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Recovery in aqueous two-phase systems of nanoparticulates applied as surrogate mimics for viral gene therapy vectors

Geneviève M.F. Braas, Simon G. Walker, Andrew Lyddiatt*

Biochemical Recovery Group, Centre for Bioprocess Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Abstract

The partition behaviour of nanoparticulate inclusion bodies (IBs) in various states of purity were evaluated as surrogate mimics for adenovirus and retrovirus products in method scouting experiments with aqueous two-phase systems (ATPSs). Such systems are proposed for effective, high capacity downstream processing (DSP) of viral gene therapy vectors. Studies with mimics provided simple descriptions of particle partition which may benefit the field of vector DSP, where experimental material is rarely available for research and development in quantities and concentrations representative of clinical manufacture. Polyethylene glycol (PEG)–salt and PEG–dextran ATPSs were screened in respect of the partition recovery of IBs from crude feedstocks. Select candidate systems were similarly evaluated with limited preparations of adenovirus and retrovirus with respect to fractional recoveries of infectivity and particle number. Maintenance of the former was good, whilst comparison of the latter with quantitation of unwashed and washed IBs indicated poor utilisation of the inherent high capacities of ATPSs in viral experiments. This is discussed in the context of the volumetric throughput and capacities reported in the literature for the recovery of infective viruses in ultracentrifugation and chromatographic processes.

Keywords: Aqueous two-phase systems; Gene therapy vectors; Nanoparticles; Adenoviruses; Retroviruses

1. Introduction

A growing interest continues in the development of viral and non-viral vectors for applications in human gene therapy [1–4]. Inevitably, particular emphases have been focused upon the molecular biology and physiology of vectors and their producer or packaging cell lines. However, production titres for many vectors are currently modest, whilst the volumetric capacities and throughputs associated with the methods of ultracentrifugation and chromatography commonly adopted for the manufacture of clinical trial lots are physically limited. This raises doubts concerning the capabilities of conventional downstream processing technologies to service the manufacturing needs and markets projected for successful gene therapy products (discussed in Braas et al. [5] and Lyddiatt and O'Sullivan [6]). For example, current estimates of doses (10^{10} to 10^{14} particles for viral vectors) and projected annual demands for successful therapeutics (10^{12} to 10^{20} viral particles) sit uneasily with cell culture titres (10^{6} and 10^{11} per ml for retrovirus and adenovirus, respectively) and the theoretical and recorded capacities of commonly used chromatographic adsorbents ($<10^{12}$ particles per ml adsorbent [5,6]). Many of these adsorbents

*Corresponding author. Tel.: +44-121-4145-278; fax: +44-121-4145-278/324.

E-mail address: a.lyddiatt@bham.co.uk (A. Lyddiatt)

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have been developed for efficient commercial recovery of proteins (typically <5 nm in diameter), and their internal porosity is unsuited for equivalent high binding capacities and unrestricted adsorption/desorption of viral and plasmid nanoparticulate products (20 to 150 nm in diameter). In addition, adsorption chromatography may not discriminate sufficiently between whole virus particles and their component parts which may compromise their effectiveness in selectively purifying intact, infective virus.

Aqueous two-phase systems (ATPSs) have been studied in the past for the handling and recovery of a number of viral types [7,8], but appropriate technologies have not been developed for the manufacture of infective viral vaccines. Speculative comparisons regarding the mechanics of nanoparticulate partition between a liquid mobile phase (the product feedstock) and a second liquid phase (as in aqueous solvent extraction) or a second solid phase of limited porosity (as in a protein adsorption) suggest that ATPSs might possess superior volumetric capacity (i.e., product occupancy per ml of system). The process intensification recorded for the recovery of crude inclusion bodies in polyethylene glycol (PEG)–phosphate ATPS supports this view [9,10], and additionally confirms the capability of such systems to separate particles from nanoparticulate cell debris and soluble proteins. These performance characteristics invite the study of such systems for the recovery and partial purification of infective viral vectors from cell culture feedstocks where the common impurities are non-infective vectors, viral components, cell debris and variously sized molecular contaminants. However, the concentrations of virus needed to rigorously evaluate the relative selectivities and capacities of adsorption and ATPS for effective product recovery (possibly $>10^{14}$ total particles per batch) commonly exceed the economic and practical resources of most research and development laboratories.

An alternative strategy might involve the fruitful study of a surrogate material which should be readily available, possess similar properties of size, density and surface characteristics to common viral products, and be readily and accurately quantified in simple, method scouting experimentation with downstream processing technologies. Such a material, comprising small inclusion bodies (IBs) of misfolded yeast α-glucosidase expressed in a recombinant strain of *E. coli*, is available in multi-gram quantities in homogenates of microbial cell paste produced in simple, pilot scale fermentations [9]. They can be simply recovered as nanoparticulate preparations in high states of purity by the exploitation of the appropriate PEG–phosphate ATPS, wherein the product is recovered from the bottom phase as a suspension or sediment [10]. The purification of IBs from cell debris and soluble macromolecules confronts and overcomes many of the practical problems associated with the recovery of infective viral vectors. We report here the characterisation of two subclasses of such inclusion bodies (unwashed and washed) which are proposed as candidate surrogate mimics for enveloped and non-enveloped viral vectors. Method scouting experiments have been undertaken to determine the selectivities and capacities of selected ATPSs with respect to their recovery from particulate feedstocks. Methods so established have then been critically evaluated in short experimental campaigns conducted with putative adenovirus and retrovirus gene therapy vectors made available to the study in extremely limited quantities.

2. Experimental

Unless stated otherwise, all chemicals listed in Sections 2.1–2.7 were purchased from Sigma (Poole, UK). Cell culture media and related products were provided by the Institute for Cancer Studies (Birmingham, UK).

2.1. Production of inclusion body preparations

Optimised recombinant *E. coli* fermentations (37°C, pH 8, on M9 minimal medium, induced with 5 m*M* IPTG [11],) enabled the controlled production of yeast α-glucosidase expressed as cytoplasmic IBs. Cell disruption by five passes through an APV-Gaulin high-pressure homogeniser (55.2 MPa; APV Products, Crawley, UK) achieved total release of the IBs from the cell cytoplasm and micronisation of the cellular debris, yielding a particulate suspension characterised by a mean particle diameter of 150 nm [12]. Washing procedures performed on IB-rich

fractions exploited a combination of EDTA (10 m*M*), lysozyme (1.6 g/l) and Triton X-100 (2%, v/v) in Tris buffer (50 m*M*) at pH 8, 22°C [13,14].

2.2. Harvest of adenoviruses

Adenoviruses were replication-deficient adenovirus type 5 (Ad5) transformed with the firefly luciferase reporter gene under the control of a cytomegalovirus promoter. Adenoviral vectors were released and recovered from packaging cell lines, 48 h post-transduction, after three cycles of freezing and thawing in phosphate-buffered saline (PBS) and 10% (w/v) glycerol. Cellular debris was removed by centrifugation (770 *g*, 10 min, 4°C). The lysis suspension was further clarified by precipitation with *n*-butanol at a 1:100 volume ratio and centrifugation treatment as above. Further purification was achieved by continuous gradient centrifugation employing CsCl (5 ml) in 50 m*M* Tris, pH 8.0 overlaid with (3 ml) a 40% (w/v) glycerol in 10 m*M* Tris, pH 8.0 and 1 m*M* EDTA solution. The viral preparation was layered on a 5 ml linear CsCl gradient ranging in density from 1.32 to 1.45 g/ml, centrifuged at 140 000 *g*, 105 min, 16°C. Banded fractions were sequentially harvested following puncture of the tubes, and the viral suspension was diluted with an equal volume of sterile PBS and 10% (w/v) glycerol to minimise aggregation of the viral particles. Purified adenoviruses (total particle number ~10^{12}: i.e., 10^{12} viruses) were recovered after dialysis (3×1 h) against PBS supplemented with 10% (w/v) glycerol at 4°C.

2.3. Quantitation of viral particles by bicinchoninic acid assay

The total number of viral particles present in the purified supernatant or debris-free solutions was estimated using the bicinchoninic acid (BCA) assay (Microwell Plate Protocol; Pierce, Rockford, IL, USA). Samples and corresponding blank solutions (25 μl) were pipetted into 96-well plates. BCA reagent (200 μl) was added to each well and the plate was shaken for 30 s and incubated at 60°C for 30 min. Plates were cooled to room temperature and absorbance measurements were conducted in a Spectra III plate-reader (SLT Instruments, Austria) at 540 nm. The total number of viral particles present (*P*) was expressed as an equivalent BSA concentration, calculated by application of the following correlation: 1 mg/ml soluble BSA equivalent=$3.45 \cdot 10^{12}$ particles/ml (S. Longhurst, Cobra Therapeutics, Keele, UK).

2.4. Plaque assay

HER911 (human embryo retinoblast) cells were seeded into six-well plates at a density of $5 \cdot 10^5$ cells/well in 3 ml medium [Dulbecco's minimum essential medium; DMEM, 2 m*M* L-glutamine, 10% (v/v) FCS, 50 U/ml penicillin, 50 μg/ml streptomycin] and were incubated for 48 h at 37°C in a 5% CO_2 humidified atmosphere. On day 3, viral samples were diluted to yield particle numbers ranging from 10^{-6} to 10^{-10}. The growth medium was discarded and replaced with 0.5 ml of virus at each dilution and all viral concentrations were evaluated in triplicate. The plates were incubated at 37°C for 90 min with constant rocking. At the end of the adsorption period, the vector-containing medium was carefully removed by aspiration. The cells were then overlaid with 3 ml of DMEM containing 12.5 m*M* $MgCl_2$, 0.375% (w/v) sodium hydrogencarbonate, 2 m*M* L-glutamine, 2% (v/v) FCS and 0.7% (w/v) Noble agar. The plates were cultured in a humidified, 5% CO_2 atmosphere at 37°C and cells were fed every 3–4 days by overlaying 2 ml of DMEM and 0.7% (w/v) Noble agar. On days 12–14, depending on the size of plaques and the state of the cells, the final overlay included neutral red (0.1%, w/v) to enhance plaque visualisation. The plates were counted 24 h later. The number of plaques was recorded, averaged for each dilution of viruses and the original viral titer was estimated.

2.5. Luciferase assay

The number of infectious viral particles (*I*) present in the cell lysate was estimated by the luciferase assay. On day 1, A549 cells (human carcinoma, ATCC; CCL-185) were seeded in 24-well plates at a density of $1 \cdot 10^4$ cells/well and grown at 37°C in a humidified air–5% CO_2 atmosphere. On day 3, the culture medium was discarded and replaced with 1 ml of sample containing the adenoviruses. The plates

were incubated for a further 90 min with gentle shaking. The infectious supernatants were removed by aspiration and replaced with fresh medium and incubated for 48 h. On day 5, the wells were emptied of the medium and washed twice with PBS (pH 7.3). The cells were then lysed by addition of 250 μl of lysis buffer (25 m*M* Tris–phosphate, pH 7.8, 2 m*M* DTT, 2 m*M* 1,2-diaminocyclohexane-*N*,*N*,*N′*,*N′*-tetraacetic acid, 10% (w/v) glycerol and 1% (v/v) Triton X-100). Each lysate was transferred into Eppendorf vials prior to centrifugation at 11 000 *g* for 10 s. The pellets were discarded and the supernatants were analysed. Estimates of the luciferase contents were performed by direct addition of 100 μl of luciferase assay reagent (beetle luciferin, Promega, MA, USA) to 50 μl of sample in a Lumat LB 9507 luminometer (ET&G Berthold, UK). For each experiment, a calibration curve was established by measuring the luciferase present in samples recovered from cells infected with known amount of infectious viruses (determined by plaque assay).

2.6. Aqueous two-phase systems

Partition experiments were conducted in (15 ml) sterile graduated centrifuge tubes. PEG 600 and 8000 and a second phase forming chemical (ammonium sulphate or potassium phosphate, respectively) were added as sterile stock solutions. Sterile water was added to the PEG–phosphate, whilst McIlvaine type buffer (154.7 m*M* Na_2HPO_4, 22.8 m*M* citric acid and 97 m*M* KCl at pH 6.8 and ionic strength of 0.5 *M* diluted five-fold to obtain a working ionic strength of 0.1 *M*) was introduced into the PEG–ammonium sulphate systems. All systems had a final mass of 9.0 g or 9.9 g depending upon the mass of particulate suspension to be treated. Thorough mixing of systems was achieved by continuous rotation of the sample tubes for 10 min at room temperature, 20°C. The viral solutions (100 μl to 1 ml) were added to the systems and the tubes were gently inverted 20 times by hand to facilitate an equilibrium of partition between particles and phase chemical components. Centrifugation (1200 *g*, 5 min, 20°C) was used to accelerate phase separation. Samples were extracted from each phase (including interphases; see Figs. 1 and 2) in all systems and aliquoted for subsequent analysis of content.

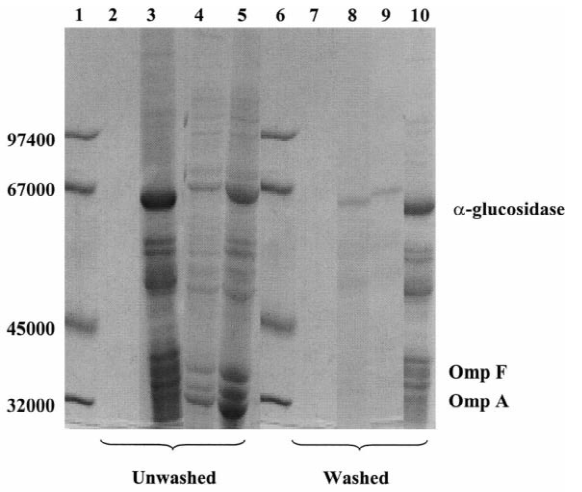


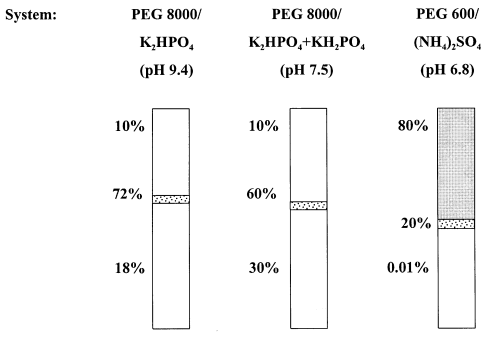
Fig. 1. Partition of α-glucosidase inclusion bodies before and after washing procedures. Inclusion body-rich feedstocks were loaded into PEG 8000–K_2HPO_4 ATPS at pH 9.4. After thorough mixing and phase separation (see Section 2.6), aliquots of phase were removed and prepared for analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (as described in Section 2.7). Lanes 2 to 5 contain the top, interphase, bottom and sediment phases, respectively of systems loaded with unwashed inclusion bodies. Lanes 7 to 10 contain the corresponding phases harvested from systems loaded with washed inclusion bodies. Lanes 1 and 6 contain Coomassie blue low-range-molecular-mass markers, having molecular masses as indicated.

All experiments were performed on samples prepared in triplicate. Although no indications of variance in individual sample analysis and associated errors are reported herein, data had a standard error of less than 5%. Full details of experimental analysis and a complete statistical treatment of the individual data points is presented in Ref. [14].

2.7. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis of the isolated fractions from ATPS experiments was conducted according to a modified method of Laemmli [15]. Samples were resuspended in denaturing buffer, 3.25% SDS and 5% β-mercaptoethanol in 62.5 m*M* Tris–HCl, pH 6.8 and boiled at 100°C for 5 min prior to electrophoretic analysis on a 7.5% T–2.65% C[1] poly-

[1] T=(g acrylamide+g Bis)/100 ml solution. C=g Bis/% T.



Phase	Volume (ml)		
TOP	3.05	3.35	4.40
INTERPHASE	0.40	0.40	0.40
BOTTOM	7.05	6.60	4.90

Fig. 2. Partition behaviour of infectious adenoviruses in ATPS loaded with viral suspensions. Adenoviral vectors were added in viral suspension as described in Experimental. The detection of infectious virions was performed by the luciferase assay. Percentages are expressed relative to the total number of infectious virions identified in the system. The accompanying Table displays the volumes of the phases (top, bottom and interphase) used in estimating the distribution of infectivity.

acrylamide separating gel and a 4% stacking gel operated in a Mini Protean vertical electrophoresis cell (Bio-Rad Labs., Hemel Hempstead, UK). Where possible protein bands were visualised by staining with 0.1% Coomassie brilliant blue R-250, 40% methanol, 10% acetic acid. Laser densitometry (Ultroscan XL; Pharmacia, Uppsala, Sweden) was used to quantify the intensity of discretely stained protein bands relative to known masses of BSA standards electrophoresed within a defined range of sample concentrations (25–250 μg/ml). Alternatively, protein bands were visualised by silver-staining (Silver Stain kit, Bio-Rad Labs.).

3. Results and discussion

3.1. Partition of inclusion bodies

The use of specific washing procedures redefined the appearance and exposed new surface characteristics of inclusion body particles (EM data not shown). Washing removed the membrane protein material commonly associated with the IBs, to present a more hydrophilic, protein surface. Quantitative SDS–PAGE analysis of unwashed IBs indicated a common banding pattern which included α-glucosidase (relative molecular mass; M_r 67 000) together with co-purifying *E. coli* outer membrane proteins (*ompF* and *ompA* with M_r 40 000 and 37 000, respectively, Fig. 1, lane 10). The *E. coli* proteins were identified by N-terminal sequencing of samples recovered from electroblot transfer to membranes [12]. Other proteins could not be so identified because of blocked N-termini. The washing procedures efficiently removed both *ompF* and *ompA*, as soluble material in the washing supernatant whilst others (particularly one at M_r 45 000, tentatively identified as *E. coli ompC*) remained associated with the IB structure.

Partitioning studies, performed with 100 mg of wet biomass, in 10/10% (w/w) PEG 8000–phosphate systems demonstrated that removal of the associated membrane proteins caused a redistribution of the washed and purified IBs from the interphase (70% recovery relative to input; based on laser densitometry data) to a discrete sediment within the bottom phase (95% recovery). Partitioning studies performed on the solubilised membrane components demonstrated that they exhibited a strong tendency to accumulate as an interphase (data not shown). The presence of membrane contaminants thus had a direct influence on the partition characteristics of the target particles. From these studies, speculative analogies for partition behaviour based upon inherent surface properties in the defined ATPS were drawn between IBs and viral particles (i.e., unwashed IBs and enveloped viruses such as retroviruses; washed IBs and non-enveloped virions such as adenoviruses) as illustrated in Table 1.

The Table indicates that all four types of particles have similar diameters (i.e., ~120 nm) and other parallel properties. The association of membrane fragments with IBs after homogenisation suggested that the membrane contaminants could be viewed as a particle coating such that the surface properties were analogous to those of enveloped viruses. Removal of the membrane fragments exposed the protein surface of the IBs, which more closely resembled the protein outer shell of non-enveloped virions such as adenoviruses. The up-scaled production of IBs was much easier than that for viral vectors. Production volumes herein were greater

Table 1
Characterisation of target nanoparticulate bioproducts[a]

	 Washed inclusion body	 Adenovirus	 Unwashed inclusion body	 Retrovirus
Shape:[1]	Irregular sphere	Icosahedral	Irregular sphere	Irregular sphere
Size:[2]	140 nm mode diameter	70–100 nm in diameter	190 nm mode diameter	80–130 nm in diameter
Buoyant density:[3]	~1.25 g/ml	~1.34 g/ml	~ 1.20 g/ml	~1.16 g/ml
Surface properties:[4]	Protein surface after washing procedure	Protein outer shell	Membrane fragments (*ompA* and *ompF*) associated to protein surface	Membrane coated with 100–300 copies of surface protein (SU)
Nature:[5]	Very resistant	Fragile	Very resistant	Extremely fragile
Production:	Easy with high yields of material (1 g/l)	Laborious with production of high titres (10^{12} particles/ml)	Easy with high yields of material (1 g/l)	Laborious with production of low titres (10^{7} particles/ml)
Product feedstock:	$6.96 \cdot 10^{14}$ particles/ml	$2.07 \cdot 10^{12}$ particles/ml	$2.78 \cdot 10^{14}$ particles/ml	$1.5 \cdot 10^{4}$ particles/ml

[a] All characteristics were established in Refs. [12,14]. In summary, [1]determined by EM studies, [2]determined by laser light scattering on Malvern Mastersizer; [3]determined by discontinuous gradient ultracentrifugation; [4]inferred from SDS–PAGE and EM studies; [5]established in shelf life experimentation.

(i.e., 20 l for *E. coli* fermentations compared to 2 l roller bottles for adenoviruses) and the production times much shorter (i.e., 16 h for the IB fermentations compared with more than 72 h for adenovirus harvests). The value of exploiting nanoparticulate protein inclusion bodies (available in gram quantities) as surrogate mimics for viral vectors in method scouting experiments to establish candidate ATPSs therefore invited further study.

3.2. Partition of adenoviruses

The criteria set for the selection of the optimal phase for particle recovery included the selective fractionation of the target product and a simple integration of the product stream with subsequent downstream processing operations. The polymeric content and elevated viscosity of top phases, and the difficulty of physically accessing interphases, recommends the bottom phase or bottom sediment as the preferred destination compartment for target products. Such fractions, generated by single- or two-stage ATPSs [16], can be readily processed further by ultrafiltration, diafiltration and/or chromatographic operations (discussed in Ref. [6]). An additional concern for all aqueous two-phase systems studied herein was to ensure that the virions maintained their infective capacity throughout the entire process purification scheme. A common characteristic documented in the description of viral vector purification for gene therapy is the ratio of the numbers of total and infectious particles (the *P*:*I* ratio). Preparations of many animal viruses contain particles which appear to be non-functional and thus the US Food and Drug Administration, in an effort to establish a minimum acceptability standard for the use of adenovirus vectors in clinical trials, have recommended a *P*:*I* ratio of <100 for purified products (discussed in Zolotukhin et al. [17]). This corresponds to a biologically infectious fraction $>1\%$. In the present study, the number of infectious virions (I) was obtained by relating the number of luciferase units

detected by luminometry to a calibration curve established for the production of enzyme by known titres of adenovirus (determined by plaque assays). Consequently the quantity of luciferase produced in unknown samples was directly proportional to the number of infectious viruses. The total number of virus particles (*P*) was estimated using the BCA assay. In this technique the BCA reagent reacted with the capsid proteins of the viral entities to generate a coloured response which (within prescribed limits; 10–400 μg soluble BSA equivalents) was correlated as being directly proportional to the total number of viral particles present in the sample (S. Longhurst, personal communication, Cobra Therapeutics).

The ATPS extraction experiments were performed with 100 μl of viral suspension (i.e., $\sim 2.07 \cdot 10^{11}$ particles/system $= 1.65 \cdot 10^{11}$ infectious viruses/system). In addition to the three PEG–salt ATPSs screened (illustrated in Fig. 2), a PEG 6000–dextran T500 system was also evaluated but subsequently eliminated from further study due a surprising total loss of viral infectivity. Systems constructed with PEG 600–$(NH_4)_2SO_4$ partitioned particles to the top phase (~80%), but the presence of PEG and ammonium sulphate appeared to severely distort quantitative assays of *P*:*I* ratios (see Table 2). The PEG 8000–phosphate systems demonstrated the partitioning of infectious adenoviruses predominantly to the interphase at both of the operational pH values used (7.5 or 9.4). In contrast to the IBs discussed in Section 3.1, the presence of a sediment was not detected under the maximal loading conditions available ($< 2.07 \cdot 10^{12}$ particles/ml ~600 μg protein equivalents of soluble BSA). At pH 9.4 most of the infectious virions were recovered in the interphase (72% of loading) as were most of the cellular contaminants (see Fig. 2). The *P*:*I* ratio (Table 2) suggested that the working conditions of the system (high salt and high pH) did not affect the infective capacity of the virions. However, the presence of visible contaminants (e.g., protein and debris) in the interphase indicated that the purification factor was low. Although the recovery of infectious particles in the bottom phase was lower (~18%) the improved *P*:*I* ratio, combined with absence of cellular contaminants, suggested that this phase was more effective for the selective recovery and subsequent purification of vectors. Similar trends in partition behaviour were observed at pH 7.5 (interphase 60%, bottom phase 30%). However, the *P*:*I* ratios suggested that optimal recovery of infectious particles would be best achieved by harvesting the interphase rather than the bottom phase.

The current technical difficulties which arise with the recovery and further processing of such interphase fractions led to the decision to define the bottom phase of PEG 8000–phosphate systems as

Table 2
Summary of adenovirus partition data[a]

System	Phase	Total No. particles (*P*)	No. infectious particles (*I*)	*P*:*I* ratio
PEG 8000–K_2HPO_4 (10/10%, w/w, pH 9.4)	Top	$7.27 \cdot 10^9$	$9.43 \cdot 10^8$	7.71
	Interphase	**$8.08 \cdot 10^{10}$**	**$7.10 \cdot 10^9$**	**11.38**
	Bottom	*$1.15 \cdot 10^{10}$*	*$1.82 \cdot 10^9$*	*6.32*
PEG 8000–K_2HPO_4+KH_2PO_4 (10/10%, w/w, pH 7.5)	Top	$9.77 \cdot 10^{10}$	$2.07 \cdot 10^9$	47.19
	Interphase	**$1.04 \cdot 10^{11}$**	**$1.05 \cdot 10^{10}$**	**9.90**
	Bottom	*$1.27 \cdot 10^{11}$*	*$4.99 \cdot 10^9$*	*25.45*
PEG 600–$(NH_4)_2SO_4$ (20/20%, w/w, pH 6.8)	***Top***	**–**	***$8.24 \cdot 10^9$***	***N/A***
	Interphase	$1.54 \cdot 10^{10}$	$1.90 \cdot 10^9$	8.11
	Bottom	$5.27 \cdot 10^{11}$	$1.41 \cdot 10^6$	372957.4

[a] Estimation of the total particle number (*P*) was achieved by application of the BCA assay and correlation quoted in the text. The number of infectious particles (*I*) was derived from the luciferase assay (full details of both techniques are given in Experimental). In the PEG 600–salt system phase chemical interference with the BCA assay reagent inhibited the determination of the total particle number. The bold type indicates the phases where the greatest number of infectious particles were recovered. The proposed ideal phase for particle location and recovery by further processing is indicated by italics.

the optimal target phase for the selective fractionation and recovery of infectious vectors for gene therapy. The isolation of such a liquid phase or sediment from which many impurities (debris and macromolecules) have been removed facilitates the direct introduction of this feedstock into conventional filtration or chromatographic based processing operations.

3.3. Comparison between washed inclusion bodies and adenovirus partition behaviour

The partition behaviour of washed inclusion bodies in 10/10% (w/w) PEG 8000–phosphate systems, when performed with 100 mg wet mass of material (i.e., ~$5.58 \cdot 10^{13}$ total particles), indicated that the nanoparticles preferentially partitioned to a sediment (95% of the initial IB content) beneath the bottom phase. The formation of such a sediment was not observed in extraction processes involving adenoviruses. This was attributed in part to the quantity of particles loaded in the system in these experiments. In fact, the restricted availability of viral vectors (discussed earlier) currently limits the scale of viral particle loading in ATPS experiments to the picogram to microgram range of protein concentrations (equivalent to 10^4 to 10^{10} viruses/ml). Consequently, to better understand the partition behaviour of virus and IB particles at equivalent loadings, ATPS were assembled containing $2.07 \cdot 10^{11}$ particles of washed IBs. At such a loading, the partition of washed IBs (quantitatively detected by BCA assay; where 1 mg/ml soluble BSA equivalent=$2.76 \cdot 10^{12}$ IB particles/ml; [14]) in 10/10% (w/w) PEG 8000–phosphate systems at pH 9.4 occurred mainly between the interphase and bottom phase (i.e., 60% and 30%, respectively). At pH 7.5 a redistribution of the particles was observed from the interphase (35%) to the bottom phase (35%) and a sediment (20%) formed due to saturation of the bottom phase (illustrated schematically in Fig. 3). Although the particle numbers loaded were equivalent, discrepancies were observed in the particle distribution. However the partition behaviour was similar for both particle types, i.e., decreasing the apparent pH of the system induced a redistribution from the interphase to the bottom phase leading to the eventual formation of a sediment (see Table 3). It

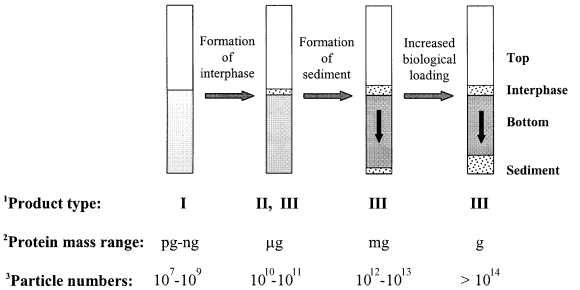


Fig. 3. Impact of biomass loading upon predicted partition behaviour of nanoparticulates. The schematic illustrates the impact of loaded biomass upon the distribution of particulate products in ATPSs. As particle loading levels exceed a defined threshold level a discrete interphase appears, between the liquid phases. Further additions of biomass beyond the saturation levels of the interphase and liquid phases results only in an increased mass of sediment beneath the lower phase. [1]Product types refer to retrovirus (I), adenovirus (II) and unwashed/washed inclusion bodies (III) classified with respect to the protein mass and particle number available in experimental feedstocks. [2]Protein masses were estimated from BCA assays of purified material and/or quantitation of SDS–PAGE analyses. [3]Particle numbers were derived from BCA assays and converted into equivalent concentrations of soluble BSA by application of the following relationship: 1 mg/ml soluble BSA equivalents=$3.45 \cdot 10^{12}$ particles/ml (S. Longhurst, Cobra Therapeutics, personal communication).

was concluded that when the capacity of the interphase for biomass reached its maximum, the presence of additional particles exceeded the resistance of the surface tension of the bottom phase and some of the particles were then recovered as a sediment. Such a phenomenon has often been observed at high loadings of unwashed IBs (200 mg–2000 mg wet mass equivalent to particle numbers of $4.36 \cdot 10^{13}$–$4.36 \cdot 10^{14}$) in these systems (S.G. Walker, unpublished experiments). The interphase commonly appeared to reach a maximal volume, whereas the volume of the sediment increased as the biomass input increased.

3.4. Partition of retroviruses

The partition behaviour of the retroviral particles (murine leukaemia virus) was examined in a limited feasibility study with 10/10% (w/w) PEG 8000–K_2HPO_4 (pH 9.4) systems. Retrovirions ($3.2 \cdot 10^5$) were introduced into the system with the sterile stock

Table 3
Washed inclusion body and adenovirus partition data in ATPSs loaded with equivalent particle numbers[a]

System	Phase	IB (% recovered)	Adenovirus (% recovered)
PEG 8000–K_2HPO_4 (10/10%, w/w, pH 9.4)	Top	10	10
	Interphase	60	72
	Bottom	25	18
	Sediment	5	Trace
PEG 8000–K_2HPO_4+KH_2PO_4 (10/10%, w/w, pH 7.5)	Top	10	10
	Interphase	35	60
	Bottom	35	30
	Sediment	20	Trace

[a] Tabulated data indicates the number of particles (expressed as a percentage of the total recovered) present in each phase. The experimental particle loading used for IBs and adenovirus were $2.07 \cdot 10^{11}$ particles (equivalent to masses of 371 μg and 60 μg, respectively).

solutions of PEG 8000 and K_2HPO_4. After gentle mixing, the individual phases were harvested and tested on target NIH3T3 cells to assess the infectivity of the virions contained in the different phases. The analyses indicated that the viruses partitioned preferentially into the interphase (~80%) where a titre of $6.6 \cdot 10^5$ cfu/ml (colony forming units/ml) was recovered. This titre was harvested in a volume of 400 μl which represents at least a concentration factor of 2.5 for the viral supernatant. The remaining viral particles appeared to partition evenly between the top and bottom phases.

In this system, the analysis of the partitioning behaviour of the main components of the viral supernatant indicated that the virions partitioned mainly to the interphase where 60% of the cells and cell debris and 47% of the BSA present in the serum-based culture medium was present. This suggested that such a system operated with these extraction conditions could only be used to concentrate the virions. The co-concentration of contaminants limited the purification performance of such a system.

3.5. Comparison between the partition of non-washed inclusion bodies and retroviruses in ATPSs

In the 10/10% (w/w) PEG 8000–K_2HPO_4 (pH 9.4) systems, non-washed inclusion bodies in the presence of soluble and insoluble contaminants were shown to partition mainly to the interphase where 70% of the initial inclusion body input was harvested (for biomass loads 100 mg wet mass ~$2.18 \cdot 10^{13}$ total particles). The remainder of these particles (30%) were identified in the bottom phase as a sediment.

Direct application of the infectivity test to an estimate of retroviral numbers in ATPSs, indicated that only $3.2 \cdot 10^5$ viruses were present in the total system (equivalent to a protein mass of 93 pg soluble BSA equivalents). Therefore the 80% of the infectious virions harvested in the interphase, represent a mass of only 75 pg, which was considerably lower than any of the IB loadings studied herein. Thus in systems loaded with retrovirus, the interphase could not have approached material saturation and no sediment would be expected. However, assuming that working titres of 10^{13} to 10^{14} could be achieved in ATPSs for the retroviruses, then the formation of the sediment (predicted by partition studies on unwashed IBs) would generate the optimal phase to harvest product for further processing. This assumes that particles maintain their infective capacity and are unaffected by aggregation on sedimentation.

4. Conclusions

The limited availability of bona fide gene therapy viral particles and their high production costs has invited the adoption of surrogate models to mimic the behaviour of viral vectors during method scouting for selective purification processes. To a first

approximation, it was concluded that non-washed IBs displayed surface similarities with enveloped viruses (i.e., retrovirus). In contrast, the washed IBs presented surface characteristics more closely similar to those of non-enveloped virions (i.e., adenovirus) as summarised in Table 1. The partitioning of both types of surrogate mimics were extensively studied in PEG–salt systems. The results of these investigations were then compared in more limited experiments with the partition behaviour of representative viral vectors.

Any discrepancies observed between the partition behaviour of the viral particles and their potential surrogate mimics suggested that the maximum loadings of feedstocks studied herein (~93 pg and 60 μg for retrovirus and adenovirus, respectively) were far too low to closely reproduce the partitioning behaviour predicted by the IB studies (see Fig. 2). Comparison between the partition behaviour of the surrogate mimics and the viral vectors suggested that the biomass loading of the system was as critical as the surface properties of the particles in directing partitioning towards the most useful compartments of bottom phase or bottom phase sediment.

The IB data suggested that ATPSs have a volumetric capacity which significantly exceeds, by at least 10-fold that of the conventional porous chromatographic matrices (e.g., 10^9–10^{12} particles/ml) when operated with nanoparticulate bioproducts (discussed in Refs. [5,6]). The capability for handling high particle numbers in ATPSs, both in the presence and absence of contaminant material, offers a significant processing advantage over currently used purification technologies of ultracentrifugation and chromatography. The ever growing portfolio of biological particulate products requires the development of suitable fractionation procedures specifically designed and optimised for the fractionation of particulates. The general reluctance of the bioindustries to embrace novel separation techniques such as ATPSs is understandable considering the complex interplay of physicochemical parameters responsible for the partition of proteins and particles. However the continued development of laboratory and pilot scale ATPS processes using industrially relevant particulate models and representative product feedstocks is essential. This will aid the advance of a practical understanding of the molecular basis for particulate fractionation and recovery.

5. Abbreviations

Ad5	Adenovirus type 5
ATCC	American tissue and cell culture
ATPS	Aqueous two phase system
BCA	Bicinchinonic acid assay
BSA	Bovine serum albumin
cfu	Colony forming units
CsCl	Caesium chloride
DMEM	Dulbecco's minimal essential medium
DSP	Downstream processing
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
FCS	Foetal calf serum
HER	Human embryonic retinoblast
IB	Inclusion body
IPTG	Isopropyl β-D-thiogalactopyranoside
M_r	Relative molecular mass
ng	Nanogram (10^{-9} g)
ompA	Outer membrane protein A
ompF	Outer membrane protein F
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pg	Picogram (10^{-12} g)
SDS	Sodium dodecyl sulphate
T500	Molecular mass of dextran (i.e., 500 000 rel. mol. mass)
TEM	Transmission electron microscopy
w/v	Weight per volume
ww/v	Wet weight per volume

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