre, serum-resistant murine leukemia virus vectors. Gene Ther 8(10):737-45.

Prior C, Bay P, Ebert B, Gore R, Holt J, Irish T, Jensen F, Leone C, Mitschelen J, Stiglitz M, Tarr C , Trauger RJ, Weber D, Hrinda M. 1995. Process development for the manufacture of inactivated HIV-1. BioPharmacology 8:25-35.

Richieri SP, Bartholomew R, Aloia RC, Savary J, Gore R, Holt J, Ferre F, Musil R, Tian HR, Trauger R and others. 1998. Characterization of highly purified, inactivated HIV-1 particles isolated by anion exchange chromatography. Vaccine 16(2-3):119-29.

Scherr M, Battmer K, Eder M, Schule S, Hohenberg H, Ganser A, Grez M, Blomer U. 2002. Efficient gene transfer into the CNS by lentiviral vectors purified by anion exchange chromatography. Gene Ther 9(24):1708-14.

Schidlovsky G. 1977. Structure of RNA Tumor Viruses. In: Gallo RC, editor . Recent Advances in Cancer Research: Cell Biology, Molecular Biology, and Tumor Virology. CRC press, Vol I, p 189-245.

Sharma S, Murai F, Miyanohara A, Friedmann T. 1997. Noninfectious virus-like particles produced by Moloney murine leukemia virus-based retrovirus packaging cells deficient in viral envelope become infectious in the presence of lipofection reagents. Proc Natl Acad Sci U S A 94(20):10803-8.

Smith RG, Lee SA. 1978. Large-scale isolation and partial purification of type C RNA viruses on hydroxyapatite. 1. Biochemical characterization. Anal Biochem 86(1):252-63.

Transfiguracion J, Jaalouk DE, Ghani K, Galipeau J, Kamen A. 2003. Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications. Hum Gene Ther 14(12):1139-53.

Walker SJ, Pizzato M, Takeuchi Y, Devereux S. 2002. Heparin binds to murine leukemia virus and inhibits Env-independent attachment and infection. J Virol 76(14):6909-18.

Weiss R. 1993. Cellular receptor and viral glycoproteins involved in retrovirus entry. In: Levy JA, editor. The Retroviridae. New York: Plenum Press. Vol 2, p. 1-108.

Williams DA, Baum C. 2003. Medicine. Gene therapy--new challenges ahead. Science Oct 17;302(5644):400-1.

Yamada K, McCarty DM, Madden VJ, Walsh CE. 2003. Lentivirus vector purification using anion exchange HPLC leads to improved gene transfer. Biotechniques 34(5):1074-8, 1080.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6(6):973-85.

Chapter III

Purification and characterization of retrovirus vector particles by rate zonal ultracentrifugation

María de las Mercedes Segura [1,2], Alain Garnier [2], and Amine Kamen [1*]

1- Biotechnology Research Institute, NRC, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2; phone: 514-496-2264 ; fax: 514-496-7251; e-mail: amine.kamen@cnrc-nrc.gc.ca

2- Department of Chemical Engineering, Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines, Université Laval, Québec, Canada, G1K 7P4

1 Résumé

L'ultracentrifugation jusqu'à équilibre sur gradient de sucrose demeure la technique la plus largement utilisée pour la purification des rétrovirus. Cependant, les préparations de virus purifiés obtenues avec cette méthode standard contiennent habituellement des quantités considérables de vésicules membranaires contaminantes. De plus, les solutions de sucrose sont très visqueuses et hyperosmotiques compromettant l'intégrité et la fonctionnalité des particules rétrovirales. Dans le but de surmonter ces problèmes, une technique alternative de purification a été développée, utilisant l'ultracentrifugation zonale transitoire et l'iodixanol pour générer un gradient. Les particules rétrovirales recombinantes ont été produites en utilisant des cellules 293-GPG en suspension en présence de 10% FBS. Les surnageants concentrés ont été purifiés par sédimentation transitoire dans un gradient d'iodixanol continu de 10 à 30%. Les particules virales localisées dans les fractions médianes du gradient étaient intactes et actives. En utilisant cette stratégie, des niveaux élevés de purification ont été obtenus, sans contamination avec des vésicules membranaires cellulaires comme indiqué par les études de traitement à la subtilisine. Le niveau de pureté des préparations rétrovirales est plus grand que 95% comme le montre l'analyse par électrophorèse sur gel de polyacrylamide en présence de SDS et par chromatographie sur tamis moléculaire. Les particules purifiées ont des dimensions et des formes homogènes selon les résultats de microscopie électronique à coloration négative. De plus, de grandes quantités de particules rétrovirales défectueuses produites dans les cellules 293-GPG peuvent être séparées des particules rétrovirales fonctionnelles en utilisant cette stratégie de purification.

2 Abstract

Sucrose equilibrium density ultracentrifugation remains the most widely used technique for retrovirus purification. However, purified virus preparations obtained by this routine method usually contain considerable amounts of contaminating cell membrane vesicles. In addition, sucrose solutions are highly viscous and hyperosmotic which jeopardizes the integrity and functionality of the retrovirus particle. In order to overcome these limitations, an alternative purification technique using rate zonal ultracentrifugation and iodixanol as gradient medium was developed. Recombinant retrovirus particles were produced by 293-GPG packaging cells grown in suspension in the presence of 10% FBS. Concentrated supernatants were purified by rate zonal sedimentation on a 10-30% continuous iodixanol gradient. Virus particles were recovered intact and active from the central fractions of the gradient. By using this strategy, high levels of purification were achieved, with no evident contamination with cell membrane vesicles as indicated by subtilisin treatment studies. The level of purity of the retrovirus preparation is over 95% as shown by SDS-PAGE analysis and size-exclusion chromatography. Purified particles appear homogenous in size and morphology according to negative stain electron microscopy. In addition, large amounts of defective retrovirus particles produced by 293-GPG packaging cells can be separated from functional retrovirus particles using this purification strategy.

3 Introduction

Highly purified virus preparations are required for characterization studies, immunological studies, gene transfer purposes and as "gold standards" for downstream processing. These preparations are difficult to obtain, particularly in cases where viruses occur at low titers and the viral particles are unstable, which is the case for retroviruses.

Mature retrovirus particles are composed of the cleavage products of three precursor polyproteins: Gag, Gag-Pol and Env-protein. Retrovirus Gag structural proteins alone represent about ¾ of the total virion protein content. The Env-proteins make up the vast majority of the remaining protein mass while the viral enzymes, derived from proteolytic cleavage of the Gag-Pol fusion polyprotein, are represented to a lesser extent. Additionally, even after successive rounds of equilibrium density centrifugation, purified virus preparations show several faint bands of cellular polypeptides detectable by SDS-PAGE. While some of these cellular proteins are an integral part of the virus (Ott, 1997; Ott, 2002), others are associated with cell membrane vesicles from broken or intact cells that have a density similar to that of the virions and thus co-purify with them by equilibrium (isopycnic) density centrifugation (Bess et al., 1997; Gluschankof et al., 1997).

Centrifugation processes are widely used for the isolation of viral particles. 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, this method lacks resolving capacity and is usually coupled with density gradient ultracentrifugation. The most widely used gradient media for virus purification are sucrose and cesium chloride (CsCl). Both media are hyperosmotic at the densities used to band retrovirus particles. Sucrose solutions are very viscous and thus require longer sedimentation times for separation. Moreover, the high viscosity of sucrose has been associated with loss of surface structures and thus loss of infectivity upon purification. Iodixanol (OptiPrep[TM]) offers several advantages over these two media. Iodixanol can be diluted in iso-osmotic buffers to form iso-osmotic solutions that help preserve retrovirus particle integrity and functionality (Dettenhofer and Yu, 1999; Moller-

Larsen and Christensen, 1998). In addition, it is less viscous than sucrose resulting in shorter processing times. Moreover, this medium, originally designed as an X-ray contrast solution, is non-toxic to cells and allows for subsequent viral infectivity assays directly without need for removal.

Large amounts of contaminating host protein-laden membrane vesicles, either microvesicles or exosomes, are found in density-gradient purified virus preparations. Between 2 to 4 fold more cell membrane vesicles than virions were found by electron microscopy in HIV-1 purified preparations from lymphoid cells (Bess et al., 1997; Gluschankof et al., 1997). 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 (Abe et al., 1998). 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. A possible way to remove these cellular vesicles is to employ immunoaffinity chromatography provided that a surface protein is found to be exclusively incorporated into either the virions or the vesicles. Taking advantage of the differential incorporation of CD45 into HIV-1 and cell membrane vesicles (Esser et al., 2001), Trubey et al. (2003) developed an immunoaffinity approach to deplete selectively membrane vesicles from density-purified retrovirus preparations. However, non-hematopoietic cells (i.e. HEK 293) are not expected to express CD45, limiting the usefulness of this technique. The separation of defective and functional virus particles poses an even more serious challenge. Although well documented for other types of viruses and viral vectors, a method that allows the separation of defective from functional retrovirus particles has not yet been described in the literature.

To date most highly purified retrovirus preparations used in retrovirus characterization studies were obtained by equilibrium ultracentrifugation on sucrose density gradients. Using this technique, retrovirus particles are isolated from a band at a density of ~1.16 g/mL, corresponding to 35% (w/w) sucrose. However, the large number of cell membrane vesicles that co-purify with the virus when this standard technique is used greatly complicates the identification and quantification of cellular proteins incorporated

into retrovirus particles and it is undesirable for gene therapy applications. The goal of this work was to develop a centrifugation procedure that yielded highly purified active retrovirus preparations free of contaminating cell membrane vesicles and thus, suitable for the analysis of the exterior virus particle proteins. Since these vesicles show a wider range of size (50-500nm), higher levels of purification could be achieved by rate zonal ultracentrifugation. In this case, separation of viral particles from contaminants is based on size and density, in contrast to the standard equilibrium ultracentrifugation procedure in which irrespective of the size, particles are separated according to their buoyant density alone. Iodixanol was selected as gradient medium because of the advantages it offers over sucrose mentioned above. This iodinated medium showed to be effective for the purification of Moloney murine leukaemia virus (MoMLV) Gag retrovirus-like particles (Hammarstedt et al., 2000) and other retroviruses, including HIV-1 and HTLV-1 (Dettenhofer and Yu, 1999; Moller-Larsen and Christensen, 1998).

A novel, efficient and reproducible method for the purification of functional MoMLV-derived retrovirus particles that employs a combination of membrane filtration and rate zonal ultracentrifugation is described in this work. This strategy resulted in highly purified virus preparations with no evident contamination with cell membrane vesicles and proved to separate defective from functional retrovirus particles.

4 Materials and methods

2.1 Retrovirus vector, cell lines and virus stock preparation

The retrovirus used in this study is a MoMLV-derived vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped retrovirus vector produced by 293-GPG packaging cells (Ory et al., 1996). This cell line, a generous gift from Dr. J. Galipeau (Lady Davis Institute for Medical Research, Montreal, QC, Canada), was stably transfected to generate a retrovirus vector encoding a fusion protein between the herpes simplex virus thymidine kinase protein (TK) and the green fluorescent protein (GFP) (Paquin et al., 2001). Suspension-adapted cultures were grown in calcium free DMEM medium supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, UT) and tetracycline (1 µg/mL; Fisher Scientific, Nepean, ON, Canada) until the cell density reached 2×10^6 cells/mL, at which point VSV-G expression was induced by entirely removing tetracycline from the culture medium. Retrovirus supernatants were harvested every 24 h during 7 days as previously described (Segura et al., 2005). Infective particles were detectable at day 3 post-induction and the maximum peak of virus production was detected on day 5 post-induction $(1 \times 10^7$ IVP/mL). The quality of harvested supernatants during vector production varies in terms of the viral titer as well as DNA concentration. The latter was found to increase continuously during production (Segura et al., 2005). Since the purification performance can be affected by DNA viscosity, a representative feed generated by pooling equal volumes of supernatants harvested from day 4 to day 7 post-induction was used for the purification experiments. Sequential microfiltration and ultrafiltration steps were selected for clarification and concentration of virions from crude supernatants (Figure 14). This strategy resulted in concentrated and partially purified virus stocks with minimal losses of infective particles as previously described (Segura et al., 2005). Briefly, supernatants were filtered using a dual HT Tuffryn® polysulfone membrane (0.45/0.2 µm) capsule filtration device to remove any cells and cell debris. Clarified permeates were subjected to ultra/diafiltration processes using an Omega™ polyethersulfone membrane disc filter (MWCO = 300 kDa, Pall Life Sciences, Mississauga, ON, Canada) and a 2 L stirred cell ultrafiltration unit (Amicon® 2000; Millipore, Etobicoke, ON, Canada). Retrovirus

supernatants (1 L) were concentrated 20-fold under constant nitrogen pressure (30 psi) and tip speed (33.5 cm/sec). Retrovirus-enriched retentate was diafiltered against cold storage buffer (150 mM NaCl in 20 mM Tris-HCl buffer, pH 7.5). The diafiltration step was repeated 3 times with 100 mL buffer in discontinuous mode. Virus stocks were aliquoted and stored at -80ºC prior to ultracentrifugation. A removal of 30% of the total proteins and 65% of small molecular weight DNA fragments was achieved during the ultrafiltration step (Segura et al., 2005). Target 143B cells used for virus titration were graciously provided by M. Caruso (Centre de recherche en cancérologie de l'Université Laval, Québec, QC, Canada). These cells were maintained in DMEM (GIBCO-BRL, Gaithersburg, MD) medium supplemented with 10% FBS at 37°C.

2.2 Retrovirus vector titer determination

Quantification of infective particles by flow cytometric analysis has been previously reported (Segura et al., 2005). Briefly, 143B target cells in 6-well plates were exposed to 1 mL aliquots of serial dilutions of virus in DMEM containing 8 µg/mL of polybrene during 3 h at 37ºC. After the addition of 1 mL of DMEM containing 20% FBS, cells were further 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 FACS analysis and viral titers were calculated as previously described (Segura et al., 2005).

2.3 Protein and DNA analysis

Total protein concentration was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions using Bovine Serum Albumin (BSA) as the standard. Cellular double-stranded DNA was detected and quantified using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR). The microassay was performed according to the manufacturer's instructions using lambda DNA as the standard. Before electrophoresis, virus samples were mixed 3:1 with 4x NuPage® sample buffer (Invitrogen Life Technologies, Burlington, ON, Canada) containing 50 mM DTT and heated at 70°C for 10 min. Proteins were fractionated by

electrophoresis on Novex® 4-12% Tris-Glycine gradient gels (Invitrogen™) or 4-20% Tris-HCl Ready Gels (Bio-Rad) and run under reducing conditions (SDS-PAGE). Mark12 MW standard (Invitrogen™) was applied to each gel to determine molecular weights. Protein bands were visualized by silver staining (Silver Stain Plus Kit, Bio-Rad) or by staining with Coomassie brilliant blue R250. For Western blot, proteins were transferred to a Protran® nitrocellulose membrane (Schleicher & Schuell Bioscience Inc., Keene, NH) using a Mini Trans-Blot® transfer cell (Bio-Rad). Viral proteins bound to the membrane were identified by indirect immunostaining using a monoclonal antibody against the C-terminus of VSV-G envelope protein (mouse Mab anti-VSV-G; Roche Diagnostics, Indianapolis, IN), a polyclonal antibody raised against wild type MoMLV (goat antiserum to MoMLV; ATCC VR-1522AS-Gt) or a polyclonal antibody for host proteins (goat anti-HEK 293 cell HRP conjugate, Cygnus Technologies Inc, Southport, NC). Primary antibodies were incubated overnight at room temperature. Appropriate secondary antibodies coupled to horseradish peroxidase (Jackson Immunochemicals, Mississauga, ON, Canada) allowed visualization by chemiluminescence using the BM Chemiluminescence Blotting Substrate (Roche Diagnostics, Indianapolis, IN). Images were obtained with a Kodak Digital Science Image Station 440cf equipped with Kodak Digital Science 1D image analysis software version 3.0 (Eastman Kodak Co., Rochester, NY).

2.4 Rate zonal ultracentrifugation

A 10-30% continuous iodixanol gradient was made in a 25 × 89 mm Beckman UltraClearTM tube (Beckman Instruments, Inc., Palo Alto, CA) using a two-chamber gradient maker. For this purpose, two solutions, 10% and 30% iodixanol (w/v) in 20 mM Tris-HCl, 1 mM EDTA, 0.85 w/v NaCl (pH 7.5) were prepared using a 60% (w/v) sterile stock solution of OptiPrep™ (AXIS-SHIELD PoC AS, Oslo, Norway). Retrovirus particles were isolated by carefully layering 3 mL of the concentrated virus stock on top of a 34 mL continuous iodixanol gradient (Figure 14). Ultracentrifugation was carried out in a Beckman ultracentrifuge using a SW28 rotor and spinning at 100,000×g for 4 h at 4ºC. Fifteen fractions of 2.5 mL (corresponding to a bandwidth of 5 mm) were collected by puncturing the bottom of the tube. The density of each fraction was determined by weight

of 200 µL aliquots on an analytical balance. Gradient fractions were immediately titrated or kept at -80°C for subsequent analysis. Concentration of a pool of virus-containing fractions (6 to 9) was required for virus detection in certain experiments as specified. Concentration was carried out by ultrafiltration using a 10 mL stirred cell unit and a YM-100 kDa membrane at 10 psi and low tip speed (20 cm/sec). Retentates were diafiltered against PBS and the final retentate volume was adjusted to achieve a final concentration of 10-fold.

2.5 Size-exclusion chromatography

Size-exclusion chromatography was used to remove iodixanol and evaluate the purity of the virus preparation (Figure 14). Sepharose CL-4B gel (Amersham Biosciences, Piscataway, NJ) was packed into a XK 16/40 glass column (1.6 cm diameter x 30 cm bed height). A 10-fold concentrated pool of iodixanol purified virus-containing fractions was passed through a 0.45 µm GHP Acrodisc® Syringe Filter (Pall Life Sciences) and 2.5 mL of feed were loaded onto the PBS equilibrated column. Size-exclusion chromatography was performed at room temperature using a low-pressure liquid chromatography system (GradiFrac; Amersham Biosciences, Uppsala, Sweden) and monitoring protein elution by UV absorbance at 280 nm. Elution was performed with PBS at a linear flow rate of 24 cm/h and 1.5 mL fractions were collected.

2.6 Enzymatic treatments

The purity of virions was further evaluated by the use of enzymatic treatments. Benzonase® and subtilisin digestions were employed to rule out the presence of contaminating nucleic acids and cell membrane vesicles respectively. Digestion with Benzonase® of a 10-fold concentrated pool of iodixanol purified virus-containing fractions was performed by adding 30 µL per mL of sample of a working solution containing 10,000 U/mL of the endonuclease in 20 mM Tris-HCl, 1 mM $MgCl2.6H2O$, 20 mM NaCl (pH 8) buffer. The sample was incubated at room temperature with gentle shaking for 30 min and filtered using 0.45 µm GHP Acrodisc® Syringe Filter (Pall Life Sciences) before being loaded onto the size exclusion chromatography column. Subtilisin treatment of a 10-fold concentrated pool of iodixanol purified virus-containing fractions was carried out as previously

described (Ott et al., 1995) using a 5X stock solution containing 25 mg/mL of subtilisin (Fluka Biochemika, Buchs, Switzerland) in 100 mM Tris-HCl, 5 mM CaCl2 (pH 8) for a final concentration of 5 mg of protease/mL. The reaction was stopped by the addition of 5 µg/mL phenylmethylsulfonyl fluoride (Fluka BioChemika). Digested retrovirus preparations were then subjected to dead end filtration and size exclusion chromatography as previously described in section 2.5. Chromatographic and electrophoretic profiles of treated and untreated samples were then compared.

2.7 Electron microscopy

Retrovirus morphology, size and total virus particle counts were determined by negative stain electron microscopy (NSEM). Particles in suspension were mixed with a known concentration of latex beads, mounted onto grids, negatively stained with uranyl acetate and examined in a transmission electron microscope at the Armand-Frappier Institute (Laval, QC, Canada) as previously described (Alain, 1997; Alain et al., 1987).
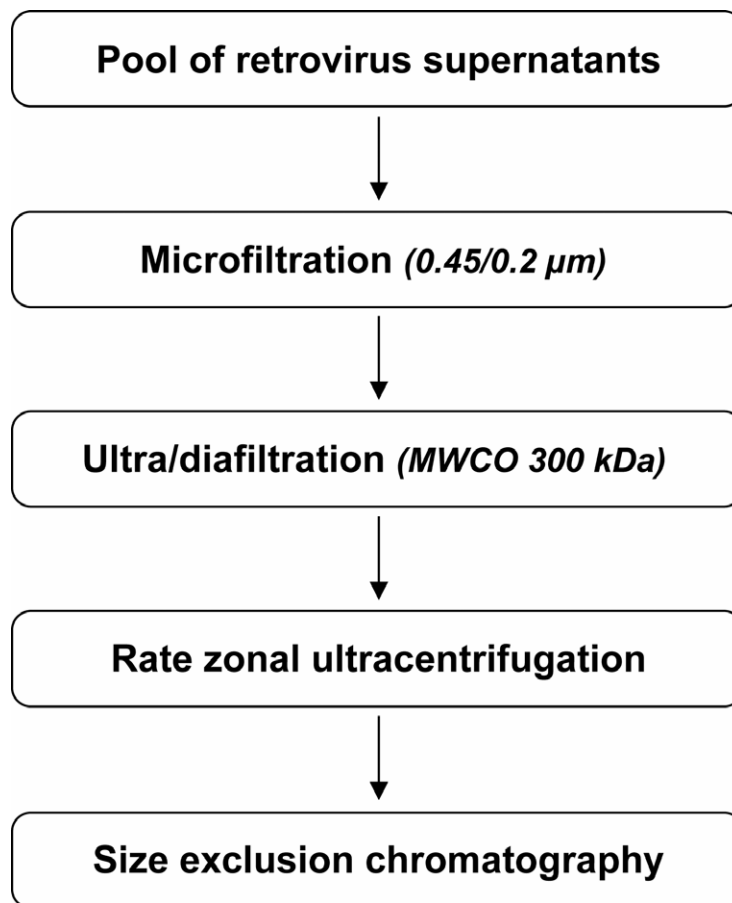
Figure 14 Scheme for the purification process of retroviral particles.
Harvested supernatants from 293 GPG cell cultures were pooled (day 4 to day 7 post-induction) and subjected to a series of downstream processing steps. Microfiltration and ultrafiltration were selected for clarification and concentration of crude supernatants. Retrovirus particles were isolated by carefully layering 3 mL of the 20-fold concentrated virus stock on top of a 10-30% continuous iodixanol gradient and spinning at 100,000×g for 4 h at 4ºC. Size-exclusion chromatography was further introduced to remove iodixanol and evaluate the purity of the pool of virus-containing fractions.

5 Results

3.1 Purification of retrovirus particles by rate zonal ultracentrifugation

Although seldom used for the purification of retrovirus particles, rate zonal ultracentrifugation seemed to be a promising tool for the separation of the virions from closely related particles. For the method to be efficient, retrovirus particles must be layered on top of a continuous density gradient as a narrow band and allowed to move down through the gradient, but not reach equilibrium. In this way, virions can be separated from particles with the same or very similar densities but different size such as some cell membrane vesicles.

For 3 out of 11 runs performed, 15 gradient fractions per run were collected and thoroughly analyzed (Figure 15A). The density gradient formed was continuous from 1.00 to 1.16 g/mL (Figure 15B). The virus banded approximately halfway down the gradient (~1.08 g/mL). According to FACS analysis, viral activity was detected in the 4 central fractions (6 to 9) of the 15 fractions collected. The recovery of infective viral particles in these 4 virus-containing fractions was 36.5 ± 3% (n=3). Only 0.1% of the total proteins originally loaded on top of the gradient were recovered in these fractions. Retrovirus particles were well separated from nucleic acids as well as the bulk of contaminating serum proteins that remained at the top of the tube (Figure 15B). A low amount of infective viral particles remained at the top of the centrifugation tube, possibly associated with protein aggregates.
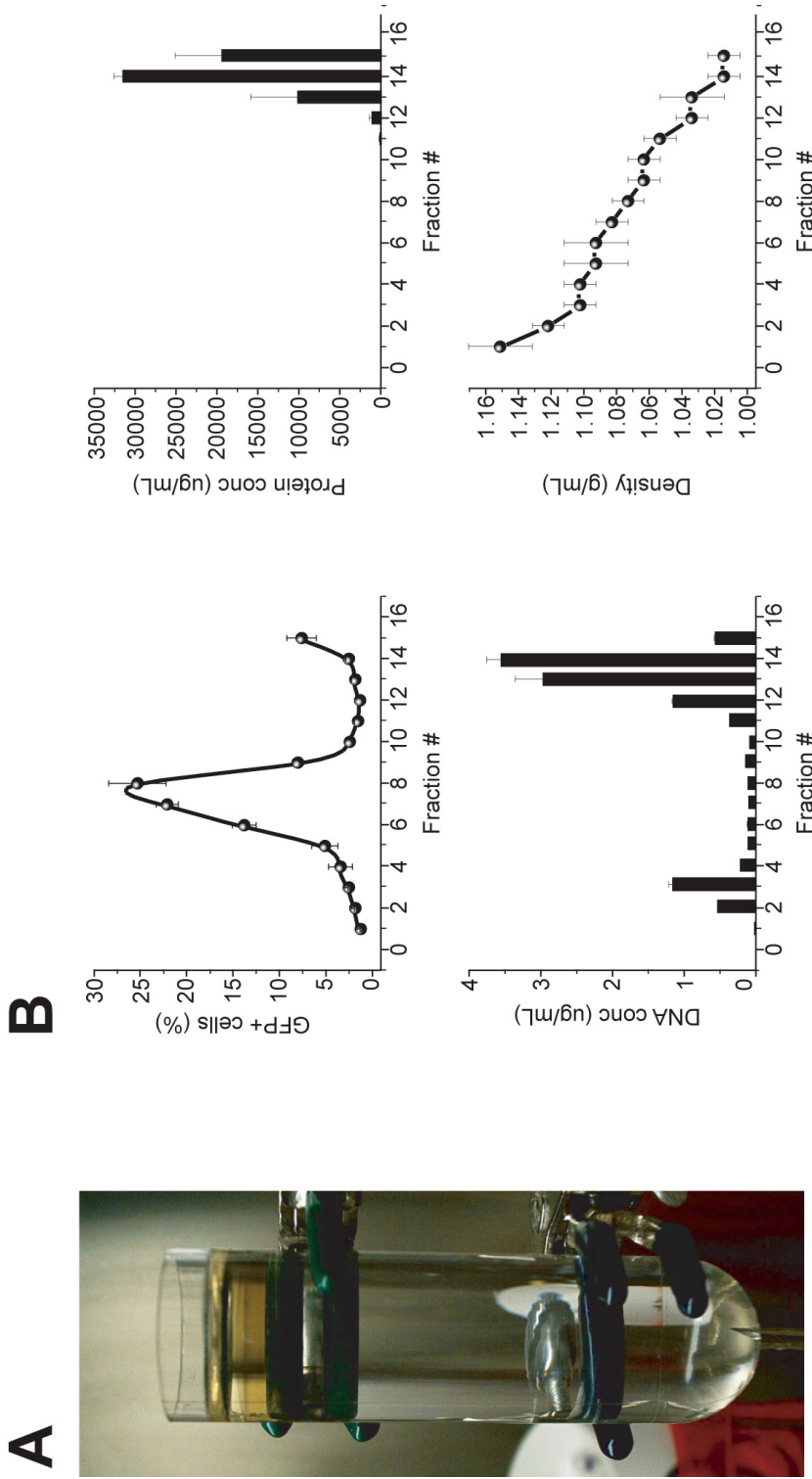
Figure 15 Rate zonal sedimentation in continuous iodixanol gradients.

(A) Gradient at the end of the ultracentrifugation run. The virus was collected by dripping 2.5 mL fractions from the bottom of the centrifugation tube. Infective virus particles were consistently detected in fractions 6 to 9. (B) Analysis of the 15 gradient fractions collected. The percentage of GFP + cells upon virus titration (fraction dilution 1/10), protein concentration (μg/mL), DNA concentration (μg/mL) and density (g/mL) from 3 runs was determined. Values represented are the mean ± standard deviation of these 3 runs.

Electrophoretic analyses confirmed the presence of the virus in fractions 6 to 9 (Figure 16). Bands corresponding to the major viral proteins, including VSV-G (68 kDa), capsid protein (p30CA), matrix protein (p15MA) and a ~10 kDa polypeptide migrating with the dye front (p12 MoMLV and the nucleocapsid protein p10NC), are visible in the 4 central lanes of the silver stained gel (Figure 16A). This result was confirmed by Western blot analyses using anti-virus antibodies (Figure 16B). A very faint band corresponding to the Gag polyprotein (65 kDa) was detected using a polyclonal antibody raised against wild type MoMLV (Figure 16B). The low levels of this protein detected in the purified preparation indicate that the vast majority of particles produced are mature virions. A ~23 kDa band was also recognized by this polyclonal antibody. The intensity of the viral bands increased continuously from fractions 6 to 9, suggesting that most viral particles banded on the top fraction. The isolation of retrovirus particles in these 4 central fractions of the gradient was consistent as demonstrated by SDS-PAGE in the 11 centrifugation runs (data not shown).

Most serum proteins remained at the top of the centrifugation tube in fractions 10 to 15. Cross-reactivity of the polyclonal antibody with BSA and other serum proteins in the top fractions was observed in this and past experiments (Segura et al., 2005). Furthermore, separation of particles with a higher sedimentation coefficient than the virus in the bottom fractions (1 to 5) was observed. These particles show a protein profile that somewhat differs from the profile of the virus.
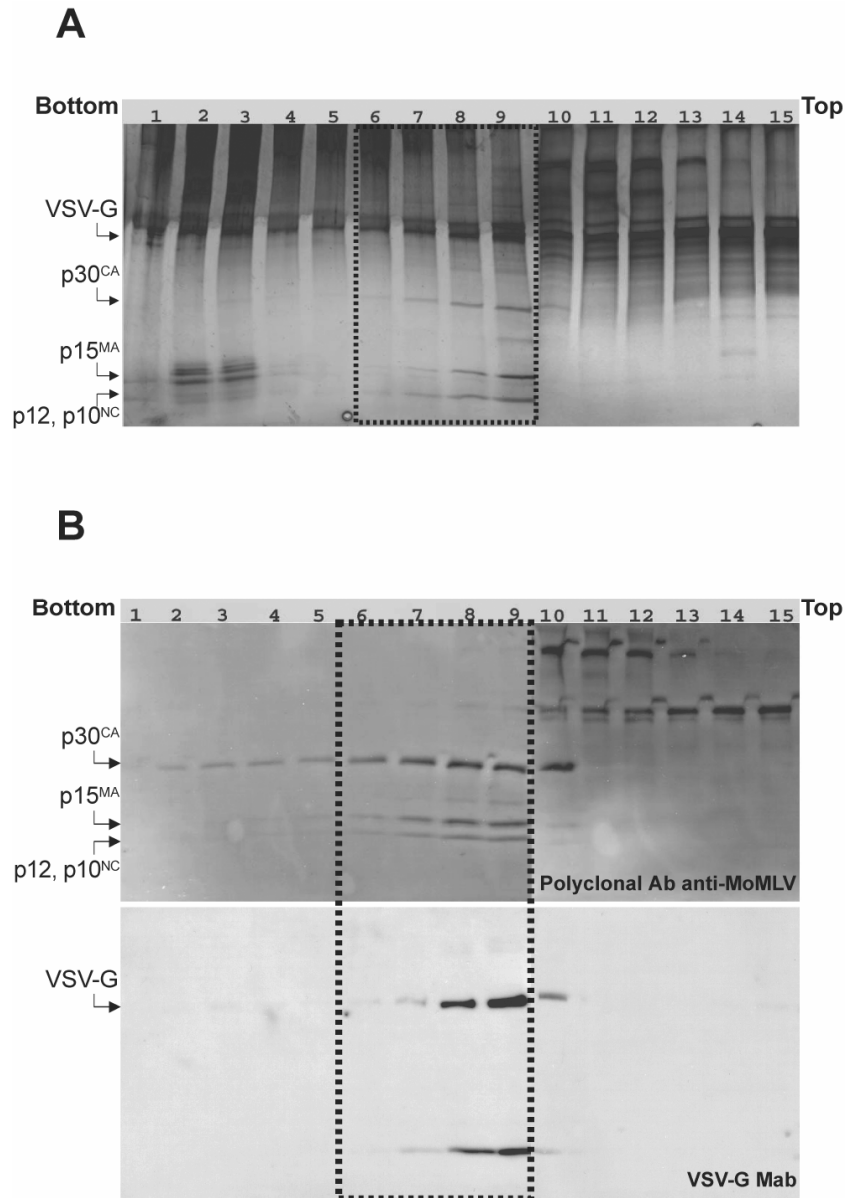
Figure 16 Electrophoretic analyses of iodixanol gradient fractions.
A) SDS-PAGE. Gradient fractions (1 to 15) were analyzed on 4-20% gradient polyacrylamide silver stained gels (Bio-Rad). Fractions 1 to 9 were loaded undiluted (25 uL/lane). The protein concentration in fractions 10-15 was adjusted to load 250 ng of protein per lane. B) Western Blot. Fractions were fractionated as described in Figure 16A and transferred to a Protran® nitrocellulose membrane. Viral proteins bound to the membrane were analyzed by indirect immunostaining using a polyclonal anti-MoMLV antibody or a monoclonal anti-VSV-G antibody. Virus-containing fraction lanes are delimited by solid black squares (■). All major viral proteins were observed in fractions 6 to 9.

3.2 Size-exclusion chromatography

Size-exclusion chromatography, typically used as a polishing step for retrovirus particles (Segura et al., 2005; Transfiguracion et al., 2003), was used in this work as an analytical tool to evaluate the purity of the virus-containing fractions and remove iodixanol. Iodixanol strongly absorbs at 244 nm. Since the virus has a very high molecular weight (~216,000 kDa) it was excluded from the gel pores thereby eluting in the void volume (Vo), while iodixanol molecules were retained inside the gel pores and eluted later. A single virus peak eluting at the expected retention volume (Vo = 23 mL) was observed (Figure 17). Iodixanol molecules (retention volume, Vr = 54 mL) were well separated from the virus peak. Similar chromatographic profiles were obtained for the virus samples treated with Benzonase® and subtilisin prior to chromatography (data not shown). There was no significant difference in the peak height after these enzymatic treatments indicating that the preparations are free of nucleic acid and cell membrane vesicle contamination.

Due to iodixanol interference at 280 nm, purity determination directly from the chromatogram could not be performed. Instead, retrovirus purity in a 10-fold concentrated pool of virus-containing fractions was evaluated by comparing electrophoretic profiles and band intensities before and after size exclusion chromatography (Figure 18). The Gag protein bands are clearly visible by Coomassie blue staining (Figure 18A). This gel shows 2 prominent bands, one corresponding to the capsid protein, p30CA, and another one that contains the remaining Gag proteins in a single non-resolved band (p15MA, p12 and p10NC) migrating with the dye front at the bottom of the gel (Figure 18A). A faint ~68 kDa band corresponding to the VSV-G envelope protein is also visible. These viral-encoded proteins alone account for >90% of the total protein content as assessed by visual inspection. No significant differences in the electrophoretic profile of the virus samples before and after size exclusion chromatography were detected in this gel.

Silver staining revealed the presence of several additional bands in the pool of purified virus fractions (Figure 18A). It should be noted that some protein bands, including the VSV-G band and a ~23 kDa band, were disproportionately more stained by silver than Coomassie blue. The electrophoretic patterns obtained before and after size exclusion

chromatography remained identical using this sensitive staining method. However, the intensity of the VSV-G envelope protein band (68 kDa) is markedly reduced after size exclusion chromatography. A ~135 kDa band (dimer form also recognized by the anti-VSV-G Mab, Figure 18B) was eliminated by size exclusion chromatography as well. The authors have no convincing hypothesis to explain this loss of VSV envelope protein during chromatography. Finally, the intensity of a ~194 kDa band is also reduced after size exclusion chromatography.

A pool of iodixanol-containing fractions eluting from the size exclusion chromatography column was also analyzed by SDS-PAGE. No protein bands were detected in these fractions, with the exception of a single ~30 kDa band, nonreactive to anti-MoMLV antibodies, when Benzonase® treated virus samples were loaded onto the column (data not shown). The authors believe this band correspond to the Benzonase® nuclease used for the treatment since the enzyme consists in two subunits of 30 kDa each.
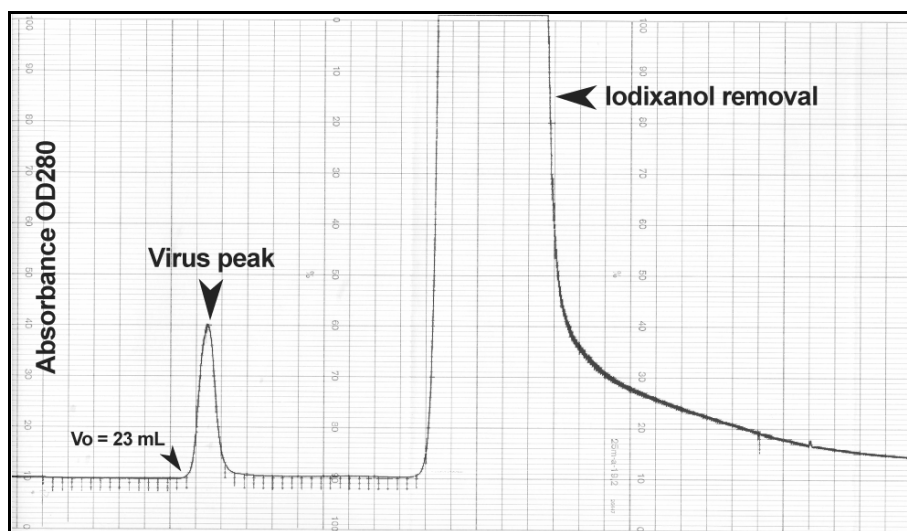
Figure 17 Size-exclusion chromatography elution profile.
Purity determination of rate zonal purified retrovirus. 2.5 mL of a 10-fold concentrated pool of virus-containing fractions were loaded onto a Sepharose CL-4B column (60 mL bed volume). The virus was eluted at 24 cm/h in PBS buffer. Elution was monitored at 280 nm. The virus was recovered in the void volume of the column (Vo) whereas iodixanol molecules eluted later.
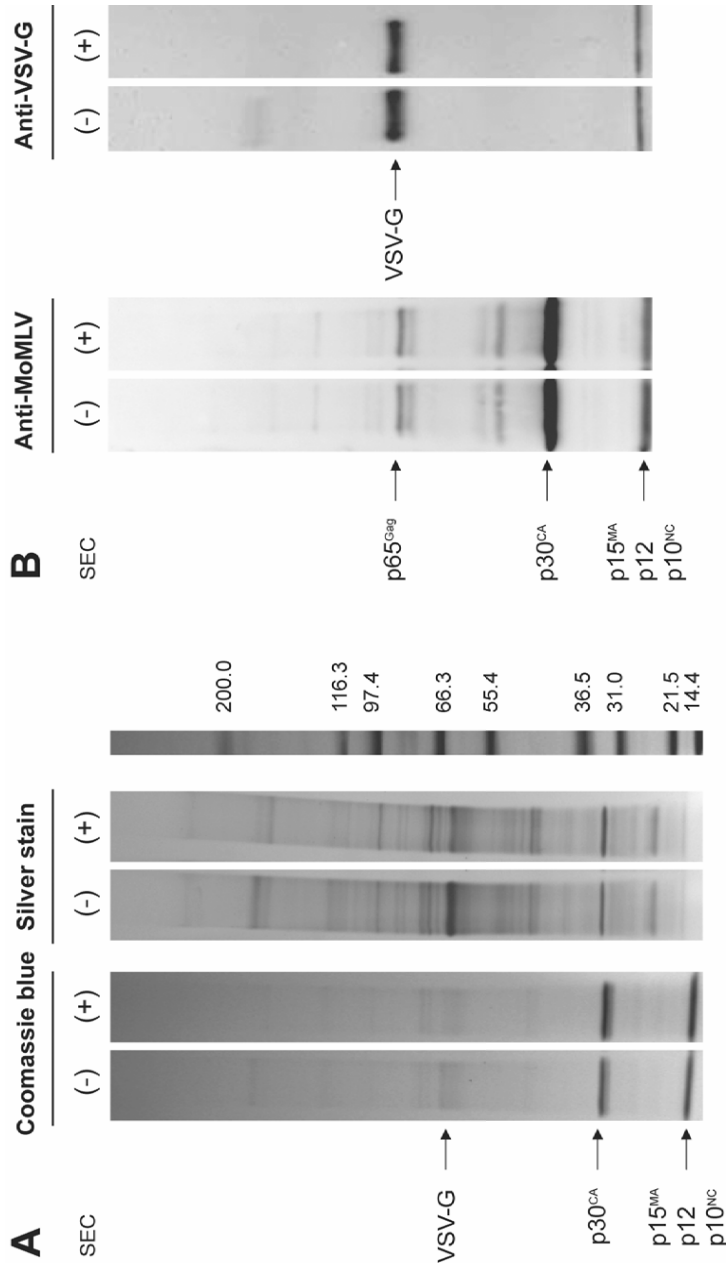
Figure 18 Electrophoretic analyses of size exclusion chromatography purified retrovirus particles.

A) SDS-PAGE. Rate zonal purified samples before (-) and after (+) size exclusion chromatography were analyzed on 4-12% gradient polyacrylamide gels (Invitrogen[TM]) stained with Coomassie brilliant blue R-250 or silver stain. A wide range molecular weight standard lane (Invitrogen[TM]) is shown (MW). B) Western blot. Samples were fractionated as described in Figure 18A and transferred to a Protran® nitrocellulose membrane. Viral proteins bound to the membrane were analyzed by indirect immunostaining using a polyclonal anti-MoMLV antibody or a monoclonal anti-VSV-G antibody. No major differences in the protein profiles before and after size exclusion chromatography were observed demonstrating the high purity of the retrovirus preparation obtained by rate zonal centrifugation.

3.3 Subtilisin treatment analysis

Subtilisin is a serine protease extensively used by HIV-1 researchers to study the location of cellular proteins in retrovirus particles (Ott, 1997; Ott et al., 1995; Ott et al., 1996). This non-specific protease is able to digest all extravirion proteins including external virus proteins, proteins simply adhered to the virus exterior and, interestingly, proteins found in contaminating cell membrane vesicles. Interior virus proteins are protected from digestion by the viral lipid envelope. The loss in protein content after subtilisin treatment is considered an indication of the amount of cell membrane vesicles contaminating a virus preparation. To evaluate the potential contamination of rate zonal purified virions with cell membrane vesicles, a pool of virus-containing fractions was submitted to subtilisin digestion. Protease-treated virions were then separated from the digestion mixture and other protein fragments by size exclusion chromatography and analyzed by SDS-PAGE and Western blot.

Electrophoretic analysis of treated and untreated virus preparations revealed that most proteins are resistant to subtilisin digestion (Figure 19) and thus should be located in the interior of the virions. This demonstrates that most proteins observed in purified preparations are associated with virions and not with contaminating cell membrane vesicles. The complete removal of VSV-G surface protein (Figure 19B) indicates that the enzymatic treatment was efficient. An intensely silver-stained 23 kDa band was also digested by subtilisin (Figure 19A). On the other hand, a ~21 kDa fragment was generated by the protease treatment. This fragment was specifically recognized by the anti-VSV-G Mab and therefore could be a cleavage product of the VSV-G that remains on the viral membrane. Western blot using polyclonal anti-MoMLV antibody provided a further indication of purity, since this antibody, that cross-reacts with BSA and other serum proteins, did not detect any protein susceptible to degradation by subtilisin. The presence of host cell derived proteins in the virions was investigated using a polyclonal antibody against 293 HEK proteins. Three proteins (~32, 50 and 71 kDa) resistant to subtilisin treatment and thus located inside the virions were detected in the purified fractions using this antibody. Very faint bands co-migrating with the 50 and 71 kDa polyproteins are

observed in lane C (HEK 293 control provided by Cygnus technologies). The origin of the 32 kDa band is less clear since although it reacted to the anti-293 HEK host cellular protein antibody, no corresponding band could be visualized in the control lane and the possibility that p30CA cross-reacted with the antibody can not be completely discarded.

Electron microscopy studies

Rate zonal purification efficiency was also monitored by transmission electron microscopy (Figure 20). Low magnification electron micrographs of the starting material ($59100\times$) showed a significant amount of protein aggregates (Figure 20A). Typical type-C retrovirus particles were observed. The background became clearer after rate zonal ultracentrifugation (Figure 20B), thus indicating an efficient removal of contaminating proteins. Numerous roughly spherical particles in a size range of 80-120 nm in diameter were observed in the purified preparation. The particles appeared uniform in shape, except for the presence of some broken particles. Fractions 6 to 9 were further analyzed separately by electron microscopy in an attempt to explain the broad distribution of virus particles along the gradient. No significant difference in size or morphology of the virions in the different gradient fractions was observed (data not shown). Using a higher magnification ($207000\times$), the round condensed core centered in the middle of the particle, characteristic of mature type-C retrovirus particles, was clearly observed (Figure 20C). Retrovirus particles appeared intact following treatment with subtilisin (Figure 20D) demonstrating that the protease digestion eliminates exterior virion proteins without affecting the integrity of the retrovirus particles as previously shown for HIV-1 (Ott et al., 1995).
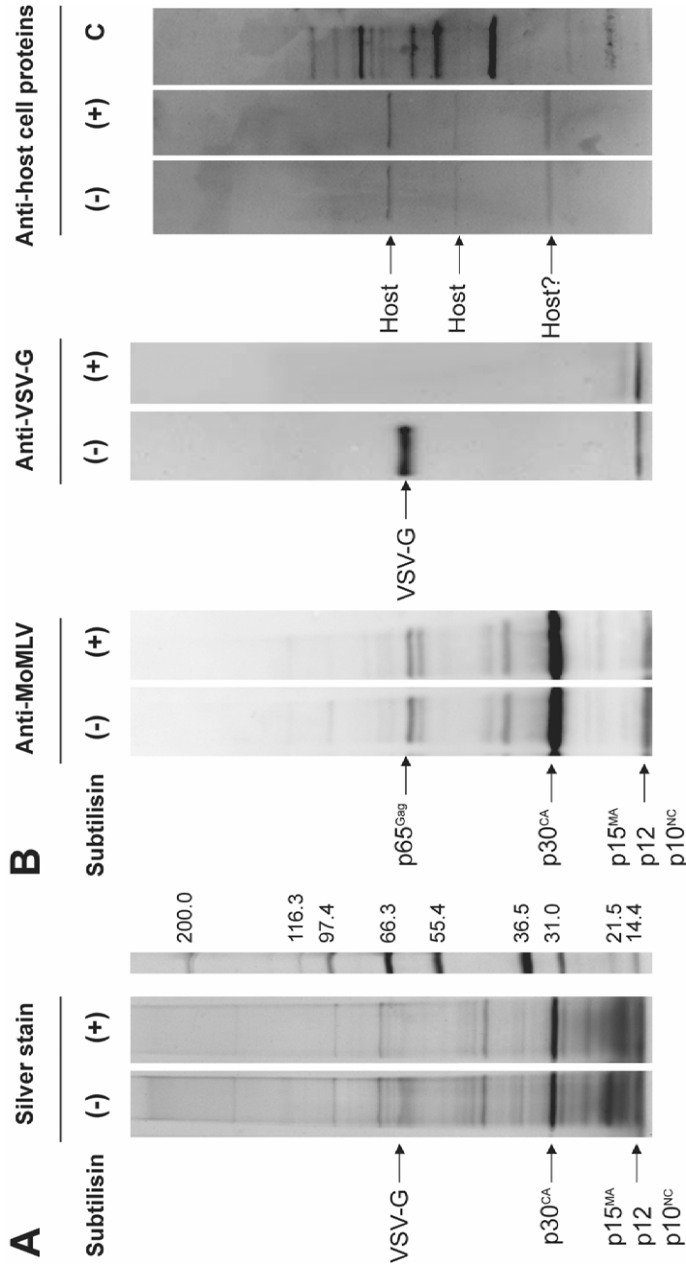
Figure 19 Electrophoretic analyses of subtilisin treated *vs* untreated retrovirus particles.

A) SDS-PAGE. Rate zonal purified virus particles before (–) and after (+) subtilisin treatment were isolated by size exclusion chromatography and analyzed on 4-12% gradient polyacrylamide (Invitrogen[TM]) silver stained gels. A wide range molecular weight standard lane (Invitrogen[TM]) is shown. B) Western Blot. Samples were fractionated as described in Figure 19A and transferred to a Protran® nitrocellulose membrane. Proteins bound to the membrane were analyzed by indirect immunostaining using a polyclonal anti-MoMLV antibody, a monoclonal anti-VSV-G antibody or a polyclonal against 293 HEK host cellular proteins antibody. Lane C corresponds to the HEK 293 control provided by Cygnus Technologies. No significant loss in protein content after subtilisin treatment is observed (except for the VSV-G protein as expected and other faint bands that possibly correspond to exterior virus proteins) indicating that the preparations are free of contaminating cell membrane vesicles. Most proteins found in purified preparation, both virus-encoded and cellular proteins, are resistant to subtilisin digestion and thus are part of the virions. Complete removal of VSV-G was observed after subtilisin digestion demonstrating the efficiency of the treatment.
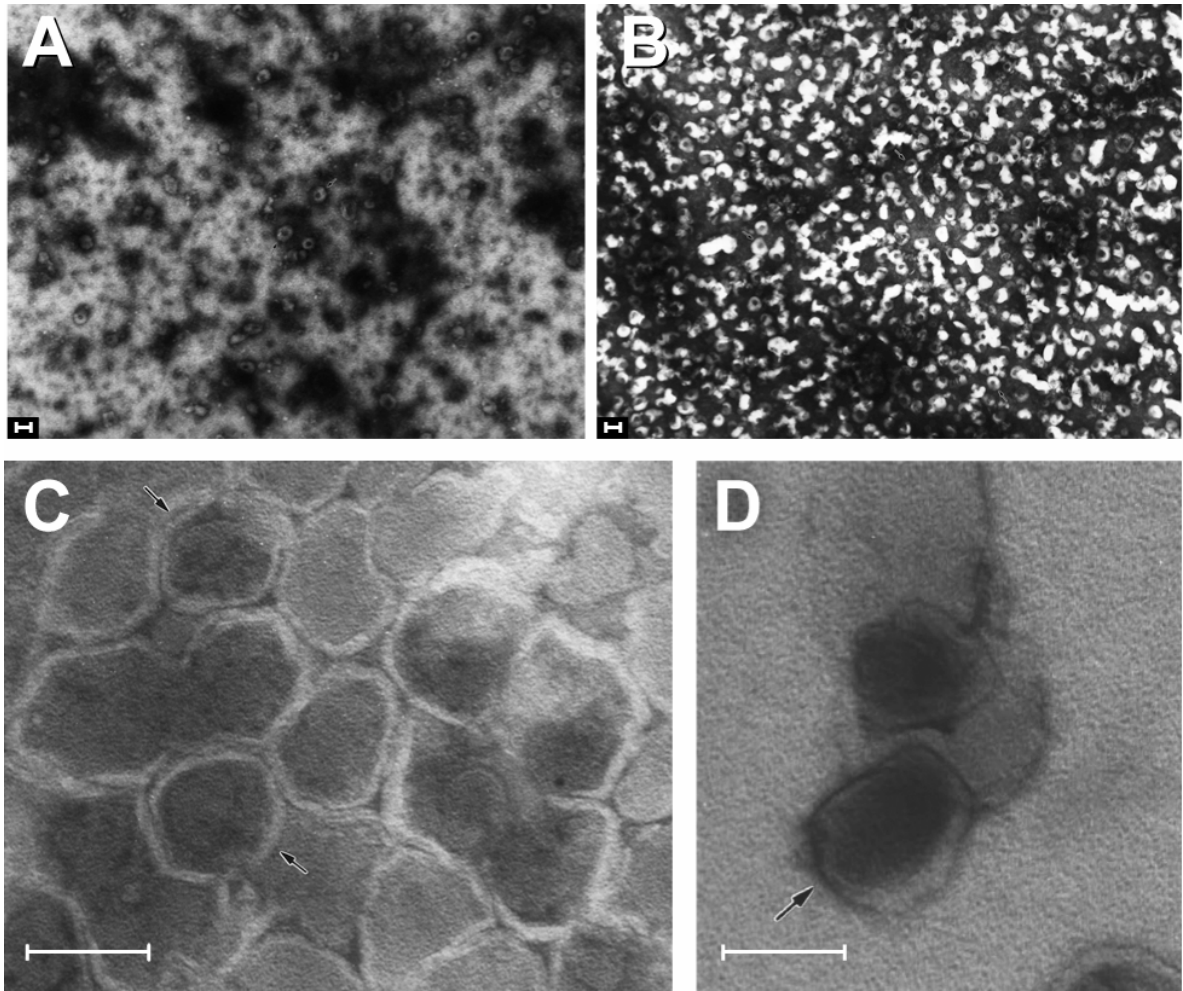
Figure 20 Transmission electron micrographs of negatively stained virions using uranyl acetate staining.

The bars represent 100 nm. (A) The 20-fold concentrated supernatant used as feed for rate zonal ultracentrifugation contained intact retrovirus particles (magnification 59100×). A significant amount of contaminating protein aggregates is visualized as a grey background. (B) Pool of purified rate zonal fractions containing uniformly shaped virus particles, free of protein aggregates (magnification 59100×). (C) Retrovirus particles purified by rate zonal sedimentation at a 207000× magnification. (D) Subtilisin digested retrovirus particles purified by rate zonal sedimentation at a 207000× magnification.

3.5 Separation of defective from functional retrovirus particles

Analysis of the gradient fractions by SDS-PAGE showed that retrovirus particles were distributed over a wide range of densities (from 1.06 to 1.09 g/mL) occupying almost 1/3 of the gradient (Figure 16A). The amount of viral proteins in these fractions increased continuously from fraction 6 to 9. However, the highest amount of infective particles was found in fractions 7 and 8. Curiously, the fraction that showed the highest amount of viral proteins (fraction # 9) also contained the lowest amount of infective viral particles (Figure 16A). This result suggests that incomplete defective viral particles band at lower densities (1.06 g/mL) and thus, can be separated from complete functional particles that move further down through the gradient using this centrifugation method.

To confirm this hypothesis, the distribution of infective viral particles and total virus particles counted by negative stain electron microscopy was compared in the purified gradient fractions. Although fractions in the middle of the gradient presented the highest infective virus titers, negative stain electron microscopy analysis confirmed that the number of total retrovirus particles increased continuously from the bottom (fraction # 6) to the top (fraction # 9) of the ultracentrifugation tube (Figure 21). Most retrovirus particles were isolated in the top fraction 9. However, this fraction showed 1 log less infectivity than the other fractions, reflecting the poor quality of the particles banding at lower densities. In fact, fraction 9 could be excluded from the purified pool without significantly affecting the recovery of infective particles. By doing so, the overall quality of the virus preparation could be improved.
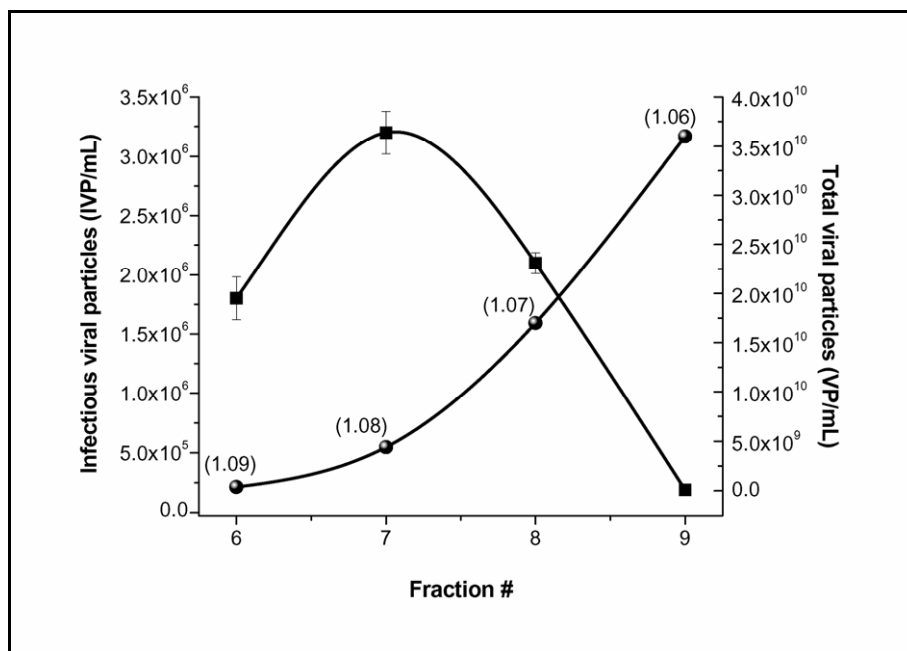
Figure 21 Distribution of infectious virus particles and total virus particles throughout the gradient.
The concentration of total virus particles (VP)(-•-) and infectious virus particles (IVP)(-■-) in fractions 6 to 9 is plotted. Viral titer values shown are the mean ± standard deviation of triplicate samples. The mean density (g/mL) for each fraction is indicated in parenthesis.

6 Discussion

The isolation of MoMLV-derived retrovirus vector particles by rate zonal ultracentrifugation is here described. The high level of purity achieved by this method was demonstrated using a variety of techniques including electrophoretic analysis, size-exclusion chromatography, subtilisin digestion and electron microscopy. Analysis by Coomassie blue stained SDS-PAGE gels is particularly valuable to evaluate the overall composition of the virus particles since Coomassie blue staining intensity is proportional to the amount of protein in each band whereas silver staining presents a high degree of protein-to-protein variability and its intensity is dependent on each polypeptide sequence and degree of glycosylation. Considering the overall composition of the retrovirus particle, analysis of the Coomassie blue stained gel indicates that the level of purity of the retrovirus preparation is greater than 95%. Although several additional minor polypeptide bands could be visualized by silver staining, these proteins were clearly associated with the particles as indicated by size exclusion chromatography and subtilisin analyses. The inability of size exclusion chromatography to further purify the pool of virus-containing fractions strongly suggests that the unidentified polypeptides visualized by electrophoresis are an integral part of the virions. Analysis of subtilisin digestion strengthens this conclusion since this treatment did not result in a significant reduction of the protein content indicating that most of these bands correspond to internal components of the virions. Some of these subtilisin resistant protein bands were identified as host proteins using an anti-HEK 293 cell polyclonal antibody. A few others were recognized by a polyclonal antibody raised against wild type MoMLV and could be incomplete Gag cleavage products and viral enzymes. Finally, NSEM studies provide further evidence that supports the level of purity observed by electrophoretic analysis. Transmission electron micrographs of rate zonal purified fractions showed homogenous fields of similar sized particles free of protein aggregates.

The identification of host cell derived proteins incorporated into the retrovirus envelope has been complicated by the presence of contaminating cell membrane vesicles containing a similar array of proteins on their membrane. HIV-1 researchers have

previously attempted the use of rate zonal sedimentation for the removal of cell membrane vesicles from virus preparations (Dettenhofer and Yu, 1999). In their protocol, viral particles were loaded on top of a 6-18% iodixanol step gradient, centrifuged for 1.5 h at $250,000 \times g$ and recovered in the 4 bottom fractions of the tube. However, the ability of this protocol to separate efficiently vesicles from virions has been questioned (Trubey et al., 2003). The authors believe that recovering the virus from the middle instead of the bottom fractions of the tube could play a role in the efficiency of separation. In fact, the separation of retrovirus vector particles from contaminating particles present in the bottom fractions observed in this work would not have been possible if the virus would have been allowed to reach the bottom of the tube. By setting the separation conditions in order to collect the virus from the middle of the gradient, the authors have shown that retrovirus preparations could be free of any significant cell membrane vesicle contamination. Thus, the virus preparations obtained by rate zonal ultracentrifugation using the described method are suitable for the study of both interior and exterior virus particle proteins.

The recovery of infective particles (37%) is reasonable considering the instability of the retrovirus particles. Moreover, it is comparable to recoveries reported for MoMLV-derived vectors using other purification methods (Kuiper et al., 2002; Transfiguracion et al., 2003; Williams et al., 2005; Ye et al., 2004). The study of the distribution of infective viral particles and total virus particles throughout the gradient is presented here for the first time to show that defective retrovirus particles can be separated from functional particles by centrifugation methods in a similar way to what was shown for other types of wild-type viruses and viral vectors (Laughlin et al., 1979; Ruchti et al., 1991; Toth et al., 1982; Vellekamp et al., 2001; Zhou et al., 1994). Retrovirus vector supernatants were found to be contaminated with a high amount of defective virus particles. Most of these particles remain in the top fraction 9 after centrifugation and their concentration decrease towards the bottom fractions. Since no significant difference in the particle size was found by electron microscopy in the 4 iodixanol purified virus-containing fractions, the authors believe that these particles slightly differ in their densities. A possibility is that the defective particles on the top fraction contain incomplete genomic RNA species while functional particles, containing complete genomic RNA species, would migrate further

down the gradient. Separation of incomplete defective viral vector particles from complete functional viral particles is highly desirable for gene therapy applications since this procedure should facilitate the production of retrovirus vector preparations with optimal purity, potency and safety. Additionally, this procedure could enable the study of the biological and physicochemical properties of different retrovirus populations.

In conclusion, rate zonal centrifugation is a reliable, fast and efficient tool for the purification of retrovirus vector particles. The method renders highly purified preparations with reasonable infectivity recoveries and no evident contamination with cell membrane vesicles. Moreover, it allows for the separation of incomplete defective retrovirus particles. Using this method, the maximum volume of vector stock that can be processed per run using SW28 rotors is 18 mL of 20-fold concentrated supernatant. The concentration step was strategically placed before ultracentrifugation to allow the processing of higher volumes of supernatant per run (360 mL). The authors encourage the use of this method for other types of retrovirus particles including HIV-1 and lentiviral vectors. The technique may be particularly attractive for investigators either attempting to identify host proteins incorporated into the virus membrane or interested in studying differences between defective and functional virus populations. The authors are currently using this technique to obtain highly purified retrovirus vector preparations for the identification of proteins on the virus surface that play a role in the unspecific virus attachment to target cells.

7 Acknowledgements

8 References

Abe, A., Miyanohara, A. and Friedmann, T., 1998. Enhanced gene transfer with fusogenic liposomes containing vesicular stomatitis virus G glycoprotein. J Virol 72, 6159-63.

Alain, R., 1997. Quantitation of virus particles by negative stain electron microscopy. Microscopy Today 97, 20.

Alain, R., Nadon, F., Seguin, C., Payment, P. and Trudel, M., 1987. Rapid virus subunit visualization by direct sedimentation of samples on electron microscope grids. J Virol Methods 16, 209-16.

Bess, J.W., Jr., Gorelick, R.J., Bosche, W.J., Henderson, L.E. and Arthur, L.O., 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology 230, 134-44.

Dettenhofer, M. and Yu, X.F., 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. J Virol 73, 1460-7.

Esser, M.T., Graham, D.R., Coren, L.V., Trubey, C.M., Bess, J.W., Jr., Arthur, L.O., Ott, D.E. and Lifson, J.D., 2001. Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation. J Virol 75, 6173-82.

Gluschankof, P., Mondor, I., Gelderblom, H.R. and Sattentau, Q.J., 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology 230, 125-33.

Hammarstedt, M., Wallengren, K., Pedersen, K.W., Roos, N. and Garoff, H., 2000. Minimal exclusion of plasma membrane proteins during retroviral envelope formation. Proc Natl Acad Sci U S A 97, 7527-32.

Kuiper, M., Sanches, R.M., Walford, J.A. and Slater, N.K., 2002. Purification of a functional gene therapy vector derived from Moloney murine leukaemia virus using membrane filtration and ceramic hydroxyapatite chromatography. Biotechnol Bioeng 80, 445-53.

Laughlin, C.A., Myers, M.W., Risin, D.L. and Carter, B.J., 1979. Defective-interfering particles of the human parvovirus adeno-associated virus. Virology 94, 162-74.

Moller-Larsen, A. and Christensen, T., 1998. Isolation of a retrovirus from multiple sclerosis patients in self-generated Iodixanol gradients. J Virol Methods 73, 151-61.

Ory, D.S., Neugeboren, B.A. and Mulligan, R.C., 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A 93, 11400-6.

Ott, D.E., 1997. Cellular proteins in HIV virions. Rev Med Virol 7, 167-180.

Ott, D.E., 2002. Potential roles of cellular proteins in HIV-1. Rev Med Virol 12, 359-74.

Ott, D.E., Coren, L.V., Johnson, D.G., Sowder, R.C., 2nd, Arthur, L.O. and Henderson, L.E., 1995. Analysis and localization of cyclophilin A found in the virions of human immunodeficiency virus type 1 MN strain. AIDS Res Hum Retroviruses 11, 1003-6.

Ott, D.E., Coren, L.V., Kane, B.P., Busch, L.K., Johnson, D.G., Sowder, R.C., 2nd, Chertova, E.N., Arthur, L.O. and Henderson, L.E., 1996. Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. J Virol 70, 7734-43.

Paquin, A., Jaalouk, D.E. and Galipeau, J., 2001. Retrovector encoding a green fluorescent protein-herpes simplex virus thymidine kinase fusion protein serves as a versatile suicide/reporter for cell and gene therapy applications. Hum Gene Ther 12, 13-23.

Ruchti, F., Siegl, G. and Weitz, M., 1991. Identification and characterization of incomplete hepatitis A virus particles. J Gen Virol 72 (Pt 9), 2159-66.

Segura, M., Kamen, A., Trudel, P. and Garnier, A., 2005. A novel purification strategy for retrovirus gene therapy vectors using heparin affinity chromatography. Biotechnol Bioeng 90, 391-404.

Toth, M., Tarodi, B. and Beladi, I., 1982. Preparative separation of intact incomplete and empty adenovirus type 2 particles. Acta Virol 26, 217-20.

Transfiguracion, J., Jaalouk, D.E., Ghani, K., Galipeau, J. and Kamen, A., 2003. Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications. Hum Gene Ther 14, 1139-53.

Trubey, C.M., Chertova, E., Coren, L.V., Hilburn, J.M., Hixson, C.V., Nagashima, K., Lifson, J.D. and Ott, D.E., 2003. Quantitation of HLA class II protein incorporated into human immunodeficiency type 1 virions purified by anti-CD45 immunoaffinity depletion of microvesicles. J Virol 77, 12699-709.

Vellekamp, G., Porter, F.W., Sutjipto, S., Cutler, C., Bondoc, L., Liu, Y.H., Wylie, D., Cannon-Carlson, S., Tang, J.T., Frei, A., Voloch, M. and Zhuang, S., 2001. Empty capsids in column-purified recombinant adenovirus preparations. Hum Gene Ther 12, 1923-36.

Williams, S.L., Eccleston, M.E. and Slater, N.K., 2005. Affinity capture of a biotinylated retrovirus on macroporous monolithic adsorbents: towards a rapid single-step purification process. Biotechnol Bioeng 89, 783-7.

Ye, K., Jin, S., Ataai, M.M., Schultz, J.S. and Ibeh, J., 2004. Tagging retrovirus vectors with a metal binding peptide and one-step purification by immobilized metal affinity chromatography. J Virol 78, 9820-7.

Zhou, L., Yu, Q. and Luo, M., 1994. Characterization of two density populations of feline calicivirus particles. Virology 205, 530-3.

Chapter IV

Heparin-binding properties of MoMLV-based retrovirus vectors and its implications for gene therapy

María de las Mercedes Segura [1,2], Amine Kamen [2], Marie-Claude Lavoie [1] and Alain Garnier [1,2*]

1- Department of Chemical Engineering, Centre de Recherche sur la Fonction, la Structure et l'Ingénierie des Protéines, Université Laval, Québec, Canada, G1K 7P4; phone : 418-656-3106; fax: 418-656-5993; e-mail: alain.garnier@gch.ulaval.ca

2- Biotechnology Research Institute, NRC, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

1 Résumé

La chromatographie est considérée comme étant la technologie la plus prometteuse pour la purification à grande échelle des vecteurs viraux. Les auteurs ont précédemment démontré que la chromatographie d'affinité sur colonne héparine pouvait être utilisée avec succès pour purifier les vecteurs dérivés du virus Moloney de la leucémie murine (MoMLV) pseudo-typés avec VSV-G, donnant d'excellents résultats en terme de récupération de particules actives, reproductibilité et sélectivité. Dans cette étude, les auteurs ont examiné si la capacité des particules retrovirales de se lier spécifiquement à l'héparine était restreinte au pseudo-type VSV-G produit dans les cellules 293. Il a été démontré que les particules rétrovirales déficientes en VSV-G étaient capturées par chromatographie d'affinité sur colonne héparine aussi efficacement que les particules contenant VSV-G. Également, les particules rétrovirales pseudo-typées avec RD114, dérivées de la lignée cellulaire HT1080, se sont liées à l'héparine avec la même affinité que les pseudo-types VSV-G dérivés des cellules 293, avec des récupérations de particules fonctionnelles de 43%. Ces résultats indiquent que la chromatographie d'affinité sur colonne d'héparine peut être utilisée pour purifier des vecteurs rétroviraux produits par différentes lignées cellulaires indépendamment de la protéine rétrovirale d'enveloppe utilisée pour le pseudo-typage. Les implications de ces découvertes sur le mécanisme d'attachement des vecteurs sur les cellules cibles et le design des vecteurs rétroviraux reciblés pour la thérapie génique sont discutés.

2 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. The implications of these findings on mechanism of vector attachment to target cells and the design of retargeted retroviral vectors for gene therapy are discussed.

3 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 purification of alternative vector pseudotypes. Retroviral vectors are labile enveloped viruses that require the strategic design of gentle purification processes. Chromatography plays an important role in the purification of high value bioproducts since it enables fast, efficient, 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 (Debelak et al., 2000; Kaludov et al., 2002; Zolotukhin et al., 2002; Arcand et al., 2003; Smith et al., 2003; Davidoff et al., 2004; Segura et al., 2005).

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 (McGrath et al., 1978; Slepushkin et al., 2003; Transfiguracion et al., 2003). 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 (Smith and Lee, 1978; Prior et al., 1995; Kuiper et al., 2002; Scherr et al., 2002; Yamada et al., 2003). 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 (Hammarstedt et al., 2000) 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 (Andreadis et al., 1999). 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) (Ye et al., 2004). Additionally, chemically biotinylated retrovirus particles have shown to bind streptavidin coated adsorbents in batch experiments (Williams et al., 2005). 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 (Katane et al., 2002; Palù et al., 2000; Tai et al., 2003).

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) (WuDunn and Spear, 1989; Shieh et al., 1992; Spear et al., 1992), foot and mouth disease virus (FMDV type O) (Jackson et al., 1996; Chen et al., 1997), dengue 2 virus (Chen et al., 1997) and adeno-associated virus (AAV-2) (Summerford and Samulski, 1998). For these viruses, heparin affinity chromatography constitutes a valuable tool for purification and serves to study virus-heparin interactions (Navarro del Cañizo et al., 1996; O'Keeffe et al., 1999; Zolotukhin et al., 1999). 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 (Herold et al., 1991; Chen et al., 1997; Fry et al., 1999; Kern et al., 2003; Opie et al., 2003). 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 (Roderiquez et al., 1995; Mondor et al., 1998; Cladera et al., 2001; Jinno-Oue et al., 2001; Vives et al., 2005).

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 (Segura et al., 2005). 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.

4 Materials and methods

Packaging cell lines and retroviral vectors

Two packaging cell lines that produce Moloney murine leukemia virus (MoMLV) vector particles pseudotyped 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 (Ory et al., 1996), 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) (Paquin et al., 2001). 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 (Cosset et al., 1995) produces GFP3 vector (Qiao et al., 2002). 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. 293 GPG culture medium additionally contained tetracycline (1 µg/mL; Fisher Scientific, Nepean, ON, Canada) to repress the expression of VSV-G gene.

Retroviral vector production

VSV-G pseudotyped vector production was carried out in a 250 mL shake flask (50 mL working volume) inoculated at 2×10^5 293-GPG 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×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×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 ($\sim8\times10^5$ 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^{TM} 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 (Segura et al., 2005). 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 -80ºC.

Infective retroviral vector titer determination

Quantification of infective particles by flow cytometric analysis has been previously reported (Segura et al., 2005). Briefly, 293 (for HT1080-derived vector particles) or 143B (for 293GPG-derived vector particles) target cells were seeded in 6-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 37ºC. 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 determined as previously described (Segura et al., 2005).

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 and collaborators (Pizzato et al., 1999). 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.

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

Equal volumes of retrovirus supernatant were incubated for 30 minutes 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.

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 (Segura et al., 2005). 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.

5 Results

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 (Baba et al., 1988; Schols et al., 1990) and retroviral vector transduction (Le Doux et al., 1996; Le Doux et al., 1999; Jinno-Oue et al., 2001; Guibinga et al., 2002; Walker et al., 2002). 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 (Figure 22). Treatment of MoMLV particles with heparin in concentration higher than 2 U/mL almost completely abolished the virus ability to tranduce target cells with a 96% inhibition of transduction.
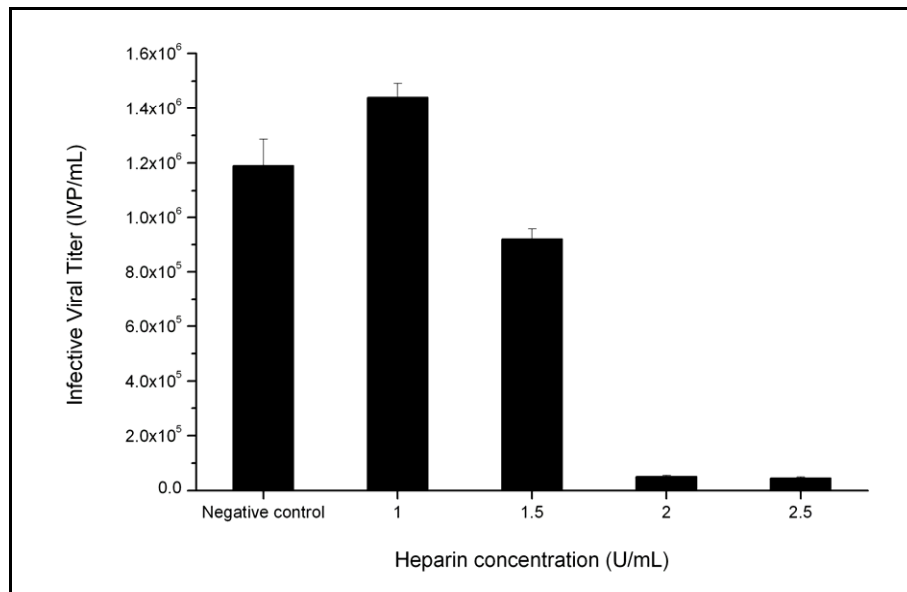
Figure 22 The effect of heparin on VSV-G retrovirus transduction to target cells. Retrovirus supernatants were incubated in the presence of various concentrations of heparin as described in Materials and Methods. A negative control was incubated without heparin. Infectious titers were determined by flow cytometric analysis for GFP expression. Titer values presented are the mean ± standard deviation of triplicate samples. Abbreviations: IVP infective virus particles

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, rather than repulsion (Segura et al., 2005).

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 (Jinno-Oue et al., 2001). 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.

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 (Segura et al., 2005). 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 (Figure 23). Production of either VSV-G containing or deficient particles was performed by taking advantage of the 293 GPG 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 293 GPG 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 (Rabenstein, 2002). 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.
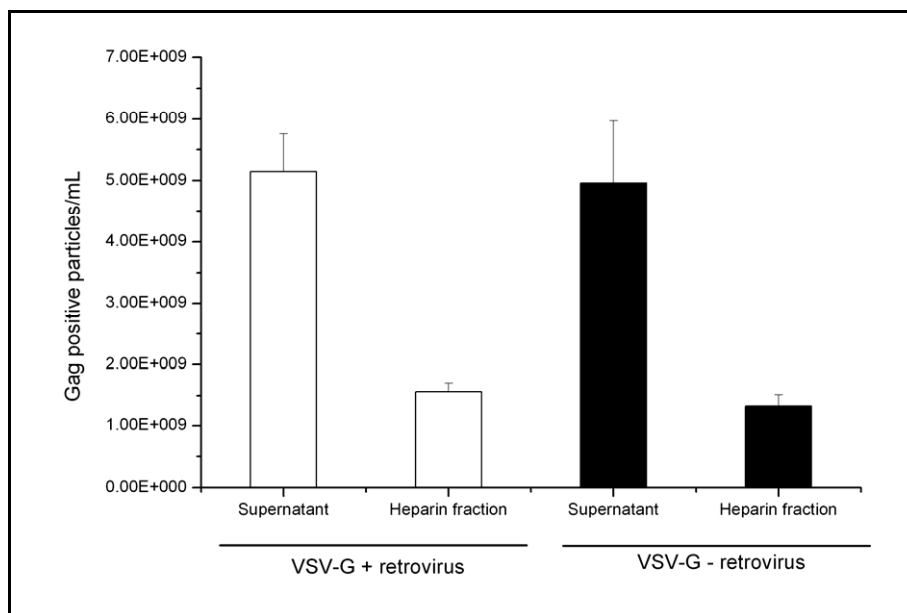
Figure 23 Binding of Env-protein deficient particles to heparin ligands.
Supernatants were produced in parallel with or without the addition of tetracycline generating comparable concentrations of VSV-G deficient retrovirus particles and VSV-G containing particles respectively. Concentrated virus stocks were loaded onto a Fractogel® EMD Heparin (S) column and elution was carried out using a step NaCl gradient as described in Materials and Methods. 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 5 counts.

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 (Merten, 2004). The most common human cell types used for vector production are HEK 293 and HT1080 cells (Merten, 2004). 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. Figure 24 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 (Figure 24). 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.

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 (Segura et al., 2005). Chromatography was performed at room temperature and completed within 1.5 h. The 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) (Figure 25). 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 VII). 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 (Segura et al., 2005).
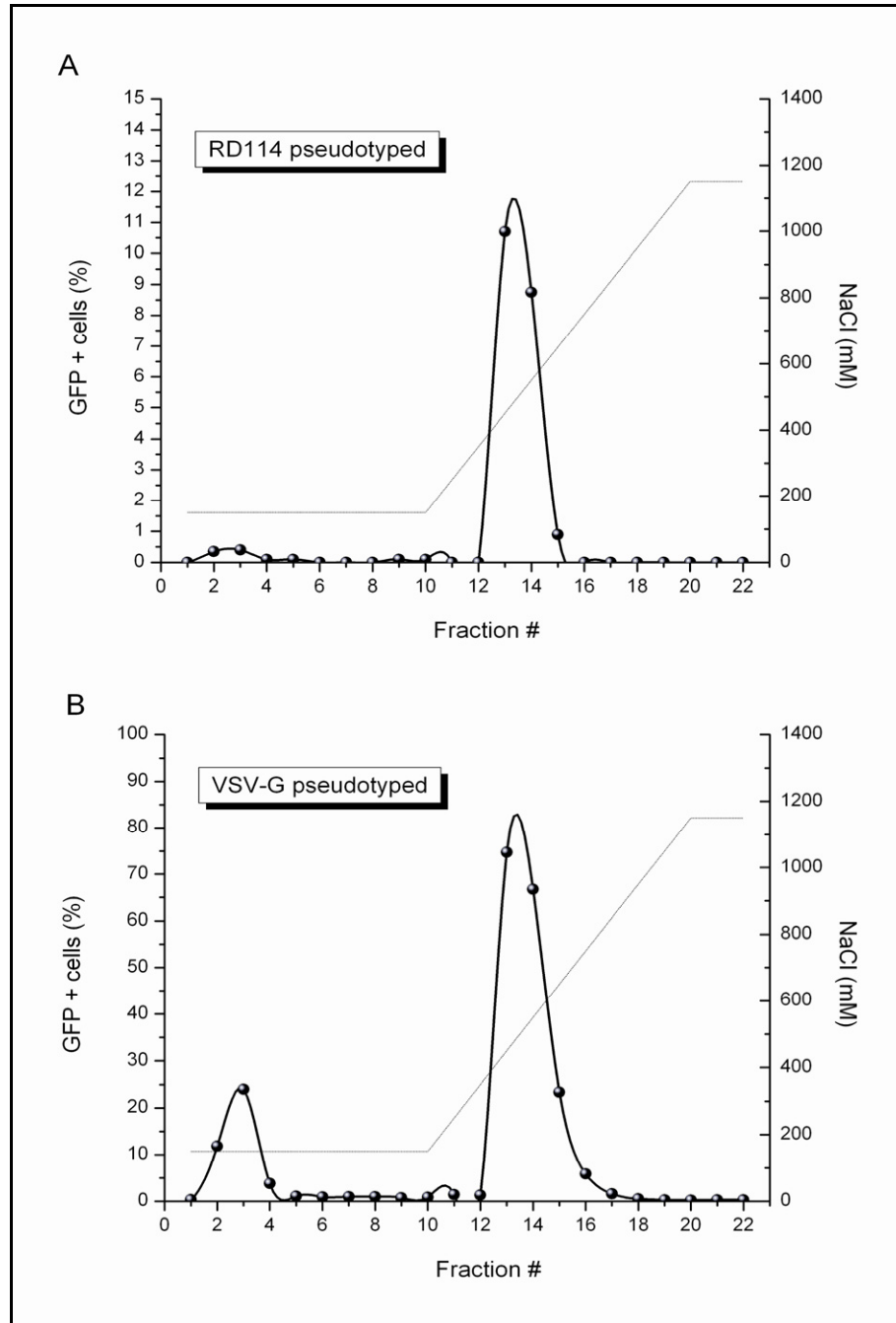
Figure 24 Binding affinity of RD114 (A) and VSV-G (B) pseudotyped retrovirus particles to heparin affinity ligands.

VSV-G and RD114 pseudotyped concentrated virus stocks were loaded onto a Fractogel® EMD Heparin (S) column and elution was carried out using a linear NaCl gradient (150-1150 mM) as described in Materials and Methods. Eluting fractions were collected throughout the run and subjected to flow cytometry analysis for GFP expression. Values presented are the mean ± standard deviation of duplicate samples.
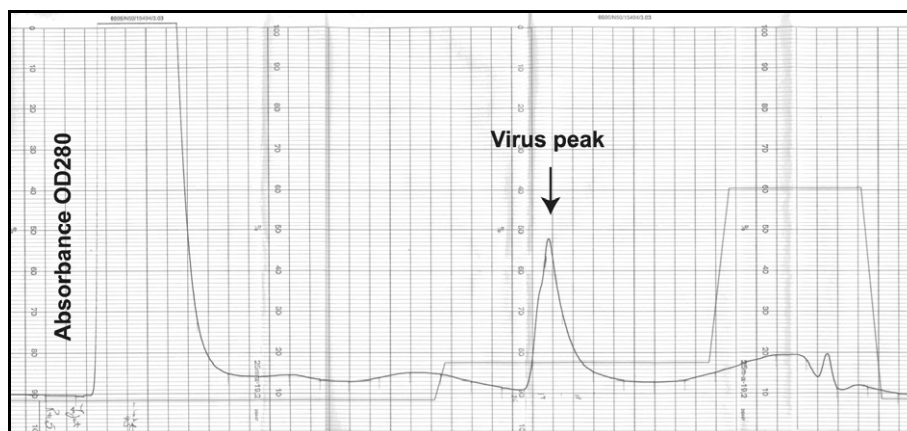
Figure 25 Heparin affinity chromatography step gradient elution profile for RD114 pseudotyped vector.
3 mL of a 20-fold concentrated virus containing $2.2×10^6$ IVP/mL were loaded onto a 1 mL Fractogel® EMD Heparin (S) column. The virus was eluted by addition of 350 mM NaCl into the mobile phase. A similar 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.

Table VII Heparin affinity chromatography recoveries for RD114 pseudotyped vector

Fraction	Volume (mL)	Titer (IVP/mL)			Average titer (IVP/mL)	Average recovery ± sd (%)
		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 developed step-wise elution strategy. Titer values presented for each fraction are the mean of duplicate determinations. Average titer and recovery values for the 3 runs are shown in the table. Abbreviations: IVP infective virus particles; sd standard deviation.

6 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 contrary to what was reported for most viruses that interact with heparin, the viral Env-protein (VSV-G in our case) does not appear to be involved in retrovirus-heparin interactions. Instead, most likely unidentified cellular component(s) on the virus surface play a role for the observed heparin-binding activity. 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.

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 (Kaludov et al., 2002; Zolotukhin et al., 2002; Zolotukhin, 2005). 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 (Aboud et al., 1982; Andreadis et al., 1999; Segura et al., 2005). 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 the poorer

stability of RD114 Env-protein or could result from suboptimal vector titers in the starting material. 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 (Kuiper et al., 2002; Ye et al., 2004; Segura et al., 2005; Williams et al., 2005).

The reported heparin-binding activity of MoMLV-based retroviral vectors also has important implications in the mechanism of virus attachment to target cells. This initial step in the virus life cycle is generally believed to be mediated solely by the viral Env-proteins which upon specific, high-affinity binding with their corresponding receptors trigger fusion of the viral and cellular membrane allowing virus entry to susceptible cells (Figure 26). However, an increasing body of evidence suggests that the mechanisms of retrovirus attachment and entry to target cells are distinct (Sharma et al., 2000; Pizzato et al., 2001). Most strikingly, Env-protein deficient retrovirus particles were found to attach to target cells as efficiently as complete virus particles (Pizzato et al., 1999; Guibinga et al., 2002). These observations suggested the presence of other molecules on the virus surface other than the viral Env-protein capable of interacting with the cells. Therefore, it is currently believed that initial attachment would involve Env-protein independent interactions between the virus and yet unidentified co-receptors at the cell surface (Davis et al., 2002; Walker et al., 2002).

Many host-derived membrane proteins were identified on the well-characterized HIV-1. Some of these proteins were proposed to enhance virus attachment to target cells either via interactions between adhesion molecules (LFA-1 and ICAM-1) (Orentas and Hildreth, 1993; Fortin et al., 1997; Fortin et al., 1998; Paquette et al., 1998; Liao et al., 2000) or with heparan sulfate moieties on the cell surface (Cyclophilin A) (Saphire et al., 1999). As for HIV-1, it seems possible that MoMLV could incorporate host-derived components on its membrane that play a role in virus attachment to target cells (Figure 26).

For instance, the incorporation of heparin-binding proteins may enable virus binding to heparin-like structures on the cell surface. Unlike heparin, which only occurs in mast cells, its structural analog heparan sulfate proteoglycan is expressed and secreted by most mammalian cells and is ubiquitously distributed on cell surfaces and in the extracellular matrix (Rabenstein, 2002). Guibinga and collaborators (2002) have previously proposed that heparan sulfate proteoglycan may be used by retrovirus particles as a receptor for attachment. Our findings strongly support this hypothesis. Another possibility is that secreted heparan sulfate molecules may serve as a molecular bridge between the heparin-binding domains on the virus and heparin-binding structures at the cell surface as previously reported for F-MuLV (Jinno-Oue et al., 2001). As for this virus, a concentration dependent dual effect of soluble heparin on the transduction efficiency of MoMLV-derived vectors was observed in this work suggesting that a similar mechanism might be taking place. For instance, an indirect interaction between the virus and fibronectin, a widely distributed multiadhesive matrix glycoprotein that coats the cell membrane, is possible by using heparan sulfate molecules contained in the producer cell line supernatant or present in the extracellular matrix as a molecular bridge. In support of this theory, fibronectin fragments have been shown to improve retrovirus gene transfer efficiency and are frequently used in *ex vivo* human gene therapy protocols (Hanenberg et al., 1996; Moritz et al., 1996; Hacein-Bey et al., 2001). Moreover, studies have shown that specific heparin-binding domains (HepII) within the fibronectin molecule are required to attain this enhancing effect on transduction (Hanenberg et al., 1996; Carstanjen et al., 2001; Lei et al., 2002). Finally, we do not exclude the possibility that other yet unidentified adhesion molecules may be present on the MoMLV virion membrane increasing the avidity of attachment by allowing direct interaction between the virus and adhesion proteins on the cell surface (Figure 26).

From the downstream processing point of view affinity of retroviruses to heparin is convenient since it allows for efficient purification of the vectors by heparin affinity chromatography. However, the ability of retroviruses to bind heparin and consequently heparin-related structures (i.e. heparan sulfate) at physiological pH and ionic strength as described herein may complicate the design of targeted retroviral vectors. Efforts for

targeting retroviral vectors to specific cell types have been centered on attempts to engineer the MoMLV Env-protein; however, little success has been attained (Pizzato et al., 1999; Russell and Cosset, 1999; Pizzato et al., 2001; Katane et al., 2002; Tai et al., 2003). Although the Env-protein is the primary determinant of the virus host range since it allows virus-cell membrane fusion and entry to target cells, it does not dictate virus biodistribution *in vivo*. Following systemic injection, virus particles showing heparin-binding activity may bind to the first population of cells encountered being wasted in non-relevant body compartments (Pizzato et al., 1999). Thus, virus particles with such broad adhesion properties may not be well suited to target specific cell types *in vivo*. A way to overcome this limitation would be to identify cell adhesion component(s) such as heparin-binding molecules on the viral membrane and remove them by genetic engineering techniques (Pizzato et al., 2001). However, the removal of heparin-binding components will be associated with the impossibility of using heparin affinity chromatography for retrovirus purification and may be associated with poor transduction efficiency. Ultimately, advantages and disadvantages related to producing vectors' that lack heparin-binding activity should be weight in a case-by-case analysis considering each particular application. In this line of thoughts, we are currently working towards the identification of host-derived cell adhesion component(s) on the virus membrane.

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. These results demonstrate that it is possible to have a common 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 under physiological conditions may have important implications in the mechanism of virus attachment to target cells and the design of targeted retrovirus vectors.
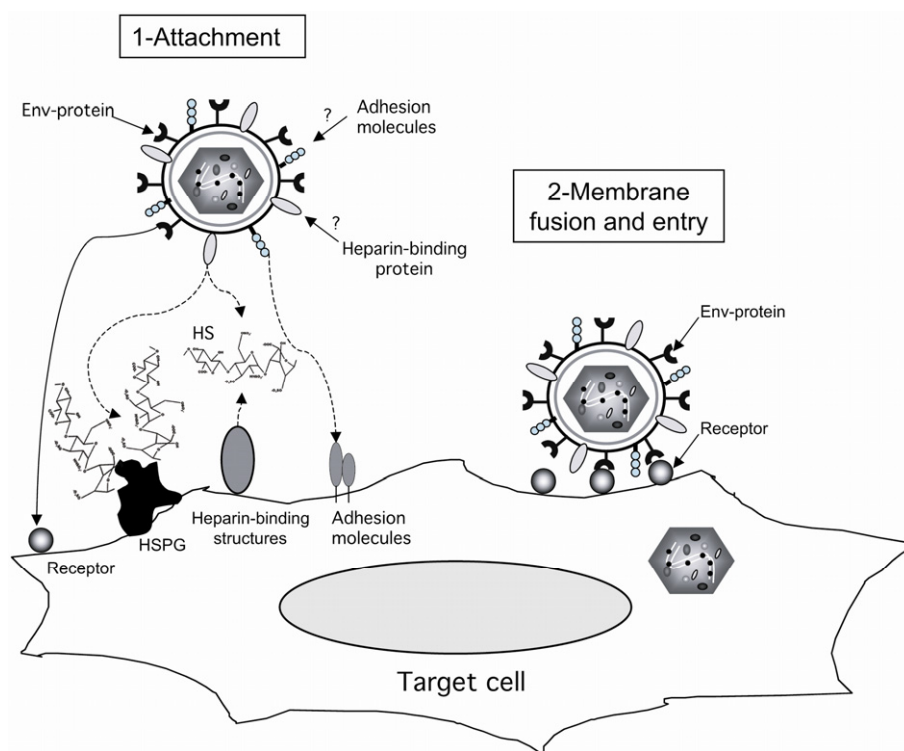
Figure 26 Potential attachment mechanisms for MoMLV-derived vectors.
The Env-protein-receptor interaction is considered the main mechanism of MoMLV vector attachment and entry to target cells. More recently, several studies suggested that at early steps of retrovirus transduction, initial virus adsorption via unidentified co-receptors at the cell surface may occur independently of the Env-protein and viral receptors. The incorporation of host-derived heparin-binding components and/or cell adhesion molecules on the virus surface would allow virus particles to use cell-cell and/or cell-matrix interaction mechanisms for attachment to target cells prior to membrane fusion and entry. Abbreviations: HSPG heparan sulfate proteoglycan; HS heparin sulfate chains.

7 Acknowledgements

8 References

Aboud, M., Wolfson, M., Hassan, Y. and Huleihel, M., 1982. Rapid purification of extracellular and intracellular Moloney murine leukemia virus. Arch Virol 71, 185-95.

Andreadis, S.T., Roth, C.M., Le Doux, J.M., Morgan, J.R. and Yarmush, M.L., 1999. Large-scale processing of recombinant retroviruses for gene therapy. Biotechnol Prog 15, 1-11.

Arcand, N., Bernier, A., Transfiguracion, J., Jacob, D., Coelho, H. and Kamen, A., 2003. Adenovirus type 5 (Ad5) chromatographic purification process at the 20L scale. Bioprocessing J 2, 72-75.

Baba, M., Snoeck, R., Pauwels, R. and de Clercq, E., 1988. Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human immunodeficiency virus. Antimicrob Agents Chemother 32, 1742-5.

Carstanjen, D., Dutt, P. and Moritz, T., 2001. Heparin inhibits retrovirus binding to fibronectin as well as retrovirus gene transfer on fibronectin fragments. J Virol 75, 6218-22.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J. and Marks, R.M., 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med 3, 866-71.

Cladera, J., Martin, I. and O'Shea, P., 2001. The fusion domain of HIV gp41 interacts specifically with heparan sulfate on the T-lymphocyte cell surface. Embo J 20, 19-26.

Cosset, F.L., Takeuchi, Y., Battini, J.L., Weiss, R.A. and Collins, M.K., 1995. High-titer packaging cells producing recombinant retroviruses resistant to human serum. J Virol 69, 7430-6.

Davidoff, A.M., Ng, C.Y., Sleep, S., Gray, J., Azam, S., Zhao, Y., McIntosh, J.H., Karimipoor, M. and Nathwani, A.C., 2004. Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock. J Virol Methods 121, 209-15.

Davis, H.E., Morgan, J.R. and Yarmush, M.L., 2002. Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophys Chem 97, 159-72.

Debelak, D., Fisher, J., Iuliano, S., Sesholtz, D., Sloane, D.L. and Atkinson, E.M., 2000. Cation-exchange high-performance liquid chromatography of recombinant adeno-associated virus type 2. J Chromatogr B Biomed Sci Appl 740, 195-202.

Fortin, J.F., Cantin, R., Lamontagne, G. and Tremblay, M., 1997. Host-derived ICAM-1 glycoproteins incorporated on human immunodeficiency virus type 1 are biologically active and enhance viral infectivity. J Virol 71, 3588-96.

Fortin, J.F., Cantin, R. and Tremblay, M.J., 1998. T cells expressing activated LFA-1 are more susceptible to infection with human immunodeficiency virus type 1 particles bearing host-encoded ICAM-1. J Virol 72, 2105-12.

Fry, E.E., Lea, S.M., Jackson, T., Newman, J.W., Ellard, F.M., Blakemore, W.E., Abu-Ghazaleh, R., Samuel, A., King, A.M. and Stuart, D.I., 1999. The structure and function of a foot-and-mouth disease virus-oligosaccharide receptor complex. Embo J 18, 543-54.

Guibinga, G.H., Miyanohara, A., Esko, J.D. and Friedmann, T., 2002. Cell surface heparan sulfate is a receptor for attachment of envelope protein-free retrovirus-like particles and VSV-G pseudotyped MLV-derived retrovirus vectors to target cells. Mol Ther 5, 538-46.

Hacein-Bey, S., Gross, F., Nusbaum, P., Hue, C., Hamel, Y., Fischer, A. and Cavazzana-Calvo, M., 2001. Optimization of retroviral gene transfer protocol to maintain the lymphoid potential of progenitor cells. Hum Gene Ther 12, 291-301.

Hammarstedt, M., Wallengren, K., Pedersen, K.W., Roos, N. and Garoff, H., 2000. Minimal exclusion of plasma membrane proteins during retroviral envelope formation. Proc Natl Acad Sci U S A 97, 7527-32.

Hanenberg, H., Xiao, X.L., Dilloo, D., Hashino, K., Kato, I. and Williams, D.A., 1996. Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. Nat Med 2, 876-82.

Herold, B.C., WuDunn, D., Soltys, N. and Spear, P.G., 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol 65, 1090-8.

Jackson, T., Ellard, F.M., Ghazaleh, R.A., Brookes, S.M., Blakemore, W.E., Corteyn, A.H., Stuart, D.I., Newman, J.W. and King, A.M., 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparan sulfate. J Virol 70, 5282-7.

Jinno-Oue, A., Oue, M. and Ruscetti, S.K., 2001. A unique heparin-binding domain in the envelope protein of the neuropathogenic PVC-211 murine leukemia virus may contribute to its brain capillary endothelial cell tropism. J Virol 75, 12439-45.

Kaludov, N., Handelman, B. and Chiorini, J.A., 2002. Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. Hum Gene Ther 13, 1235-43.

Katane, M., Takao, E., Kubo, Y., Fujita, R. and Amanuma, H., 2002. Factors affecting the direct targeting of murine leukemia virus vectors containing peptide ligands in the envelope protein. EMBO Rep 3, 899-904.

Kern, A., Schmidt, K., Leder, C., Muller, O.J., Wobus, C.E., Bettinger, K., Von der Lieth, C.W., King, J.A. and Kleinschmidt, J.A., 2003. Identification of a heparin-binding motif on adeno-associated virus type 2 capsids. J Virol 77, 11072-81.

Kuiper, M., Sanches, R.M., Walford, J.A. and Slater, N.K., 2002. Purification of a functional gene therapy vector derived from Moloney murine leukaemia virus using membrane filtration and ceramic hydroxyapatite chromatography. Biotechnol Bioeng 80, 445-53.

Le Doux, J.M., Morgan, J.R., Snow, R.G. and Yarmush, M.L., 1996. Proteoglycans secreted by packaging cell lines inhibit retrovirus infection. J Virol 70, 6468-73.

Le Doux, J.M., Morgan, J.R. and Yarmush, M.L., 1999. Differential inhibition of retrovirus transduction by proteoglycans and free glycosaminoglycans. Biotechnol Prog 15, 397-406.

Lei, P., Bajaj, B. and Andreadis, S.T., 2002. Retrovirus-associated heparan sulfate mediates immobilization and gene transfer on recombinant fibronectin. J Virol 76, 8722-8.

Liao, Z., Roos, J.W. and Hildreth, J.E., 2000. Increased infectivity of HIV type 1 particles bound to cell surface and solid-phase ICAM-1 and VCAM-1 through acquired adhesion molecules LFA-1 and VLA-4. AIDS Res Hum Retroviruses 16, 355-66.

McGrath, M., Witte, O., Pincus, T. and Weissman, I.L., 1978. Retrovirus purification: method that conserves envelope glycoprotein and maximizes infectivity. J Virol 25, 923-7.

Mondor, I., Ugolini, S. and Sattentau, Q.J., 1998. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. J Virol 72, 3623-34.

Moritz, T., Dutt, P., Xiao, X., Carstanjen, D., Vik, T., Hanenberg, H. and Williams, D.A., 1996. Fibronectin improves transduction of reconstituting hematopoietic stem cells by retroviral vectors: evidence of direct viral binding to chymotryptic carboxy-terminal fragments. Blood 88, 855-62.

Navarro del Cañizo, A.A., Mazza, M., Bellinzoni, R. and Cascone, O., 1996. Foot and mouth disease virus concentration and purification by affinity chromatography. Appl Biochem Biotechnol 61, 399-409.

O'Keeffe, R.S., Johnston, M.D. and Slater, N.K., 1999. The affinity adsorptive recovery of an infectious herpes simplex virus vaccine. Biotechnol Bioeng 62, 537-45.

Opie, S.R., Warrington, K.H., Jr., Agbandje-McKenna, M., Zolotukhin, S. and Muzyczka, N., 2003. Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. J Virol 77, 6995-7006.

Orentas, R.J. and Hildreth, J.E., 1993. Association of host cell surface adhesion receptors and other membrane proteins with HIV and SIV. AIDS Res Hum Retroviruses 9, 1157-65.

Ory, D.S., Neugeboren, B.A. and Mulligan, R.C., 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. Proc Natl Acad Sci U S A 93, 11400-6.

Palù, G., Parolin, C., Takeuchi, Y. and Pizzato, M., 2000. Progress with retroviral gene vectors. Rev Med Virol 10, 185-202.

Paquette, J.S., Fortin, J.F., Blanchard, L. and Tremblay, M.J., 1998. Level of ICAM-1 surface expression on virus producer cells influences both the amount of virion-bound host ICAM-1 and human immunodeficiency virus type 1 infectivity. J Virol 72, 9329-36.

Paquin, A., Jaalouk, D.E. and Galipeau, J., 2001. Retrovector encoding a green fluorescent protein-herpes simplex virus thymidine kinase fusion protein serves as a versatile suicide/reporter for cell and gene therapy applications. Hum Gene Ther 12, 13-23.

Pizzato, M., Blair, E.D., Fling, M., Kopf, J., Tomassetti, A., Weiss, R.A. and Takeuchi, Y., 2001. Evidence for nonspecific adsorption of targeted retrovirus vector particles to cells. Gene Ther 8, 1088-96.

Pizzato, M., Marlow, S.A., Blair, E.D. and Takeuchi, Y., 1999. Initial binding of murine leukemia virus particles to cells does not require specific Env-receptor interaction. J Virol 73, 8599-611.

Prior, C., Bay, P., Ebert, B., Gore, R., Holt, J., Irish, T., Jensen, F., Leone, C., Mitschelen, J., Stiglitz, M., Tarr, C., Trauger, R.J., Weber, D. and Hrinda, M., 1995. Process development for the manufacture of inactivated HIV-1. BioPharmacology 8, 25-35.

Qiao, J., Roy, V., Girard, M.H. and Caruso, M., 2002. High translation efficiency is mediated by the encephalomyocarditis virus internal ribosomal entry sites if the natural sequence surrounding the eleventh AUG is retained. Hum Gene Ther 13, 881-7.

Rabenstein, D.L., 2002. Heparin and heparan sulfate: structure and function. Nat Prod Rep 19, 312-31.

Roderiquez, G., Oravecz, T., Yanagishita, M., Bou-Habib, D.C., Mostowski, H. and Norcross, M.A., 1995. Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120-gp41. J Virol 69, 2233-9.

Russell, S.J. and Cosset, F.L., 1999. Modifying the host range properties of retroviral vectors. J Gene Med 1, 300-11.

Saphire, A.C., Bobardt, M.D. and Gallay, P.A., 1999. Host cyclophilin A mediates HIV-1 attachment to target cells via heparans. Embo J 18, 6771-85.

Scherr, M., Battmer, K., Eder, M., Schule, S., Hohenberg, H., Ganser, A., Grez, M. and Blomer, U., 2002. Efficient gene transfer into the CNS by lentiviral vectors purified by anion exchange chromatography. Gene Ther 9, 1708-14.

Schols, D., De Clercq, E., Balzarini, J., Baba, M., Witvrouw, M., Hosoya, M., Andrei, G., Snoeck, R., Neyts, J., Pauwels, R., Nagy, M., Gyorgyi-Edelenyi, J., Machovich, R., Horvath, I., Low, M. and Gorog, S., 1990. Sulphated polymers are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, respiratory syncytial virus, and toga-, arena- and retroviruses. Antivir Chem Chemother 1, 233–240.

Segura, M., Kamen, A., Trudel, P. and Garnier, A., 2005. A novel purification strategy for retrovirus gene therapy vectors using heparin affinity chromatography. Biotechnol Bioeng 90, 391-404.

Sharma, S., Miyanohara, A. and Friedmann, T., 2000. Separable mechanisms of attachment and cell uptake during retrovirus infection. J Virol 74, 10790-5.

Shieh, M.T., WuDunn, D., Montgomery, R.I., Esko, J.D. and Spear, P.G., 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol 116, 1273-81.

Slepushkin, V., Chang, N., Cohen, R., Gan, Y., Jiang, B., Deausen, E., Berlinger, D., Binder, G., Andre, K., Humeau, L. and Drupolic, B., 2003. Large-scale purification of a lentiviral vector by size exclusion chromatography or mustang Q ion exchange capsule. Bioprocessing J 2, 89-95.

Smith, R.G. and Lee, S.A., 1978. Large-scale isolation and partial purification of type C RNA viruses on hydroxyapatite. 1. Biochemical characterization. Anal Biochem 86, 252-63.

Smith, R.H., Ding, C. and Kotin, R.M., 2003. Serum-free production and column purification of adeno-associated virus type 5. J Virol Methods 114, 115-24.

Spear, P.G., Shieh, M.T., Herold, B.C., WuDunn, D. and Koshy, T.I., 1992. Heparan sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex virus. Adv Exp Med Biol 313, 341-53.

Summerford, C. and Samulski, R.J., 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol 72, 1438-45.

Tai, C.K., Logg, C.R., Park, J.M., Anderson, W.F., Press, M.F. and Kasahara, N., 2003. Antibody-mediated targeting of replication-competent retroviral vectors. Hum Gene Ther 14, 789-802.

Transfiguracion, J., Jaalouk, D.E., Ghani, K., Galipeau, J. and Kamen, A., 2003. Size-exclusion chromatography purification of high-titer vesicular stomatitis virus G glycoprotein-pseudotyped retrovectors for cell and gene therapy applications. Hum Gene Ther 14, 1139-53.

Vives, R.R., Imberty, A., Sattentau, Q.J. and Lortat-Jacob, H., 2005. Heparan sulfate targets the HIV-1 envelope glycoprotein gp120 coreceptor binding site. J Biol Chem 280, 21353-7.

Walker, S.J., Pizzato, M., Takeuchi, Y. and Devereux, S., 2002. Heparin binds to murine leukemia virus and inhibits Env-independent attachment and infection. J Virol 76, 6909-18.

Williams, S.L., Nesbeth, D., Darling, D.C., Farzaneh, F. and Slater, N.K., 2005. Affinity recovery of Moloney Murine Leukaemia Virus. J Chromatogr B Analyt Technol Biomed Life Sci 820, 111-9.

WuDunn, D. and Spear, P.G., 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol 63, 52-8.

Yamada, K., McCarty, D.M., Madden, V.J. and Walsh, C.E., 2003. Lentivirus vector purification using anion exchange HPLC leads to improved gene transfer. Biotechniques 34, 1074-8, 1080.

Ye, K., Jin, S., Ataai, M.M., Schultz, J.S. and Ibeh, J., 2004. Tagging retrovirus vectors with a metal binding peptide and one-step purification by immobilized metal affinity chromatography. J Virol 78, 9820-7.

Zolotukhin, S., 2005. Production of recombinant adeno-associated virus vectors. Hum Gene Ther 16, 551-7.

Zolotukhin, S., Byrne, B.J., Mason, E., Zolotukhin, I., Potter, M., Chesnut, K., Summerford, C., Samulski, R.J. and Muzyczka, N., 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6, 973-85.

Zolotukhin, S., Potter, M., Zolotukhin, I., Sakai, Y., Loiler, S., Fraites, T.J., Jr., Chiodo, V.A., Phillipsberg, T., Muzyczka, N., Hauswirth, W.W., Flotte, T.R., Byrne, B.J. and Snyder, R.O., 2002. Production and purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. Methods 28, 158-6.

Conclusion and perspective

Human gene therapy is a revolutionary approach to medicine that holds great promise for the cure of many devastating diseases that presently have no therapies. But gene therapy, like every other major innovative technology, needs time to develop and mature. Retroviral vectors are valuable tools for gene transfer technology and have shown great potential as vectors for gene therapy. Despite the progress made in the field of retroviral gene therapy in the past 20 years (Sinn et al., 2005) and despite the numerous positive results obtained in pre-clinical and clinical trials (Aiuti et al., 2002; Cavazzana-Calvo et al., 2000), further investigation will be required before retrovirus vectors can be used efficiently and safely in a clinic setting. Further advancements in vector design are required to improve the efficiency and specificity of gene delivery and expression for *in vivo* applications. To support these efforts, a better understanding of retrovirus-cell interactions is essential for the coherent design of engineered targeted vectors. In addition, a thorough evaluation of the vectors' integration site preferences and the risks for insertional mutagenesis is needed. These studies should be coupled with the development of strategies to direct vector integration to specific sites in the genome for increased biosafety. Finally, the wide clinical application of these vectors for gene therapy will also depend on the availability of efficient large-scale manufacturing procedures. Hence, the optimization of upstream bioprocesses, the adaptation of packaging cells to serum-free media, the development of downstream processing strategies and the establishment of quality control tests for clinical-grade vector stocks are critical to advances in retroviral gene therapy.

Downstream processing of retroviral vectors is particularly challenging because of the low titers generated by most commonly used packaging systems and the instability of the viral particle. This thesis describes the development of two innovative and valuable methods for retroviral vector purification: heparin affinity chromatography and rate zonal ultracentrifugation. These methods were included in two complete strategies for the purification of the retroviral particles from crude supernatants to highly purified vector preparations which are described in detail in chapters II and III. A comparative analysis of these strategies is presented in this section followed by perspectives for future work.

Both strategies presented render high levels of purity as revealed by SDS-PAGE analysis. The electrophoretic profiles of the purified products showed the main viral proteins and a few other minor bands, many of which are likely to be host proteins incorporated into the viral particle or intermediate viral products as confirmed in Chapter III. Based on the visual inspection of the gels, the levels of purity reached using either strategy were estimated to be over 90%. Although the exact composition of the retroviral particle is still unknown, the impossibility to further purify the rate zonal purified virus preparation by size-exclusion chromatography or to digest proteins in this preparation with subtilisin treatment allowed us to safely estimate the levels of purity in the absence of a purified standard.

In terms of process time, both strategies are comparable. Microfiltration and ultra/diafiltration of a 2-L batch using the described methodology requires 6 hours of processing. These initial filtration steps were employed in both strategies resulting in the clarification, 20-fold concentration and partial purification and crude viral stocks with no significant losses of infectivity. To reach high levels of purity either rate zonal ultracentrifugation or 2 steps of chromatography were needed. While rate zonal centrifugation required 4 h of processing, chromatography purification was carried out in 5 h (1.5 h for heparin affinity chromatography and 3.5 h for size-exclusion chromatography). The total processing time for either strategy was around 10 h. Therefore, a coordinated effort would allow completion of either process within 1 working day.

Surprisingly, both strategies resulted in roughly the same overall recovery of infective retroviral particles which was approximately 40%. These overall recoveries are superior to those reported using other purification techniques (Kuiper et al., 2002; Williams et al., 2005; Ye et al., 2004). Few reports have shown a complete purification strategy for MoMLV-derived vectors where final overall recoveries are presented. An example was provided by Kuiper and collaborators who estimated that a final overall recovery of 5% would be obtained using their 3-step downstream processing protocol based on hydroxyapatite chromatography (Kuiper et al., 2002).

Both strategies presented here should be useful for retroviral particles regardless of the gene or Env-protein carried by the vector or packaging cell type used for production. None of these variables greatly affects the size and density of the viral particles which should allow the separation of various vectors by rate zonal ultracentrifugation. The general applicability of heparin affinity chromatography was demonstrated in Chapter IV. The usefulness of this methodology was not affected by the presence or absence of different Env-proteins. Moreover, vectors derived from 293 and HT1080 cells bind heparin columns with similar efficiency and affinity. The fact that these cell lines are currently the most attractive cell lines for retrovirus production (Merten, 2004) further supports our conclusions on the general utility of this method.

Each strategy also offers distinct advantages. Adsorptive chromatography can be easily scaled-up by increasing the column diameter. However, the possibility to scale-up the ultracentrifugation protocol depends on the availability of costly high capacity ultracentrifuge equipment suitable for retrovirus purification purposes that unfortunately, at the present time is not available in most laboratories. Additionally, chromatography is a very simple and reproducible method. Compared to chromatography, rate zonal centrifugation is labor-intensive and prone to inter-operator variability. These features make chromatography technologies ideal for use at industrial scale.

On the other hand, most chromatography methods are unlikely to remove significant amounts of defective vector forms and/or cell membrane vesicles (Vellekamp et al., 2001). The ability of rate zonal ultracentrifugation to separate these closely-related structures from retrovirus preparations was clearly shown in Chapter III. To date, no other technique used for MoMLV purification has shown the ability to separate these contaminants from vectors stocks. Moreover, these contaminants are extremely difficult to detect because they possess a composition similar to that of the retroviral particles, making their detection by SDS-PAGE analysis impossible in practice. This limitation can be circumvented by the use of electron microscopy which can successfully distinguish cell membrane vesicles from viral particles (active or inactive) in the hands of expert microscopists (Bess et al., 1997; Gluschankof et al., 1997).

The possible implications of having defective vector forms or cell membrane contaminants in retroviral clinical-grade preparations are rarely discussed in the literature. Moreover, to the best of my knowledge, there are no specific regulations that demand the quantitation or elimination of these contaminants from retroviral vector stocks for clinical studies. In contrast, the "Guidance for Human and Somatic Cell Therapy and Gene Therapy" first issued by the FDA's Center for Biologics Evaluation and Research (CBER) in March 1998 (CBER, 2001), specifically states that the measurement of total vs. infectious particle ratios is required for adenoviral vectors and they recommend that it does not exceed 100:1 in the final product. These considerations for the use of adenoviral vectors are largely due to the fact that adenoviral particles are potentially toxic themselves. It should be also noted that a CsCl density gradient method that efficiently separates complete from empty adenoviral particles has been available for a long time (Niiyama et al., 1975), which allows researchers to meet this specifications.

Given that both strategies presented are comparable in terms of processing time, recovery of infective particles, ability to purify various types of retroviral vectors and reach high levels of purity that are acceptable for clinical applications, the choice of strategy may ultimately depend on the process scale and availability of specific laboratory equipment required. As a result, the rate zonal ultracentrifugation method will be particularly useful in academic laboratories equipped with ultracentrifuges while chromatography will be more attractive for large-scale vector production facilities.

In addition to the preparation of clinical-grade vectors for gene therapy, the methods developed in this work could be applied to fundamental research. The ability of rate zonal ultracentrifugation to separate defective virus forms may be particularly attractive for investigators interested in studying differences between defective and functional virus populations. Additionally, this method is extremely useful for obtaining highly purified virus preparations suitable for studying the composition of the poorly characterized retroviral membrane. These studies have been greatly complicated by the presence of contaminating cell membrane vesicles that co-purify with virions using standard ultracentrifugation techniques (Ott, 1997; Ott, 2002; Trubey et al., 2003). As described in

Chapter IV of this thesis, retroviral particles are able to specifically interact with heparin ligands under physiological conditions regardless of the vectors' cellular origin or Env-protein used for pseudotyping. These intriguing results may motivate further investigation on the nature of retrovirus-heparin interactions using heparin affinity chromatography.

By taking advantage of the expertise gained on virus purification, we are currently attempting to identify host proteins on the retroviral membrane that could bring light to the mechanism of virus attachment to target cells and contribute to the development of targeted retroviral vectors for *in vivo* gene therapy applications. For this purpose, a detailed characterization of the composition of the purified viral particles by proteomic analysis was performed in our laboratory. Three host-derived cell adhesion proteins were identified by 1-D-electrophoresis coupled with mass spectrometry analysis. The presence of these proteins on the virus membrane was confirmed by immunogold electron microscopy studies. Virus neutralization assays are being conducted in order to establish whether these proteins influence virus infectivity.

This thesis represents a framework from which to direct future research in retrovirus purification as there exist numerous opportunities for further optimization. First, taking into account that the vast majority of impurities contained in the vector supernatant come from the serum added to the culture media, progress towards development of serum-free adapted packaging cell lines would greatly facilitate downstream processes. To date, reports demonstrating successful production of retroviral vectors in serum-free media are scarce (McTaggart and Al-Rubeai, 2002). Alternatively, production of vectors in very low serum media helps reduce the levels of contamination and more importantly, often results in a higher specific vector productivity than with the 10% concentrations of serum typically used (McTaggart and Al-Rubeai, 2002; Merten, 2004). It would be interesting to test the performance of the strategies presented in this thesis for the downstream processing of supernatants produced in serum-free or low protein media. However, because serum is believed to play a role in the protection of viral particles, particularly when exposed to stressful conditions such as those encountered during concentration, it wouldn't be

surprising that the presence of low concentrations of serum at these early stages, as opposed to serum-free conditions, result in improved vector recoveries.

Ultrafiltration is the preferred method for large-scale processing of retroviral particles because it allows processing large volumes of supernatant in a relatively short period of time. Although stirred cell technologies are particularly attractive for ultrafiltration because they are very gentle and thus, result in high recovery of infective particles as shown in this work, the maximum capacity of a stirred cell unit is 2-L. Larger volumes of retroviral vectors (8 to 10 L) could be processed by tangential-flow filtration (Kotani et al., 1994; Makino et al., 1994; Paul et al., 1993) and thus, it would be appealing to test these technologies for large-scale production.

Due to the high amounts of heparin-binding contaminants contained in serum, purification of retroviral vectors by heparin affinity chromatography in a single step was not possible. In this work, efficient removal of heparin-binding proteins was achieved by size-exclusion chromatography. Alternatively, one could achieve removal of these contaminants by using the rate zonal centrifugation strategy described in this work. This approach is very attractive since it would combine the advantages of both purification strategies developed in this thesis, namely the scalability of chromatography and the high resolution of rate zonal ultracentrifugation. Although this would be an ideal situation in terms of purity and process scalability, it should also be noted that the theoretical overall recovery of infective particles, calculated as a factor of the recoveries obtained in each individual step, is expected to be lower (26.4%) than that obtained with the purification strategies described in this thesis.

Another way to optimize the heparin affinity chromatography method would be to use membrane chromatography instead of column chromatography. Membrane chromatography is attractive because it provides higher binding capacities for large bioproducts such as viruses and higher flow rates than the traditional column chromatography. Processes using membrane chromatography devices can be easily, cost-effectively, and linearly scaled up using multiple cartridges in parallel. Mustang Q anion-

exchange membrane capsules have been successfully employed for the purification of lentiviral vectors (Slepushkin et al., 2003).

Engineering vectors by inserting tags or chemically modifying the viral Env-protein structure without reducing or eliminating the viruses' ability to transduce cells has proved to be difficult (Katane et al., 2002; Palù et al., 2000; Pizzato et al., 2001; Tai et al., 2003). This has been partly attributed to the inability of the Env-protein to provide fusogenic functions for viral entry once its structure has been modified. Provided the composition of MoMLV particles was more deeply characterized, proteins other than the viral Env-protein could be chosen to insert tags for purification or targeting purposes. For instance, host proteins incorporated into the MoMLV could be genetically modified to contain such tags possibly without compromising virus infectivity. To date, this approach has not been tested likely due to our poor current understanding of the viral membrane composition and the lack of suitable purification methods for virus characterization studies.

Finally, developments in lentiviral vector manufacture and design have greatly benefited from the many years of experience and advances made with retroviral vectors. Additionally, the strategies presented in this work may also be exploited for the purification of these vectors. Since both types of vectors share common physical properties, it is likely that the rate zonal ultracentrifugation technique presented here for MoMLV-derived vectors will also be useful for the purification of lentiviral vectors. Moreover, the fact that soluble heparin also inhibits lentiviral vector transduction to target cells (Guibinga et al., 2002) and that HIV-1 particles were shown to specifically interact with heparan sulfate (Cladera et al., 2001; Mondor et al., 1998; Roderiquez et al., 1995; Vives et al., 2005) strongly suggests that these particles can also be purified by heparin affinity chromatography.

To conclude, the methods we have developed represent a significant contribution to the technological progress in the field of gene therapy and may also open the door to a finer characterization of retroviral particles.

References

Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M.G. and Bordignon, C., 2002. Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science 296, 2410-3.

Anderson, W.F., Blaese, R.M. and Culver, K., 1990. The ADA human gene therapy clinical protocol: Points to Consider response with clinical protocol, July 6, 1990. Hum Gene Ther 1, 331-62.

Andreadis, S.T., Roth, C.M., Le Doux, J.M., Morgan, J.R. and Yarmush, M.L., 1999. Large-scale processing of recombinant retroviruses for gene therapy. Biotechnol Prog 15, 1-11.

Baltimore, D., 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature 226, 1209-11.

Barquinero, J., Eixarch, H. and Perez-Melgosa, M., 2004. Retroviral vectors: new applications for an old tool. Gene Ther 11 Suppl 1, S3-9.

Berns, A., 2004. Good news for gene therapy. N Engl J Med 350, 1679-80.

Bess, J.W., Jr., Gorelick, R.J., Bosche, W.J., Henderson, L.E. and Arthur, L.O., 1997. Microvesicles are a source of contaminating cellular proteins found in purified HIV-1 preparations. Virology 230, 134-44.

Bishop, J.M., 1983. Cellular oncogenes and retroviruses. Annu Rev Biochem 52, 301-54.

Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L., Bousso, P., Deist, F.L. and Fischer, A., 2000. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288, 669-72.

CBER, 2001. Guidance for human somatic cell therapy and gene therapy. Hum Gene Ther 12, 303-14.

Cladera, J., Martin, I. and O'Shea, P., 2001. The fusion domain of HIV gp41 interacts specifically with heparan sulfate on the T-lymphocyte cell surface. Embo J 20, 19-26.

Cone, R.D. and Mulligan, R.C., 1984. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. Proc Natl Acad Sci U S A 81, 6349-53.

Ellermann, V. and Bang, O., 1908. Experimentelle Leukämie bei Hühnern. Zentralbl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. Orig. 46, 595-609.

Gaspar, H.B., Parsley, K.L., Howe, S., King, D., Gilmour, K.C., Sinclair, J., Brouns, G., Schmidt, M., Von Kalle, C., Barington, T., Jakobsen, M.A., Christensen, H.O., Al Ghonaium, A., White, H.N., Smith, J.L., Levinsky, R.J., Ali, R.R., Kinnon, C. and Thrasher, A.J., 2004. Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet 364, 2181-7.

Gluschankof, P., Mondor, I., Gelderblom, H.R. and Sattentau, Q.J., 1997. Cell membrane vesicles are a major contaminant of gradient-enriched human immunodeficiency virus type-1 preparations. Virology 230, 125-33.

Guibinga, G.H., Miyanohara, A., Esko, J.D. and Friedmann, T., 2002. Cell surface heparan sulfate is a receptor for attachment of envelope protein-free retrovirus-like particles and VSV-G pseudotyped MLV-derived retrovirus vectors to target cells. Mol Ther 5, 538-46.

Gunzburg, W.H., 2003. Retroviral gene therapy--where now? Trends Mol Med 9, 277-8.

Huebner, R.J. and Todaro, G.J., 1969. Oncogenes of RNA tumor viruses as determinants of cancer. Proc Natl Acad Sci U S A 64, 1087-94.

Katane, M., Takao, E., Kubo, Y., Fujita, R. and Amanuma, H., 2002. Factors affecting the direct targeting of murine leukemia virus vectors containing peptide ligands in the envelope protein. EMBO Rep 3, 899-904.

Kotani, H., Newton, P.B., 3rd, Zhang, S., Chiang, Y.L., Otto, E., Weaver, L., Blaese, R.M., Anderson, W.F. and McGarrity, G.J., 1994. Improved methods of retroviral vector transduction and production for gene therapy. Hum Gene Ther 5, 19-28.

Kuiper, M., Sanches, R.M., Walford, J.A. and Slater, N.K., 2002. Purification of a functional gene therapy vector derived from Moloney murine leukaemia virus using membrane filtration and ceramic hydroxyapatite chromatography. Biotechnol Bioeng 80, 445-53.

Makino, M., Ishikawa, G., Yamaguchi, K., Okada, Y., Watanabe, K., Sasaki-Iwaki, Y., Manabe, S., Honda, M. and Komuro, K., 1994. Concentration of live retrovirus with a regenerated cellulose hollow fiber, BMM. Arch Virol 139, 87-96.

Mann, R., Mulligan, R.C. and Baltimore, D., 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 33, 153-9.

McTaggart, S. and Al-Rubeai, M., 2002. Retroviral vectors for human gene delivery. Biotechnol Adv 20, 1-31.

Merten, O.W., 2004. State-of-the-art of the production of retroviral vectors. J Gene Med 6 Suppl 1, S105-24.

Miller, A.D., 1997. Development and applications of retroviral vectors. Cold Spring Harbor Laboratory Press, New York.

Moloney, J.B., 1960. Biological studies on a lymphoid-leukemia virus extracted from sarcoma 37. I. Origin and introductory investigations. J Natl Cancer Inst 24, 933-51.

Mondor, I., Ugolini, S. and Sattentau, Q.J., 1998. Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. J Virol 72, 3623-34.

Niiyama, Y., Igarashi, K., Tsukamoto, K., Kurokawa, T. and Sugino, Y., 1975. Biochemical studies on bovine adenovirus type 3. I. Purification and properties. J Virol 16, 621-33.

Ott, D.E., 1997. Cellular proteins in HIV virions. Rev Med Virol 7, 167-180.

Ott, D.E., 2002. Potential roles of cellular proteins in HIV-1. Rev Med Virol 12, 359-74.

Palù, G., Parolin, C., Takeuchi, Y. and Pizzato, M., 2000. Progress with retroviral gene vectors. Rev Med Virol 10, 185-202.

Paul, R.W., Morris, D., Hess, B.W., Dunn, J. and Overell, R.W., 1993. Increased viral titer through concentration of viral harvests from retroviral packaging lines. Hum Gene Ther 4, 609-15.

Pizzato, M., Blair, E.D., Fling, M., Kopf, J., Tomassetti, A., Weiss, R.A. and Takeuchi, Y., 2001. Evidence for nonspecific adsorption of targeted retrovirus vector particles to cells. Gene Ther 8, 1088-96.

Rainov, N.G. and Ren, H., 2003. Clinical trials with retrovirus mediated gene therapy-- what have we learned? J Neurooncol 65, 227-36.

Roderiquez, G., Oravecz, T., Yanagishita, M., Bou-Habib, D.C., Mostowski, H. and Norcross, M.A., 1995. Mediation of human immunodeficiency virus type 1 binding by interaction of cell surface heparan sulfate proteoglycans with the V3 region of envelope gp120-gp41. J Virol 69, 2233-9.

Rous, P., 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med. 13, 397-411.

Shimotohno, K. and Temin, H.M., 1981. Formation of infectious progeny virus after insertion of herpes simplex thymidine kinase gene into DNA of an avian retrovirus. Cell 26, 67-77.

Sinn, P.L., Sauter, S.L. and McCray, P.B., Jr., 2005. Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors--design, biosafety, and production. Gene Ther 12, 1089-98.

Skalka, A.M. and Goff, S.P., 1993. Reverse transcriptase. Cold Spring Harbor Laboratory Press, New York.

Slepushkin, V., Chang, N., Cohen, R., Gan, Y., Jiang, B., Deausen, E., Berlinger, D., Binder, G., Andre, K., Humeau, L. and Drupolic, B., 2003. Large-scale purification of a lentiviral vector by size exclusion chromatography or mustang Q ion exchange capsule. Bioprocessing J 2, 89-95.

Tai, C.K., Logg, C.R., Park, J.M., Anderson, W.F., Press, M.F. and Kasahara, N., 2003. Antibody-mediated targeting of replication-competent retroviral vectors. Hum Gene Ther 14, 789-802.

Temin, H.M. and Mizutani, S., 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226, 1211-3.

Trubey, C.M., Chertova, E., Coren, L.V., Hilburn, J.M., Hixson, C.V., Nagashima, K., Lifson, J.D. and Ott, D.E., 2003. Quantitation of HLA class II protein incorporated into human immunodeficiency type 1 virions purified by anti-CD45 immunoaffinity depletion of microvesicles. J Virol 77, 12699-709.

Varmus, H.E., 1984. The molecular genetics of cellular oncogenes. Annu Rev Genet 18, 553-612.

Vellekamp, G., Porter, F.W., Sutjipto, S., Cutler, C., Bondoc, L., Liu, Y.H., Wylie, D., Cannon-Carlson, S., Tang, J.T., Frei, A., Voloch, M. and Zhuang, S., 2001. Empty capsids in column-purified recombinant adenovirus preparations. Hum Gene Ther 12, 1923-36.

Vives, R.R., Imberty, A., Sattentau, Q.J. and Lortat-Jacob, H., 2005. Heparan sulfate targets the HIV-1 envelope glycoprotein gp120 coreceptor binding site. J Biol Chem 280, 21353-7.

Vogt, V.M., 1997. Retroviral virions and genomes. Cold Spring Harbor Laboratory Press, New York.

Watanabe, S. and Temin, H.M., 1983. Construction of a helper cell line for avian reticuloendotheliosis virus cloning vectors. Mol Cell Biol 3, 2241-9.

Wei, C.M., Gibson, M., Spear, P.G. and Scolnick, E.M., 1981. Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. J Virol 39, 935-44.

Williams, S.L., Nesbeth, D., Darling, D.C., Farzaneh, F. and Slater, N.K., 2005. Affinity recovery of Moloney Murine Leukaemia Virus. J Chromatogr B Analyt Technol Biomed Life Sci 820, 111-9.

Ye, K., Jin, S., Ataai, M.M., Schultz, J.S. and Ibeh, J., 2004. Tagging retrovirus vectors with a metal binding peptide and one-step purification by immobilized metal affinity chromatography. J Virol 78, 9820-7.

Annex I Assay protocols

> **Important note:**
> All samples containing MoMLV-derived vectors must be handled in a Biosafety Level 2 (BSL-2) laboratory by trained personnel using the biological safety cabinet and following BSL-2 guidelines.

1. Virus titer assay

Objective:

The following procedure describes the quantification of infectious retroviral particles containing the GFP reporter gene by FACS analysis

Materials:

- Flow cytometer
- DMEM
- FBS
- Polybrene (8mg/mL stock filter sterilized)
- PBS
- Tripsin-EDTA
- Formaldehyde (16%)
- 6-well plates
- Flow cytometry tubes 12 ×75
- Syringes 3cc with blue caps (cut cap edge and insert filter)
- Nitex filter

Procedure:

1- Plate 2-3 x10^5 target cells per well in 6-well plates. Allow cells to attach overnight at 37ºC and a 5% CO_2 atmosphere in 3 mL of DMEM + 10% FBS. Include 2 wells for negative control (only cells) and 2 wells for retrovirus positive control samples.

2- The next day, prepare the required volume of DMEM + 8 ug polybrene per mL of medium for sample dilution.

3- Label sterile eppendorf tubes and add the required volume of DMEM + polybrene virus dilution medium (i.e. 900 μL per tube for dilutions 1/10 or 990 μL per tube for dilutions 1/100).

4- Thaw retrovirus samples and prepare sample dilutions in DMEM + polybrene medium (i.e. add 100 μL of sample per tube for dilutions 1/10 or 10 μL per tube for dilutions 1/100). Mix the virus dilution gently by pipetting. The dilution factor will depend on the virus titer. When the virus titer is completely unknown, serial dilutions of the virus sample should be prepared to obtain a percentage of transduced cells between 3 and 20.

5- Expose the target cells to the virus dilutions prepared. For this purpose, label the 6-well plates containing the exponentially growing target cells, gently remove the culture medium and add 1 mL of virus sample dilution per well. Count viable cells in one of the negative control wells. Incubate the cells at 37 ºC and a 5% CO_2 atmosphere for 3 h.

6- Then add 1 mL of DMEM + 20% FBS per well and incubate plates at 37 ºC and a 5% CO_2 atmosphere for 48 h.

7- Following this incubation time, prepare a 4% formaldehyde solution by diluting the 16% formaldehyde stock 4-fold in PBS. Then add 1 mL of 4% formaldehyde solution into 15 mL labelled tubes.

8- Remove the culture medium from each well, wash transduced cells with 1 mL of PBS, trypsinize the cells with trypsin-EDTA and gently resuspend them in 1 mL of PBS.

9- Add the 1 mL cell suspension to the 15 mL tubes containing formaldehyde. Let the cells fix for 20 minutes at room temperature.

10- Centrifuge the cells at 420×g for 10 min, remove the supernatant by inverting the tube in the chemical hood and resuspend the cell pellet in 1 mL of PBS.

11- Transfer the cell suspension with a Pastuer pipet into the syringes and filter through nitex fabric into FACS tubes. Cells are now ready for FACS analysis. They can be stored a couple of hours at 4ºC before analysis.

12- Before proceeding with the flow cytometric analysis of the virus samples, use the negative control sample to adjust the gate to 1% GFP + cells. Only virus dilutions giving values of GFP + cell percentage ranging from 3 to 20 are selected for virus titer determination.

13- Virus titers were calculated as follows:

Titer (IVP/mL) = (% GFP+ cells) × (number of cells at time of exposure) × (dilution factor)/ (sample volume)

Note: Samples from a given experiment should be analyzed in a single titration assay to avoid inter-assay variability and all samples should be processed in duplicate or triplicate to minimize intra-assay variability.

2. Bradford protein assay

Objective:

The following procedure describes the determination of total protein concentrations in solution using the Bio-Rad protein assay based on the method of Bradford.

Materials:

- Spectrophotometer
- BSA fraction V standard solution (2mg/mL)
- Bio-Rad Protein Assay dye reagent concentrate (Bio-Rad Laboratories)
- Visible cuvettes
- Borosilicate tubes

Procedure:

1- Prepare 500 μL BSA working solution (WS) in Milli-Q H_2O (200 μg/mL) by diluting 10-fold BSA standard solution in Milli-Q H_2O.

2- Prepare standard solutions (2 mL final volume) for the calibration curve as follows:

Final concentration of BSA (μg/mL)	Vol of WS (μL)	Vol of Milli-Q H2O (μL)
blank	0	2000
1.0	10	1990
2.5	25	1975
5.0	50	1950
10.0	100	1900
12.5	125	1875

3- Aliquot 800 μL of each standard dilution into duplicate borosilicate tubes and cover with parafilm until use.

4- Turn on the spectrophotometer to allow stabilization on the visible lamp (Tungsten) for 20 min. Set spectrophotometer at 595 nm.

5- Prepare the unknown virus samples using the biological safety cabinet. The samples with the unknowns are assayed once at three different dilutions. Add 800 μL of Milli-Q H_2O into triplicate borosilicate tubes. Then remove 10, 20 or 30 μL of Milli-Q H_2O per tube and add 10, 20 or 30 μL of virus sample or sample dilution respectively.

6- Add 200 μL of Bio-Rad dye reagent concentrate using an eppendorf repeater pipette in each tube and gently vortex.

7- Incubate tubes at room temperature for 10 min (for up to 90 min total) and transfer samples to visible cuvettes.

8- After having zeroed the spectrophotometer with the blanks, read all standards and samples at 595 nm.

3. dsDNA quantitation assay

Objective:

The following procedure describes the determination of double-stranded DNA concentrations in solution using the PicoGreen® dsDNA quantitation assay

Materials:

- Microplate fluorescence reader
- PicoGreen dsDNA Quantitation Reagent (Molecular Probes)
- TE buffer (20X) (Molecular Probes)
- Lambda ds DNA standard (100 μg/mL) (Molecular Probes)
- 96 well flat bottom plates

Procedure:

1- Dilute the 20X TE buffer 20-fold in Milli-Q H_2O (Note: Approximate volume required per plate: 25 mL of TE buffer)

2- Prepare 500 μL of ds DNA working solution (WS) in TE (2 μg/mL) by diluting 50-fold lambda DNA standard solution in TE buffer.

3- Prepare standard solutions (0.5 mL final volume) for the calibration curve as follows:

Final concentration of dsDNA (ng/mL)	Vol of WS (µL)	Vol of TE (µL)
blank	0	500
40	10	490
100	25	475
200	50	450
300	75	425
400	100	400
500	125	375

4- Prepare the virus samples using the biological safety cabinet. The samples with the unknowns are assayed in triplicates. Prepare 400 µL final volume of each sample or sample dilution in TE buffer.

5- Dilute the PicoGreen reagent 200-fold in TE buffer. Protect reagent from light and always use gloves when manipulating the reagent and discard waste in a pale dedicated for incineration. (Note: Approximate volume required per plate: 10 mL of diluted reagent).

6- In 96 well flat bottom plates add:

- 100 µL of standards and unknown samples in triplicates

- 100 µL of PicoGreen reagent using a repeater eppendorf pipet or multichannel pipet

7- Incubate tubes at room temperature for 2-5 min in the dark (for up to 60 min total)

8- Read plate using a microplate fluorescence reader at 480 nm excitation and 520 nm emission.

Annex II Downstream processing protocols

Important note:
All samples containing MoMLV-derived vectors must be handled in a Biosafety Level 2 (BSL-2) laboratory by trained personnel using the biological safety cabinet and following BSL-2 guidelines.

---

1. Microfiltration

Objective:

The following procedure describes the clarification of retroviral particles in crude harvested supernatants containing 10 % FBS by microfiltration using a dual HT Tuffryn® polysulfone membrane (0.45/0.2 µm) capsule filtration device.

Materials:

- Centrifuge Avanti^JM-25
- JLA-10.500 Fixed Angle Rotor
- Peristaltic pump and tubings
- Pressure gauge
- Dual HT Tuffryn® polysulfone membrane (0.45/0.2 µm) capsule filtration device (Pall Gelman Sciences)

Procedure:

1- Thaw retrovirus supernatants using water bath at 37ºC.

2- Pellet cells and cell debris by centrifugation at 10,000×g for 10 min. Decant the supernatant into a sterile bottle. (Note: Using this rotor the manufacturer informs that viruses with a sedimentation coefficient of ∼700S would pellet in 90min at 15,000×g or 10,000rpm).

3- Aliquot starting material samples for analyses and measure the starting volume. Keep the supernatant at 4ºC until further use.

4- Set up the filtration system in the biological safety cabinet. Flush the microcapsule membrane with 500 mL of Milli-Q H_2O and empty the tubing.

5- Filter supernatants at constant flow rate (start at 10mL/min and gradually increase to 50 mL/min). Collect the permeate in sterile bottles. Check the pressure at all times. Using the

capsule filtration device with an effective filtration area of 500 cm^2, 1-2 L of retrovirus vector supernatants can be filtered with low back pressure (below 0.01 MPa).

6- Aliquot clarified supernatant samples for analyses and measure the final volume.

7- Keep the clarified supernatant at 4°C until the next downstream processing step or store at -80°C along with the aliquoted samples.

2. Ultrafiltration

Objective:

The following procedure describes the concentration of retroviral particles in clarified supernatants containing 10% FBS by ultra-diafiltration using Omega^{TM} polyethersulfone membrane disc filters (MWCO 300,000) and stirred cell units.

Materials:

- Amicon Stirred cell units
- Nitrogen tank
- Omega^{TM} polyethersulfone membrane disc filters, MWCO 300,000 (Pall Gelman Sciences)
- DMEM
- FBS
- Diafiltration buffer: 150 mM NaCl in 20 mM Tris-HCl pH 7.5 filter sterilized buffer

Procedure:

1- Use the following table to select the stirred cell model and membrane size according to the application

Unit model	8003	8010	8050	8400	2000
Cell capacity (mL)	3	10	50	400	2000
Membrane diameter (mm)	25	25	43	76	150
Effective membrane area (cm^2)	0.9	4.1	13.4	41.8	162
Stirred minimum volume (mL)	0.75	1	2.5	10	50

2- Soak the membrane in a container with Milli-Q H$_2$O to remove trace amounts of glycerine humectants and sodium azide bacteriostat. The membrane should be kept wet at all times from this point on.

3- Clean and assembly the stirred cell unit. The glossy side of the membrane should be facing up.

4- Fill the stirred cell unit with Milli-Q H$_2$O. Prewash the membrane with Milli-Q H$_2$O and check the water flux at 10 psi to ensure the membrane is not broken or displaced. A water flux of 1-3 mL/min/cm^2 indicates the 300, 000 MWCO membrane is intact.

5- Prefoul the membrane with ∼ 0.25 mL/cm^2 of DMEM + 10% FBS media (note: the media should not contain phenol red if the supernatants to be concentrated are produced in the absence of this pH indicator). Adjust the tip speed to 33.5 cm/sec and increase the nitrogen pressure to 30 psi. Be careful not to let the membrane dry. Add PBS if necessary.

6- Aliquot starting material clarified retrovirus samples for analyses and measure the starting volume. Considering this volume, calculate the final volume desired in order to attain a predetermined concentration factor at the end of the process.

7- Add the starting material into the stirred cell unit and carry out ultrafiltration at nitrogen constant pressure (30 psi) and tip speed (33.5 cm/sec). Collect the permeate in sterile bottles. Monitor the process at different times by determining the flow rate and volume of permeate. (Note: The flow rate should drop slowly during the process).

8- Once the desired volume of retentate is reached, add cold ultrafiltration buffer into the stirred cell unit (∼1 mL/cm^2) and carry out diafiltration at nitrogen constant pressure (30 psi) and tip speed (33.5 cm/sec). Repeat diafiltration 3 times in discontinuous mode. (Note: The permeates obtained during ultrafiltration and diafiltration can be collected separately for subsequent analyses).

9- The ultra/diafiltration process is stopped once the final desired volume of retentate is reached. Slowly resuspend the virus and gently wash the membrane with diafiltration buffer. Aliquot the concentrated supernatant samples for analyses and measure the final volume.

10- Store concentrated samples at -80ºC along with the previously aliquoted samples.

3. Heparin affinity chromatography

<u>Objective:</u>

The following procedure describes the purification of retroviral particles in 20-fold concentrated supernatants by affinity chromatography using a Fractogel® EMD heparin affinity chromatography column

Materials:

- Low-pressure liquid chromatography system (GradiFrac^{TM}; GE Healthcare)
- Fractogel® EMD heparin gel (Merck, Darmstadt, Germany) packed into an HR 5/5 column (Amersham Biosciences) to a final volume of 1-mL
- 0.45 µm pore size Acrodisc syringe-mounted filters (Pall Gelman Sciences)
- Buffer A: 20 mM Tris-HCl buffer pH 7.5 filtered and degassed
- Buffer B: 2 M NaCl in 20 mM Tris-HCl buffer pH 7.5 filtered and degassed
- Storage buffer: 20 % EtOH + 150 mM NaCl in Milli-Q H_2O filtered and degassed

Note: The HR5/5 filters should be replaced with a 10 µm mesh filter for virus purification purposes

Procedure:

1- Turn on the UV lamp and rinse the GradiFrac system thoroughly with Milli-Q H_2O

2- Install the 1-mL Fractogel® EMD heparin column and remove the storage buffer with 10 column volumes (CV) of Milli-Q H_2O at 0.3 mL/min.

3- Rinse the lines A and B with the corresponding buffers and equilibrate the column with 10 CV of binding buffer (150 mM NaCl in 20 mM Tris-HCl buffer, pH 7.5) at 0.5 mL/min.

4- Thaw retrovirus 20-fold concentrated samples. Filter the samples using 0.45 µm pore size Acrodisc syringe-mounted filters with HT Tuffryn® polysulfone membrane. Aliquot starting material samples for analyses.

5- Monitor UV absorbance at 280 nm. When stable baseline is achieved, load 3 mL of the concentrated virus sample and apply a step-wise gradient elution strategy that includes a wash step at 150 mM NaCl (19.5 CV) to remove the bulk of serum contaminating proteins, followed by virus elution at 350 mM NaCl (13 CV) and a final high-stringency wash step at 1200 mM NaCl (7.5 CV) to remove tightly bound contaminants. The process is carried out at room temperature at 0.5 mL/min (or 153 cm/h linear flow rate).

6- The virus particles elute at 350 mM NaCl in a defined peak. Pool virus-containing fractions and aliquot for analyses.

7- After each run, re-equilibrate the column with binding buffer (10 CV) at 0.5 mL/min or store the column in storage buffer (10 CV) at 0.3 mL/min.

8- Store semi-purified samples at -80ºC along with the previously aliquoted samples.

4. Rate zonal ultracentrifugation

Objective:

The following procedure describes the purification of retroviral particles in 20-fold concentrated supernatants by rate zonal ultracentrifugation 10-30% continuous iodixanol gradient.

Materials:

- Two-chamber gradient maker
- Peristaltic pump and tubings
- Beckman ultracentrifuge
- SW28 rotor and buckets
- 25 × 89 mm Beckman UltraClear^{TM} tubes (Beckman Instruments)
- OptiPrep^{TM} (60% sterile iodixanol stock solution in water) (AXIS-SHIELD)
- Diluent for WS: 6 mM EDTA, 0.85% NaCl, 120 mM Tris-HCl pH 7.5 buffer
- Buffer: 1 mM EDTA, 0.85% NaCl, 20 mM Tris-HCl pH 7.5 buffer

Procedure:

1- Rotor and buckets should be pre-cooled in the ultracentrifuge to 4 ºC before the run.

2- Prepare a 50% iodixanol working solution (WS) in 1 mM EDTA, 0.85% NaCl, 20 mM Tris-HCl buffer pH 7.5 by mixing 5 parts of OptiPrep^{TM} stock with 1 part of diluent for WS.

2- Prepare a 30% iodixanol gradient solution in 1 mM EDTA, 0.85% NaCl, 20 mM Tris-HCl buffer pH 7.5 by mixing 1.5 part of WS with 1 part of buffer

3- Prepare a 10% iodixanol gradient solution in 1 mM EDTA, 0.85% NaCl, 20 mM Tris-HCl buffer pH 7.5 by mixing 1 part of WS with 4 parts of buffer

4- Thaw retrovirus 20-fold concentrated samples. Aliquot starting material samples for analyses. Keep the virus at 4ºC until use.

5- Fill one chamber of the gradient maker with 16 mL of the 30% iodixanol gradient solution and the other with 18 mL of the 10% iodixanol gradient solution. Form 10-30% continuous iodixanol gradients in 25 × 89 mm Beckman UltraClear^{TM} tubes.

6- Carefully load 3 mL of the concentrated virus stock on top of the 34 mL continuous iodixanol gradient. Insert the tubes inside of the SW28 buckets and balance the weight of the buckets using an analytical balance. The buckets must be well balanced to avoid spills and tube collapse during ultra centrifugation. Avoid mixing the formed gradient at all times.

7- Carefully place buckets in the SW28 rotor and spin at 24,000 rpm (100,000×g) for 4 h at 4ºC. (Note: Use acceleration at 1 when using swinging bucket rotors and set deceleration at 0).

8- Remove buckets from the rotor and open them inside the biological safety cabinet. A yellow broad band containing most serum proteins is observed at the top of the tube. The virus bands in the middle of the tube (from 1.06 to 1.09 g/mL). No virus band is visualized at the concentrations of retrovirus stock used.

9- Clean the outside of the tube with EtOH and collect 15 gradient fractions of 2.5 mL by puncturing the bottom of the tube with an 18G needle. The density of each fraction can be determined by weight of 200 μL aliquots on an analytical balance.

10- Aliquot and store purified samples at -80ºC along with the previously aliquoted samples for analyses.

5. Size exclusion chromatography

Objective:

The following procedure describes the final purification of retroviral particles in heparin semi-purified fractions by size exclusion chromatography using a Sepharose CL-4B chromatography column

Materials:

- Low-pressure liquid chromatography system (GradiFrac[TM]; GE Healthcare)
- Sepharose CL-4B gel (GE Healthcare) packed into a XK 16/70 (Amersham Biosciences) to a final volume of ~100-120 mL
- 0.45 μm pore size Acrodisc syringe-mounted filters (Pall Gelman Sciences)
- Running buffer: PBS pH 7.4 filtered and degassed
- Storage buffer: 20 % EtOH + 150 mM NaCl in Milli-Q H_2O filtered and degassed

Procedure:

1- Turn on the UV lamp and rinse the GradiFrac system thoroughly with Milli-Q H_2O

2- Install the Sepharose CL-4B column and remove the storage buffer with 10 column volumes (CV) of Milli-Q H_2O at 0.5 mL/min.

3- Rinse the lines A with running buffer and equilibrate the column with 5 CV at 0.5 mL/min.

4- Thaw retrovirus semi-purified samples. Filter the samples using 0.45 µm pore size Acrodisc syringe-mounted filters with HT Tuffryn® polysulfone membrane. Aliquot starting material samples for analyses.

5- Monitor UV absorbance at 280 nm. When stable baseline is achieved, load 7.5 mL of the concentrated virus sample and perform isocratic elution with PBS running buffer (1.2 CV). The process is carried out at room temperature at 0.5 mL/min. (Note: Load <10% of bed volume for best peak resolution).

6- The virus particles elute in the void volume of the column. Pool virus-containing fractions and aliquot for analyses.

7- After each run, re-equilibrate the column with running buffer (5 CV) at 0.5 mL/min or store the column in storage buffer (5 CV) at 0.5 mL/min (or 15 cm/h linear flow rate).

8- Store purified samples at -80ºC along with the previously aliquoted samples.