CONFERENCE PAPER

Downstream processing of viral vectors and vaccines

R Morenweiser

GE Healthcare, Amersham Biosciences Europe GmbH, Fast Trak Services Europe, Freiburg, Germany

Viral vectors and viral vaccines more and more play an important role in current medical approaches. Gene vectors like adenoviruses, adeno-associated viruses or retroviruses are the vehicles being developed for delivering genetic material to the target cell in gene therapy. Viral vaccines, like attenuated or inactivated rabies virus, influenza virus or hepatitis virus vaccines, are powerful tools to limit the number of serious viral infections and pandemics. Higher safety demands, that is, reduction of side effects, by regulatory authorities like Food and Drug Administration (FDA) and European Agency for the Evaluation of Medicinal

Products (EMA), nowadays force developers as well as manufacturers to improve their production and purification processes for viral vectors and vaccines. Like for influenza viral vaccines, manufacturers begin to switch from egg cultivation to mammalian cell culture systems. Also within the purification procedure, a clear trend from classical purification methods like sucrose gradient centrifugation towards more sophisticated techniques like tangential flow filtration and liquid chromatography can be observed.

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Introduction

Although technologies have advanced significantly since the first approval was given for a clinical trial of a gene therapy product in 1990, many challenges remain.¹ Among them is the need for fast, simple and inexpensive downstream processing methodologies to purify biologically active vectors for transfer of therapeutic genetic material into recipients. Both viral and nonviral vectors are in various stages of gene therapy clinical trials. The majority are viral vectors (see Table 1), and it is this category to which we will address our discussion of downstream processing.

It is still difficult to estimate the quantities of viral vectors needed to satisfy the demands of gene therapy regimens. Predictions have ranged from 10^{11} to 10^{14} particles, with annual productivity requirements variously projected in the range 10^{13} – 10^{20} .³ This figures will likely increase dramatically as the number of patients who could benefit from treatment based on gene therapy increases.⁴ The availability of large-scale processes capable of generating vectors with levels of purity and biological activity that satisfy regulatory standards is therefore an urgent prerequisite for gene therapy applications. For any purification process, maximal productivity is a key issue. Additionally, the protocol should be robust, scalable, cost effective and preferably generic in scope, with broad applicability to a variety of viral vectors.

The development and validation of large-scale viral vector downstream processing strategies will require an intimate understanding of the physical, chemical and

biological characteristics of viruses that can be exploited in these processes.⁵ Viruses far exceed the dimension of proteins used in pharmaceutical applications with respect to weight and size. Therefore, it cannot be simply a matter of 'plugging' viruses into existing protein purification schemes and expecting adequate results.

This review discusses general purification methods for viral vectors and vaccines and provides a summary of techniques used for downstream processing of recombinant retroviruses, adenoviruses and adeno-associated viruses (AAVs). Although our discussion is limited to downstream processes, it is worthwhile for the reader to keep in mind that process development for production of viral gene therapy vectors and vaccines, in fact, consists of three integrated stages – upstream processing (selection of appropriate producer cell culture lines), defining optimal growth conditions and downstream processing (isolation and purification steps).⁶ As downstream operations are greatly impacted by the presence of impurities and contaminants in the virus-containing lysates funneled into them, and because they account for as much as 70% of production costs, it is important that all phases of process development be designed in tandem.³

Purification methods based on virus properties

Downstream processing is aimed at eliminating contaminants originating from host cells or culture media and producing large volumes of concentrated, biologically active viruses that are highly efficient in gene transfer. As impurities and contaminants can induce immunological and biological responses, they must be removed to comply with strict regulatory guidelines. For viruses,

Correspondence: Dr R Morenweiser, GE Healthcare, Amersham Biosciences Europe GmbH, Fast Trak Services Europe, Munzingerstrasse 9, Freiburg D-79111, Germany

Table 1 Vectors in use for gene therapy clinical trials (modified from Journal of Gene Medicine, 2005)²

Vector	Gene therapy clinical trials	
	Number	%
AAV	27	2.6
Adenovirus	260	25.5
Gene gun	5	0.5
Herpes simplex virus	32	3.1
Lentivirus	3	0.3
Lipofection	87	8.5
<i>Listeria monocytogenes</i>	1	0.1
Measles virus	1	0.1
Naked/plasmid DNA	162	15.9
Pox virus	56	5.5
Retrovirus	261	25.6
RNA transfer	12	1.2
<i>Salmonella typhimurium</i>	2	0.2
Semliki forest virus	1	0.1
Vaccinia virus	36	3.5
Adenovirus+retrovirus	2	0.2
Pox virus+vaccinia virus	17	1.7

there are additional requirements to separate infective vectors from noninfective and/or empty capsids.³

Characteristics such as pI, surface hydrophobicity, the presence or absence of an envelope, hydrodynamic diameter and lability of virus particles can play important roles in determining what methods might be applicable for purification.⁵ Size is the characteristic most convenient for separating viruses from the cells and media in which they have been propagated.

Viruses are quite large when compared with biomolecules such as proteins, peptides, glycoproteins, sugars and nucleic acids that are inevitably present in lysates harvested from virus-infected cells. In fact, viruses really should be considered molecular assemblies. While proteins typically range from 0.005 to 0.08×10^6 Da, viruses generally exceed 5×10^6 Da and can have a size of up to 1000 nm.

Purification strategies based on viral size have included a variety of methods such as density-gradient ultracentrifugation, ultrafiltration, precipitation, two-phase extraction systems and size exclusion chromatography (SEC). Several of the more commonly used procedures are discussed below.

High-speed preparative centrifugation (ultracentrifugation), using cesium chloride (CsCl) or sucrose gradients, has traditionally been used to process many viral vector preparations. Iodixanol gradients have also been used, for example, for adenovirus vector purification.⁷ The transduction ability of some retroviruses is significantly lessened when the viruses are purified by gradient centrifugation, so relative lability of virus particles to shear forces must be considered.⁸ In general, gradient centrifugation is considered to be chemically and physically appropriate for even labile viral particles; however, it is labor-intensive, time-consuming and scale-restricted. Additionally, virus particles may suffer a cumulative loss of infectivity during the extended period of time required for fractionation of the gradients. As virus sedimentation behavior falls within a range, other particulate contaminants like cellular host cell DNA

are likely to have similar physical characteristics.⁵ For downstream purification of viral vectors, ultracentrifugation has become a less and less frequent method of choice.

For some viruses, precipitation with chemicals, such as polyethylene glycol (PEG),⁹ ammonium sulfate or calcium phosphate,¹⁰ has been useful in promoting viral aggregation with subsequent removal by low-speed centrifugation. However, impurities or polymers may co-precipitate along with viral particles,⁵ and unfortunately some viruses may lose infectivity, possibly due to changes in osmotic pressure.¹¹

Furthermore, it has been reported that aqueous two-phase separation systems with PEG, dextran or polyvinyl alcohol have been used successfully to separate viral particles from cellular components, but high costs in the recycling of the organic solutions have hindered this technique to become used widely so far.¹²

Membrane-based tangential flow filtration techniques, in particular ultrafiltration and microfiltration, are applicable in the downstream process for purification of viruses for vaccine production. Especially the open-flow path of the hollow-fiber crossflow membranes promotes gentle handling of virus particles and shear-sensitive proteins, so virus structural integrity is not compromised. This makes the downstream process especially applicable for sensitive molecules.¹³ Membrane filtration has also been used successfully for concentration and partial purification of retroviral particles.^{5,8} Using a two-step process of microfiltration through 0.45- μ m membranes, followed by ultra/diafiltration through membranes with a 300 000 MWCO, where the virus is recovered in the dialyzed retentate, Braas *et al*⁵ achieved partial purification, concentration and salt depletion of retroviral preparations. While membrane filtration is an attractive procedure, there are problems with membrane fouling and, at high pressures, loss of viral infectivity, possibly due to shear forces that may act on the viral envelope. In addition, since separation is based on size differences, large molecular weight transduction inhibitors are coconcentrated with the viral particles, resulting in a reduction in viral transduction efficiencies.¹¹

Viruses can also be separated from crude cell lysates by SEC. For example, retroviruses, which are typically in the order of 100 nm in diameter, have been separated from smaller biomolecules using crosslinked agarose SEC media. When Moloney murine leukemia virus was purified by SEC, yields were greater, viral envelope glycoprotein was conserved and virus particles retained a higher level of infectivity when compared with conventional sucrose gradient ultracentrifugation techniques.¹⁴ If used in group separation mode, feed volumes of up to 30% of the column volume can be applied, which makes SEC even suitable for the initial steps within the downstream process. As with membrane filtration, separation from high molecular weight components, such as proteoglycans or cellular genomic DNA, is difficult to achieve with SEC.¹¹

Chromatography-based virus purification strategies

Virus purification protocols that are more amenable to scale-up generally involve some type of chromatographic procedure. Adsorption of virus particles to solid phases, in fact, provides a convenient and practical choice for

fractionating and recovering viruses from cell and media contaminants. While they are somewhat imperfect in their selectivity, adsorption methods do offer several important advantages in large-scale virus purification: (1) high flow rates can be used, thus limiting the processing time, (2) biological activity of labile viruses is often preserved, since mild conditions are generally used to elute the virus from the chromatographic matrix, (3) scale-up is relatively easy, (4) large volumes of cell lysates can be processed and (5) the cost of operation is relatively low.¹¹ However, it is important to remember that the design of suitable selective chromatographic protocols for virus purification must take into account the structure and physical and chemical surface properties of the viruses.⁵ Although viruses contain proteins, they are not exclusively protein in nature. Consequently, proven protein chromatographic methods may require extensive modification to accommodate virus purification.

Solid-phase bases of current commercially available chromatography media consist of particles ranging in size from 15 to 300 μm , with pore diameters of 30–400 nm. Viruses diffuse much more slowly than proteins in solution. In fact, proteins move about two- to 100-fold more quickly in solution than large particulates like viruses and plasmid DNA.¹⁵ Unlike proteins, virus binding occurs primarily on the bead surfaces since viral particles are too large to diffuse into the pores. Any binding within the bead will only reduce yield since virus recovery is unlikely and unpredictable. Additionally, the hydrodynamic diameters of viruses are large, and some viruses (such as rotavirus with a hydrodynamic diameter of ~ 130 nm) are also very 'hygroscopic'.¹⁶ Chromatographic conditions must therefore be optimized to promote binding of slowly diffusing virus particles to the limited number of sites on the bead surface in the midst of much smaller and faster diffusing competing contaminants – for example, by carefully optimizing flow rates during adsorption and desorption. Future developments in media structure/chemistry will most likely be geared toward increasing bead capacity either through use of small diameter beads, beads with increased surface area or beads of different porosity to allow mass movement of virus particles into and out of the bead pores.

As with any virus purification method, stability problems can hamper production. Viruses differ widely in their stability. As 'giant' molecules, they are more likely to shear even at low flow velocities because they are often nonspherical and too large to fit into the 'pockets' described by the Komolgorov model for liquid shear forces. If the virus particle attaches to the matrix at multiple points, this may also contribute to shear. The lability of some viruses, such as retroviral vectors, may limit their chromatographic processing to a neutral pH range, moderate ionic strength, aqueous solvent systems and reduced operating temperatures.⁵ In contrast, nonenveloped viruses are often harder in terms of their ability to withstand conditions normally used in chromatography.

The various selective binding chemistries that have been used for chromatographic purification of viral vectors – affinity, ion exchange and hydrophobic interaction – all relate to the surface chemistry of the virus particle.⁵ For viruses that retain stable over a large pH range, adjustment of the pH of the feed suspension will modify the charge on the virus surface and affect its binding. Consequently, anion and cation exchange media

or charged membranes can be used to adsorb selectively the virus particles in the presence of other charged contaminants.¹⁷ For example, at neutral pH, the surface of adenovirus particles predominately has a negative net charge and therefore will selectively bind to anion exchange media.¹⁸ Processes based on ion exchange media have, in fact, been used in a number of virus preparations for conventional vaccine and gene therapy clinical trials.³

The relative surface charge of a viral particle at a given pH can be determined by preparing an electrophoretic titration curve or by capillary isoelectric focusing.¹⁹

Affinity chromatography is a separation method based on specific and reversible binding between two molecules. In the case of immunoaffinity chromatography, binding occurs between an antigen and a matrix-bound antibody that will recognize a structural surface component on the virus. This procedure can render high viral yield in a single purification step, thus simplifying the following downstream process.²⁰ However, the high costs associated with antibody purification and immobilization may be prohibitive for industrial-scale use.

Affinity chromatography methods have been used for purification of several viral vectors. For example, adeno-AAV was purified to $>99\%$ purity using a heparin affinity column. The procedure also resulted in a lower number of transfection-deficient viral vectors.⁷ Grimm *et al.*²¹ also reported the purification of recombinant AAV (rAAV) with an antibody that specifically recognizes only assembled virions.

In practice, chromatographic purification schemes are usually multistep in nature and are often combined with some type of size-based separation procedure. For example, by a sequence of membrane ultrafiltration, SEC in group separation mode followed by an anion exchange chromatography in negative mode, human influenza virus could be purified to a level of purity that is compatible with traditional preparations based on density-gradient centrifugation, a technique that is currently accepted by pharmaceutical regulations.¹³ By combining purification steps based on different techniques, it is often possible to achieve a high degree of virus purity combined with high concentrations.

Downstream processing of viral vectors

Viral vectors derived from retroviruses, adenovirus and AAV are used in more than half of the clinical gene therapy trials worldwide (see Table 1). The discussion below focuses on these vectors and how researchers have approached their purification. Although discussing only retroviruses, Andreadis stresses five criteria important in selecting appropriate methods for virus concentration and purification: (i) capacity for processing large volumes of viral preparations with high yield, (ii) preservation of viral stability, (iii) easy scale-up, (iv) low cost of operation and (v) production of a final product that meets the Food and Drug Administration (FDA) standards for biological therapeutics. These criteria are addressed in the discussion that follows.¹¹

Retroviruses

Retroviruses are enveloped RNA tumor viruses consisting of 80–100 nm spherical particles. Recombinant

retroviruses are the most prevalent type of vector used in gene therapy clinical trials today (see Table 1). Unlike most other gene therapy vectors, retroviruses infect only dividing cells so that their transduction efficiency is low. For this reason, large volumes of retroviral stocks containing a high proportion of infective particles are necessary for therapeutic use.¹¹

A major problem confronting large-scale retrovirus production and even *in vivo* therapeutic use is the stability of the viruses. Consequently, production schemes must be concerned with maintaining viral stability. For example, retroviral particles are sensitive to various detergents and ionic and pH conditions. Shear forces generated during purification steps involving filtration or chromatography may cause shedding of the viral envelope, thus affecting infectivity.²² Frequent freeze-thaw cycles, temperatures above 37°C and pH values either below or above 7 have been shown to destabilize MuLV and HIV-1.²³ Traditional methods for concentration and purification of retroviruses have included gradient centrifugation through sucrose or CsCl.¹¹ These methods are not only laborious and difficult to scale up but they can also destroy the transduction ability of retroviruses.⁸ Some investigators have used precipitating (PEG)⁹ and/or complexing additives in conjunction²⁴ with low-speed centrifugation to concentrate retroviruses from cell culture supernatants and thus improve titers. However, impurities are likely to co-precipitate, thus limiting the use of these methods. Membrane ultrafiltration (100 000–300 000 molecular weight cutoff) has also been used to concentrate and partially purify retroviral particles from large volumes of viral particles.^{5,8} Submicron-size retroviruses have been separated from cell and media contaminants by SEC. Studies more than 25 years ago showed that the viral glycoprotein of retroviruses was conserved when they were chromatographically purified on Sepharose 4B (Amersham Biosciences), with increased recovery of biological infectivity.¹⁴ Nowadays, this type of media has been replaced by more rigid resins like Sepharose 6 FF or Sepharose 4 FF (Amersham Biosciences).¹³

While ion exchange and affinity chromatography can be used for retroviral purification and offer high selectivity and generally mild desorption conditions, the low concentration of the virus is often unfavorable for binding to the resin. Additionally, flow rates and elution conditions are critical in maintaining structural and biological integrity of the viruses. However, Kuiper *et al*²⁵ did achieve purification of a MuLV-based gene therapy vector using microfiltration in conjunction with chromatography using a ceramic hydroxyapatite resin.

Lentiviruses

Lentiviral vectors derived from HIV are a subclass of retroviruses that can infect both proliferating and nonproliferating cells. They are extremely efficient vehicles for delivering genes into the brain and hold great promise for future gene therapy of neurodegenerative disorders.²⁶

Zhang *et al*²⁷ describes the harvesting of lentiviruses by ultracentrifugation at 50 000 g for 2 h. However, because rotor capacity at these speeds is limited, virus preparation is laborious and time-consuming, and there is a risk of vector damage associated with prolonged handling. For example, to process 3 l of viral supernatant

requires 18 rounds of centrifugation and more than 50 h. When viral supernatants from cells were pretreated with poly-L-lysine (PLL), virus particles bound to the PLL, and the complexes could be removed by low-speed centrifugation. With this procedure, 3 l of viral supernatant could be concentrated in one round of centrifugation over a period of 2 h. As the processing scheme is rapid with no deleterious effect on gene transfer efficiencies, it is likely to be relevant for *in vivo* gene transfer studies.

Using serum-free culture conditions, Reiser²⁸ prepared concentrated stocks (50- to 300-fold) of lentiviral vectors pseudotyped with alternative Env proteins. The modified virus retained full infectivity, with titers of up to 10⁹ transducing units/ml, after ultracentrifugation or ultrafiltration, thus opening up the possibility for large-scale generation of highly concentrated lentiviral vectors for gene therapeutical applications.

Purification of gp120-depleted HIV-1 particles by anion exchange chromatography resulted in the recovery of a homogeneous preparation of highly pure, intact viral particles. The chromatographic conditions had no effect on viral infectivity or structural integrity and composition of the virus, as evidenced by retention of envelope proteins, lipid bilayer and core components. Preparations derived by anion exchange were equivalent in purity and infectivity to those purified by sucrose density gradients. Large-scale processing based on ion exchange chromatography could thus provide the high titers of virus necessary for gene therapy applications.²⁹

Adenoviruses

Adenoviruses are large (~65–80 nm) nonenveloped double-stranded DNA viruses³⁰ exhibiting a wide tissue tropism and high transduction efficiencies³¹ and are currently used in about 25% of gene therapy protocols (Table 1), targeting mainly cancer diseases.³² Efficient, cost-effective, scalable production and purification processes are thus needed to satisfy the demands for adenovirus vectors for clinical use.

Adenoviruses are highly stable and have traditionally been purified by two rounds of CsCl density-gradient ultracentrifugation that follow multiple freeze-thaw cycles to lyse infected cells. Purified preparations containing 10¹² virus particles/ml can be obtained easily.³³ While gradient centrifugation can generate small-scale clinical grade lots, it is not linear scalable.³² In contrast, column chromatography is an attractive alternative that is scalable, flexible and able to meet cGMP requirements. It is considerably faster – 5 h compared with about 24 h for CsCl – so more vectors can be purified per time unit. Only relatively inexpensive buffers and salts are used, and therefore validating the removal of CsCl from the vector preparation is not an issue. Column chromatography also appears to be more effective in removing residual contaminating viral components that can induce an immune response *in vivo* and thus negate the therapeutic potential of adenovirus-based gene therapy.³¹

As discussed earlier in this review, chromatographic purification of virus particles mainly exploits their size, surface charge or specific receptor binding properties. Owing to their large size, adenovirus particles bind only to the surface of the chromatography resin. Consequently, beads with flexible, elongated spacers in

between the base matrix and the ligand such as Q Sepharose XL (Amersham Biosciences) provide high binding capacity and selectivity.³⁴

A number of chromatography-based methodologies have been reported for purification of adenoviruses, and these invariably yield product that is comparable to or even greater in purity and yield than CsCl-processed preparations.

Kamen and Henry³² combined ion exchange chromatography with SEC to recover 99% pure adenovirus from a 20-l suspension culture production. Lysates were prepared by osmotic shock, treated with DNase, centrifuged, conditioned and filtered, and then applied to the ion exchange column. Prior to the size exclusion polishing step, the column eluate was concentrated by ultrafiltration at which point some virus loss occurred, presumably due to aggregation. They successfully scaled the process up to accommodate 100 l of cell lysate.

In another report, a tandem column chromatography process was designed in which adenovirus was first captured on a DEAE anion exchange media.³¹ Any residual host and viral proteins in the column eluate were subsequently removed on a second column of PolyFlo medium (Puresyn Inc., Malvern, PA, USA). By adjusting the final concentration of isopropanol to 10% in the eluate of the DEAE step, intact virus did not bind to PolyFlo, while host and viral-free proteins did. The tandem DEAE/PF-FT purification process was efficient, scalable and yielded biologically active adenovirus suitable for clinical use. The process was complete in less than 1 day in comparison to gradient centrifugation, which required 2 days.³¹

Huyghe *et al*¹⁸ also used anion exchange chromatography for capture and purification of type 5 adenovirus, but in this case, it has been combined with immobilized metal affinity chromatography in order to produce high-quality adenovirus. In addition, the prior treatment of the cell lysates with nuclease removed contaminating genomic DNA and improved the performance of the chromatographic process in terms of yield and purity.

Adeno-associated viruses

AAV are small, single-stranded DNA viruses that establish a latent, nonproductive infection *in vivo* and require superinfection with a helper virus (eg adenovirus or herpesvirus) for efficient productive replication.³⁵ There are currently eight immunologically distinct serotypes of AAV; these vary in their tissue tropism, a feature that has been used in several purification schemes. rAAV have shown great promise in gene therapy, especially for disorders that affect the liver.³⁶ Their popularity as gene therapy vectors is based on several of their characteristics – they do not induce an immune response toward viral components; they can integrate into human chromosome 19; they do not require actively dividing cells for transduction; and they are nonpathogenic.³⁵ Consequently, many clinical trials are in progress using these vectors. As with other gene therapy vectors, the clinical demand for AAV vectors currently exceeds production capabilities.

Like for adenovirus, traditionally AAV vectors have been purified from cell lysates using several rounds of density-gradient centrifugation (CsCl or iodixanol), an approach that results in vector preparations that are often contaminated with impurities that can cause local

inflammation *in vivo*.^{35–37} Owing to these problems, a great deal of effort has gone into the development of chromatographic procedures for AAV purification. These methodologies provide AAV vectors of high purity and infectivity. The majority of the procedures described thus far have focused primarily on the use of affinity and ion exchange chromatography. Regardless of the nature of the chromatographic method, the overall goal is the development of a process that requires only minimal initial treatment of cell lysates, while yielding a final product that is pure and biologically potent.

The identification of AAV receptors and vector-specific reagents has led to the development of purification protocols for rAAV vectors by affinity matrix chromatography.³⁸ For example, heparin sulfate proteoglycan has been identified as a cellular receptor for AAV-2³⁹ and 2,3-linked sialic acid is required for AAV-5 entry.⁴⁰ Consequently, heparin and mucin affinity columns have been used to recover high yields of AAV-2 and AAV-5, respectively, from cell lysates. While some of these procedures have required pretreatment of the lysates by iodixanol gradient centrifugation⁷ or with detergents or enzymes,⁴¹ others have yielded highly pure vector in a single step.⁴⁰ Grimm *et al*²¹ also reported the use of an immunoaffinity chromatography procedure using monoclonal antibody generated against AAV structural proteins. The one-step procedure was highly selective and recognized only assembled AAV particles.

Although affinity chromatography methods are highly selective and amenable to scale-up, they are more expensive than other methods, and furthermore, the cleaning-in-place of the resin is very restricted. Additionally, they are serotype specific, which precludes application of the same process to all AAV serotypes.⁴² Consequently, much effort has been invested in the development of ion exchange chromatography processes that can be adapted for all AAV serotypes. Brument *et al*⁴² describe a sequence out of cation or anion exchange chromatography to purify AAV vectors of serotype 2 and 5 without compromising its structural integrity or infectivity.

The purity of the preparation was higher and the activity was similar to particles isolated with traditional centrifugation methods. Similar methods have been used to purify potent AAV-8 vectors for clinical use.³⁶

Some procedures have required pretreatment of cell lysates with nucleases prior to chromatography,³⁷ while others have by-passed this step in favor of a more simplistic approach.^{35,36,42,43}

Safety issues and regulatory guidance

A discussion on downstream purification of viral vectors and vaccines is not complete without addressing the critical issue of biosafety. Concerns vary by vector type, but may include risk of contamination by adventitious agents, toxicity, immunogenicity, activation or deactivation of oncogenes or tumor-suppressing genes, and development of replication-competent viruses. Indeed, at least partially because of safety concerns, nonviral, plasmid vectors have become increasingly desirable as gene therapy vehicles; plasmids or naked DNA currently constitute 15.9% of all gene therapy clinical trials (see Table 1).

Oversight and guidance on the safety of gene therapy is addressed by numerous agencies, regional, national and worldwide. The main regulatory guidance in Europe is provided by the Committee for Proprietary Medicinal Products (CPMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) and in the US by the Center for Biologics Evaluation and Research (CBER) of the FDA. In addition to the FDA's oversight role, the Recombinant DNA Advisory Committee (RAC) makes recommendations to the National Institutes of Health (NIH) on gene therapy issues. FDA decides whether individual gene therapy protocols should proceed after evaluating the information in the investigational new drug (IND) application, while NIH/RAC conducts the public scientific and ethical review and public discussion of novel applications of human gene transfer, both of which are carefully considered during FDA's review process.⁴⁴

In October 2001, Europe's CPMP implemented a 'Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products' (CPMP/BWP/3088/99).⁴⁵ This guidance note should be read in conjunction with relevant European legislation and other guidance notes as listed in the above document. In summary, guidance note CPMP/BWP/3088/99 calls for full documentation on the developmental genetics, production, purification, product characteristics, and consistency and routine batch control of the final processed gene therapy product. In addition, the guidance note calls for the provision of such items as the scientific rationale with regard to the choice of the viral vector with reference to the proposed clinical indications, including tissue tropism, transduction efficiency in the target cell population, the presence and maintenance of the viral gene sequence(s) important for anti-viral chemotherapy of the wild-type virus and the tissue specificity of replication. The note stresses that the main safety issue associated with the use of replication-deficient viral vectors concerns reacquisition of replication competency through recombination or complementation with contaminating viral nucleic acid sequences, and states that the strategy taken to render the viral vector replication deficient should be clearly documented. In the case of employing a replication-competent vector, even more detailed documentation is specified.

In November 2004, FDA released a draft guidance to industry titled 'Content and review of chemistry, manufacturing and control (CMC) information for human gene therapy IND applications'.⁴⁶ It states:

...we recommend that you, a sponsor, provide a detailed description of where and how the gene therapy product is manufactured. Include all of the components used during the manufacture of the gene therapy product, such as vector, cells or cell bank systems, and any reagents or excipients. In addition, describe all procedures used during the manufacturing process. Examples of these procedures may include production and purification of the vector, preparation of *ex vivo* gene modified cells, and final formulation of the product. This information will allow us to assess the identity quality, purity, and potency of your product. For further information, refer to the 'Guidance for Human Somatic Cell Therapy and

Gene Therapy',⁴⁷ the guidance on 'Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products'⁴⁸... and, when finalized and where relevant, the 'Draft Guidance for Reviewers: Instructions and Template for Chemistry, Manufacturing and Control (CMC) Reviewers of Human Cell Therapy Investigational New Drugs (INDs).'⁴⁹

A review by Borellini and Ostrove⁵⁰ discusses recommended quality assurance and safety testing programs from early product development through preclinical toxicology and pharmacokinetic studies, and serves as a useful complement to the governmental guidelines.

Concluding remarks

As our fundamental understanding of viral biology increases, we should see improvements in the types of downstream purification schemes used. For example, identification of virus-specific ligands would allow for more affinity-based purification systems to be developed, possibly broadly applicable to the purification of vectors derived from different virus backgrounds.

Significant advances in the design and construction of safer and more efficient viral vectors are taking place, but coupled with that, there is a need for more research directed toward the design of packaging cell lines that efficiently complement the necessary viral functions and for a significantly improved understanding of culture conditions that promote higher yields of vector production. Lyddiatt and O'Sullivan³ argue that strategic consideration should be given to the design of appropriate separations systems in tandem with the development of vectors and producer cell systems.

The field of gene therapy continues to witness great progress. Recently, for example, researchers discovered a way to overcome one of the major hurdles in gene therapy for cancer: its tendency to kill normal cells in the process of eradicating cancer cells.⁵¹ Gene therapy based on the described technique should be effective for a wide range of tumors because the virus is constructed to exploit a characteristic of all solid cancers. This and other advances will mean an ever-growing need to optimize the downstream purification of viral vectors for pre-clinical and clinical testing.

References

- 1 Blaese R *et al.* T lymphocyte-directed gene therapy for ADA deficiency SCID: initial trial results after 4 years. *Science* 1995; 270: 475–480.
- 2 The Journal of Gene Medicine, Gene Therapy Clinical Trials, Charts and Tables, Vectors, 2005; <http://82.182.180.141/trials/FMPro?-db=Trials.FP5&-format=vectors.html&-lay=Vectors&-findAll>.
- 3 Lyddiatt A, O'Sullivan D. Biochemical recovery and purification of gene therapy vectors. *Curr Opin Biotechnol* 1998; 9: 177–185.
- 4 Wu N, Ataai M. Production of viral vectors for gene therapy applications. *Curr Opin Biotechnol* 2000; 11: 205–208.
- 5 Braas G, Searle P, Slater N, Lyddiatt A. Strategies for the isolation and purification of retroviral vectors for gene therapy. *Bioseparation* 1996; 6: 211–228.

- 6 Guilherme N, Monteiro G, Prozeres D, Cabral J. Downstream processing of plasmid DNA for gene therapy and DNA vaccine applications. *TIBTECH* 2000; **18**: 380–388.
- 7 Zolotukhin S *et al.* Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy* 1999; **6**: 973–985.
- 8 Saha K, Lin Y-C, Wong P. A single method for obtaining highly viable virus from culture supernatant. *J Virol Methods* 1994; **46**: 349–352.
- 9 Aboud M, Wolfson M, Hassan Y, Huleihel M. Rapid purification of extracellular and intracellular Moloney murine leukemia virus. *Arch Virol* 1982; **71**: 185–195.
- 10 Morling F, Russell S. Enhanced transduction efficiency of retroviral vectors coprecipitated with calcium phosphate. *Gene Therapy* 1995; **2**: 504–508.
- 11 Andreadis S *et al.* Large-scale processing of recombinant retroviruses for gene therapy. *Biotechnol Prog* 1999; **15**: 1–11.
- 12 Hammar L, Gilljam G. Extraction of HIV-1 in aqueous two-phase systems to obtain a high yield of gp120. *AIDS Res Hum Retroviruses* 1990; **6**: 1379–1388.
- 13 Morenweiser R. Amersham Biosciences. *Downstream* 2004; **37**: 20–21.
- 14 McGrath M, Witte O, Pincus T, Weissman I. Retrovirus purification: method that conserves envelope glycoprotein and maximizes infectivity. *J Virol* 1978; **25**: 923–927.
- 15 Ljunglof A, Bergvall P, Bhikhabhai R, Hjorth R. Direct visualization of plasmid DNA in individual chromatography adsorbent particles by confocal scanning laser microscopy. *J Chromatogr A* 1999; **844**: 129–135.
- 16 Martin M, El Azher M, Vasseur M. Ionic strength- and temperature-induced K_{Ca} shifts in the uncoating reaction of rotavirus strains RF and SA11: correlation with membrane permeabilization. *J Virol* 2002; **76**: 552–559.
- 17 Specht R *et al.* Densitometry virus purification by ion exchange membranes. *Biotechnol Bioeng* 2004; **88**: 465–473.
- 18 Huyghe B *et al.* Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography. *Hum Gene Ther* 1995; **6**: 1403–1416.
- 19 Herzer S, Wegmann T, Beckett P. Isoelectric titration curves of viral particles as an evaluation tool for ion exchange chromatography. *Life Sci News* 2003; **13**: 16–18.
- 20 Valdes R *et al.* Hepatitis B surface antigen immunopurification using a plant-derived specific antibody produced in large scale. *Biochim Biophys Res Commun* 2003; **310**: 742–747.
- 21 Grimm D, Kern A, Rittner K, Kleinschmidt J. Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum Gene Ther* 1998; **9**: 2745–2760.
- 22 Walther W, Stein U. Viral vectors for gene transfer. *Drugs* 2000; **60**: 249–271.
- 23 Higashikawa F, Chang L. Kinetic analyses of stability of simple and complex retroviral vectors. *Virology* 2001; **280**: 124–133.
- 24 Darling D *et al.* Low-speed centrifugation of retroviral vectors absorbed to a particulate substrate: a highly effective means of enhancing retroviral titer. *Gene Therapy* 2000; **7**: 914–923.
- 25 Kuiper M, Sanches R, Walford J, Slater N. Purification of a functional gene therapy vector derived from Moloney murine leukemia virus using membrane filtration and ceramic hydroxyapatite chromatography. *Biotechnol Bioeng* 2002; **80**: 445–453.
- 26 Baekelandt V *et al.* Optimized lentiviral vector production and purification procedure prevents immune response after transduction of mouse brain. *Gene Therapy* 2003; **10**: 1933–1940.
- 27 Zhang B *et al.* A highly efficient and consistent method for harvesting large volumes of high-titre lentiviral vectors. *Gene Therapy* 2001; **8**: 1745–1751.
- 28 Reiser J. Production and concentration of pseudotyped HIV-1-based gene transfer vectors. *Gene Therapy* 2000; **7**: 910–913.
- 29 Richieri S *et al.* Characterization of highly purified, inactivated HIV-1 particles isolated by anion exchange chromatography. *Vaccine* 1998; **16**: 119–129.
- 30 Bondoc L, Fitzpatrick S. Size distribution analysis of recombinant adenovirus using disc centrifugation. *J Ind Microbiol Biotechnol* 1998; **20**: 317–322.
- 31 Green A *et al.* A new scalable method for the purification of recombinant adenovirus vectors. *Hum Gene Ther* 2002; **3**: 1921–1934.
- 32 Kamen A, Henry O. Development and optimization of an adenovirus production process. *J Gene Med* 2004; **6**: 184–192.
- 33 Smith A. Viral vectors in gene therapy. *Annu Rev Microbiol* 1995; **49**: 807–838.
- 34 Blanche F *et al.* An improved anion-exchange HPLC method for the detection and purification of adenoviral particles. *Gene Therapy* 2000; **7**: 1055–1062.
- 35 Smith R, Ding C, Kotin R. Serum-free production and column purification of adeno-associated virus type 5. *J Virol Meth* 2003; **114**: 115–124.
- 36 Davidoff A *et al.* Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock. *J Virol Methods* 2004; **121**: 209–215.
- 37 O'Riordan C, Lachapelle A, Vincent K, Wadsworth S. Scaleable chromatographic purification process for recombinant adeno-associated virus (rAAV). *Gene Med* 2000; **2**: 444–454.
- 38 Summerford C, Samulski R. Viral receptors and vector purification: new approaches for generating clinical-grade reagents. *Nat Med* 1999; **5**: 587–588.
- 39 Summerford C, Samulski R. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 vectors. *J Virol* 1998; **72**: 1438–1445.
- 40 Auricchio A, O'Connor E, Hildinger M, Wilson J. A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Mol Ther* 2001; **4**: 372–374.
- 41 Clark K, Liu M, McGrath J, Johnson P. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild type viruses. *Hum Gene Ther* 1999; **10**: 1031–1039.
- 42 Brument N *et al.* A versatile and scalable two-step ion-exchange chromatography process for the purification of recombinant adeno-associated virus serotypes-2 and -5. *Mol Ther* 2002; **6**: 678–686.
- 43 Blouin V *et al.* Improving rAAV production and purification: towards the definition of a scaleable process. *J Gene Med* 2004; **6**: 223–228.
- 44 Siegel J. Gene Therapy: Is There Oversight for Patient Safety?. Testimony before the Senate Committee on Health, Education, Labor and Pensions, Subcommittee on Public Health, February 2, 2000.
- 45 The European Agency for the Evaluation of Medicinal Products, Note for Guidance on Quality, Preclinical and clinical aspects of gene transfer medicinal products, 9CPMP/BWP/3088/99, London, April 24, 2001, pp 1–33, <http://www.emea.eu.int/pdfs/human/bwp/308899en.pdf>.
- 46 Food and Drug Administration [Docket No. 2004D-0465], Draft Guidance for Food and Drug Administration Review Staff and Sponsors: Content and Review of Chemistry, Manufacturing, and Control Information for Human Gene Therapy Investigational New Drug Applications, November 1, 2004, pp 1–4, <http://www.fda.gov/ohrms/dockets/98fr/cb0240.pdf>.
- 47 Food and Drug Administration Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy, dated March 1998, <http://www.fda.gov/cber/gdlns/somgene.pdf>.
- 48 Food and Drug Administration Guidance for Industry: Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-derived Products, dated November 1995, <http://www.fda.gov/cder/guidance/phase1.pdf>.

- 49 Food and Drug Administration Draft Guidance for Reviewers: Instructions and Template for Chemistry, Manufacturing, and Control (CMC) Reviewers of Human Somatic Cell Therapy Investigational New Drug Applications (INDs), dated August 2003, <http://www.fda.gov/cber/gdlns/cmcsomcell.pdf>.
- 50 Borellini F, Ostrove J. The transfer of technology from the laboratory to the clinic: in-process controls and final product testing. In: Meager A (ed). *Gene Therapy Technologies, Applications and Regulations*. John Wiley & Sons Ltd: New York, 1999, pp 359–373.
- 51 Su Z-Z *et al*. Targeting gene expression selectively in cancer cells by using the progression-elevated gene-3 promoter. *Proc Natl Acad Sci USA* 2005; **102**: 1059–1064.