Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 854 (2007) 13–19
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Aqueous two-phase recovery of bio-nanoparticles: A miniaturization study for the recovery of bacteriophage T4

Alejandro Negrete *, Tau Chuan Ling [1], Andrew Lyddiatt [2]

Biochemical Recovery Group, Department of Chemical Engineering, School of Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Received 26 October 2006; accepted 25 March 2007
Available online 7 April 2007

Abstract

Biotechnology industry has recently been demanding nanoparticulate products (20–200 nm) such as viruses, plasmids, virus-like particles and drug delivery assemblies. These products are mainly used as gene delivery systems in gene therapy protocols. During the process development for the manufacture of these products, it is crucial to optimize the recovery and purification steps. Unfortunately, the high value of some bio-nanoparticles complicates the optimization studies. The solvent extraction method with aqueous two-phase systems (ATPS) has been used to successfully recover bioproducts on a large scale. In this study, the potential miniaturization of ATPS is presented. The partition behavior of pure bovine serum albumin (BSA) in PEG-800-phosphate and bacteriophage T4 in PEG 8000-phosphate and PEG 600-sulphate systems were studied at three different scales (10 g, 2 g and 300 μl). The results obtained showed that the volume ratio (V_R) for BSA ($V_R = 1.0$) was comparable to the blank systems at the scales studied. Additionally, the partition coefficient (K) was also similar ($K = 0.05$) with more than 82% of BSA concentrated in the bottom phase. Same system was challenged with bacteriophage T4 showing a $V_R = 1.0$ and K greater than 5 with the infective particles concentrated in the top phase. The bacteriophage T4 was concentrated in opposite phase in the PEG-600-sulfate system with a consistent $V_R = 0.8$ and $K < 0.2$ for the scales analyzed. The partition behavior the bacteriophage T4 was comparable to that reported previously for adenoviral vectors in same system at 15 ml scale. The results obtained demonstrated that the miniaturization of ATPS is feasible and reproducible for the two models selected. This provides significant information about the miniaturization process of such ATPS for their potential generic applications in the recovery of different bio-nanoparticle products.

Keywords: Nanoparticulate; Aqueous two-phase system; Recovery; Bacteriophage T4

1. Introduction

Aqueous two-phase system (ATPS) forms when two polymers or one polymer and a salt are prepared in an aqueous solution above their critical solubility concentrations. The mixture will then separate into two immiscible phases [1], where the light phase (top phase) is rich in one polymer and the heavy phase (bottom phase) is rich in the second polymer or the salt and an interphase forms between both phases. The non-conventional recovery method, namely ATPS described by Albertson in the mid-1950s [1] has been exploited for the recovery and purification of different bioproducts [1,2]. These include proteins [3], viruses and intact cells [4] virus-like particles [5], inclusion bodies [6], plasmid DNA [7,8] surrogate mimics for viral vectors and adenoviral vectors [9] and even inorganic compounds produced by microorganisms [10]. By speculative comparisons, ATPS might possess a superior volumetric capacity (occupancy per volume of phase) for such nanoparticulate products than adsorptive chromatography density and gradient centrifugation [6,11]. Other ATPS advantages over density gradient centrifugation and chromatography include the easy removal of cell debris, scaleable, low cost of chemicals, and short processing time [12–14].

The mechanism of the partition of bioproducts in ATPS has been uncertain until now, although it can be described qualita-

* Corresponding author. Present address: National Institutes of Health, National Heart, Lung, and Blood Institute, 9000 Rockville Pike Bldg 10, Room 7D04, Bethesda, MD 20892, USA. Tel.: +1 301 496 6104; fax: +1 301 496 9985.
E-mail address: negretea@nhlbi.nih.gov (A. Negrete).
[1] Present address: Department of Process and Food Engineering, Faculty of Engineering, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.
[2] Present address: Millipore Biopharmaceutical Division, Bioprocessing Ltd., Medomsley Road, Consett DH8 6SZ, UK.

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.03.041

tively. Some mathematical models have been developed in order to predict such partition behavior [15]. However, none of these models has been applicable yet due to the limited knowledge of the complex partition behavior of biomolecules [16]. The water content in the ATPS is usually greater than 70% (w/w) and thus provides a friendly environment, which facilitates the partition of bioproducts without affecting their chemical or biological characteristics. The partition behavior of a bioproduct can be influenced by different parameters such as type and molecular weight of polymer, density, viscosity, interfacial tension, hydrophobicity, ionic strength and ionic composition, addition of affinity molecules [1,2,17], addition of a neutral salt [18], pH [18,19], temperature [20,21] and biomass [22,23]. These parameters can be manipulated in order to exploit the most favorable conditions for efficient fractionation. Due to the influence of these parameters the partition behavior of a bioproduct cannot be predicted in the presence of crude material (e.g. cells, cell debris, complex biomolecules, buffers, etc.). For this reason, ATPS relays upon trial and error experimentations.

In response of the limiting amounts of viral vectors for gene therapy applications, washed inclusion bodies as first generation mimics of nanoparticles have been used to predict the partition behavior of adenoviral vectors [9,24]. However, a biological system with similar characteristics to the viral vectors is required. In this paper, the potential miniaturization of ATPS was studied in order to develop a protocol to carry out a large number of experiments with a minimum amount of valuable bioproduct. The linearity of the scale-down procedure at different scales including 10 g, 2 g and 300 μl was evaluated by comparing volume ratio (V_R) and partition results for pure soluble BSA in PEG 8000-phosphate ATPS.

Additionally, studies were extended to the use of a nanoparticulate bioproduct, namely bacteriophage T4 in PEG-800-phosphate and PEG-600-sulfate ATPS. The bacteriophage T4 has been used for molecular biology, genetic studies and phage therapy [25]. The bacteriophage T4 was selected due to its characteristics including nanoparticle dimensions, surface characteristics, easy manipulation, easy to produce, non infection risk and short period of time of production. The use of this bacteriophage served two purposes. Firstly, miniaturization studies could be carried out challenging ATPS with crude material and with a particle with nanodimensions. Secondly, partition data for bacteriophage T4 could be compared with systems reported previously for the recovery of adenoviral vectors [9], constituting a potential second generation mimic, providing more partition data for a particular ATPS. This provided significant information about the miniaturization process of such ATPS for their generic applications in the recovery of different valuable bionanoparticle products.

2. Materials and methods

2.1. Escherichia coli

In this study, *E. coli* strain C600 (NCIMB 11549) from National Collections of Industrial and Marine Bacteria Ltd., UK was used. Cells were cultivated in 500 ml baffled shake flasks containing 270 ml of LB broth (BBL™, Becton Dickinson and Company, US). Flasks were inoculated with 30 ml of *E. coli* at a concentration of 1.4×10^9 cell forming units per milliliter (cfu/ml). Flasks were incubated at 37 °C for 24 h under constant agitation (80 rpm). Cells were harvested by centrifugation at 17700 × *g* for 30 min at 4 °C. Pellets were pooled and stored with 10% (v/v) glycerol at −80 °C for storage. Weekly subcultures of *E. coli* grown for 24 h in plates with LB agar and stored at 4 °C were also used to conserve and to check purity of the strain.

The *E. coli* concentration was determined by inoculating LB agar plates with 10-fold serial dilutions (10^{-1}–10^{-10}) of the sample in LB broth. A volume of 100 μl of each dilution was homogeneously spread on the surface of the LB agar plate. All samples were analyzed in duplicate. Plates were incubated at 37 °C for 24 h. The *E. coli* colonies were counted in plates where the number of colonies per plate was typically in the range of 30–100 and the average number was recorded. The cfu/ml was calculated by considering the dilution factor and the sample volume.

2.2. Propagation of bacteriophage T4

The wild type strain of bacteriophage T4 (NCIMB 10360) was obtained from NCIMB Ltd., UK. *E. coli* host cells were grown overnight at 37 °C with gentle shaking in LB broth supplemented with 1% (w/v) $MgSO_4$, 20% (w/v) maltose and 5 μg/ml tryptophan [26]. A volume of 400 μl of host cells containing 1.2×10^8 cfu was infected with bacteriophage T4 stock solution at multiplicity of infection (MOI) 1 for 5–10 min. Infected cells were mixed with 15 ml of melted LB agar having a temperature of 42 °C (top agar) and poured into LB agar plates. After the top agar had set, plates were incubated at 37 °C and checked every 2 h until transparency of the medium was observed (approximately 5 h after incubation), which indicated complete cell lysis. In order to recover bacteriophage T4, plates were washed for 24 h with 15 ml phage buffer (50 mM Tris pH 7.6, 100 mM $MgCl_2$) per plate with gentle rocking at 4 °C. The phage buffer was removed and the plates were rinsed with 5 ml of clean buffer. The collected buffer was pooled and cell debris removed by centrifugation at 2000 × *g* for 10 min. The supernatant was filtered using a sterile 0.22 μm pore size filter and the stock was stored at 4 °C.

2.3. Phage assay

The bacteriophage T4 titer of the produced supernatant and samples was determined by phage assay. Each infective particle, when replicated, provokes visible cell lysis in a confluent *E. coli* culture grown in agar plates, and considering that each lytic zone is caused by a single infective particle, the number of infective particles in the original sample can be estimated [27].

Using phage buffer, 10-fold serial dilutions (10^{-1}–10^{-10}) of the sample containing the bacteriophage T4 were prepared. A volume of 400 μl of host cells was mixed with 100 μl of diluted samples for 5–10 min. A total of 3 ml of melted and cooled to

42 °C top LB agar was added to the infected cells and poured onto a LB agar plate. After the top agar had set, the plates were incubated at 37 °C for 24 h. The lytic zones or plaque forming units (pfu) were counted from the duplicates where the number of pfu per plate was in the range of 20–200 and the average number was recorded. The pfu/ml was calculated by considering the dilution factor and the sample volume. For every set of experiments, internal standards for the phage assay were prepared in order to validate the experimental results. Internal standards had three different concentrations of bacteriophage T4 including 2×10^2 pfu/ml, 2×10^6 pfu/ml and 2×10^9 pfu/ml and they were analyzed under the same conditions as the experimental samples.

2.4. Aqueous two-phase systems

All manipulations were performed under sterile conditions in a Gelaire BSB3-S laminar flow cabinet (ICN Biomedicals, UK) unless stated otherwise. Two different compositions of ATPS were analyzed in this study as single determination. These included PEG 8000-phosphate (10% (w/w) PEG 8000, 10% (w/w) K_2HPO_4–KH_2PO_4, ratio 18:7, pH 7.5) and PEG 600-sulphate (20% (w/w) PEG 600, 20% $(NH_4)_2SO_4$, pH 6.8) systems. Blank systems had the above compositions but were made up with McIlvaine buffer (154.7 mM Na_2HPO_4, 22.8 mM citric acid and 97 mM KCl at pH 6.8) for the PEG 600-sulphate system and distilled water for the PEG 8000-phosphate systems. For crude partitioning studies, ATPS were made up with feedstock. Partition studies with BSA and bacteriophage T4 were carried out at different scales including 10 g, 2 g and 300 μl. The ATPS used for the partition studies included PEG 8000-phosphate for BSAs, PEG 8000-phosphate and PEG 600-sulphate for bacteriophage T4.

Systems with a total weight of 10 g were prepared in sterile 15 ml graduated conical tubes. The phase forming chemicals were added as sterile concentrated stock solutions to obtain final concentrations as described above. The buffer or water was added up to a final weight of 9 g and the 10 g system was completed with 1 g of feedstock. The partition equilibrium was achieved by gently turning the tubes 20 times by hand in order to avoid the damage of bacteriophage T4 fibers, which are the responsible for the infectivity. The tubes were centrifuged at 1200 × *g* for 5 min at 20 °C in order to accelerate phase separation. After phase separation, phase volumes were marked on the outside of the centrifuge tube and determined later by gravimetry. Each phase was carefully harvested and aliquoted for analysis. The interphase was harvested together with small volumes of top and bottom phase as a set volume of 500 μl.

For the 2 g systems, sterile 2 ml graduated Eppendorf tubes were used. The phase forming chemicals were added as stock solutions together with buffer or water (final weight 1.9 g). Blank systems were constructed by completing the 2 g system with buffer or water (PEG 8000-phosphate and PEG 600-sulphate, respectively), whilst for the partition studies the total weight was made up with 0.1 g of feedstock. The phases were mixed by gently turning the tubes and separated by centrifugation at 7570 × *g* for 1 min. Phase volumes were determined by gravimetry and the interphase was harvested as a set volume of 200 μl.

For the construction of 300 μl ATPS, sterile 96 well plates were used and densities of the chemical stock solutions were determined to make up ATPS based on volume. The units from the 96 well plates were separated one from another to obtain individual wells to facilitate the construction and visualisation of the systems. ATPS phase forming chemicals were added up to 270 μl at the established concentrations. The total volume of 300 μl was completed with water or buffer for blank systems (PEG 8000-phosphate and PEG 600-sulphate, respectively), and with 30 μl of feedstock for the partitioning studies. Systems were centrifuged at 1200 × *g* for 5 min at 20 °C using appropriate adaptors for the 96 well plates in the centrifuge. For a better visualisation of the phases during their harvest in the 300 μl systems a magnification lens was used whilst the 96 well plates were illuminated from the base and from one side. For harvesting the phases a micro-syringe was utilised. Top phase was harvested (100 μl) and 100 μl containing presumably part of the top phase, interphase and part of the bottom phase was collected. The remaining 100 μl corresponded to the bottom phase. Only top and bottom phases were analysed in these systems. The volume ratio (V_R) can be determined using Eq. (1). The distribution of the target product in the ATPS (K, partition coefficient) can be estimated from the ratio of the product concentrations in the top to bottom phase using Eq. (2) [1].

$$V_R = \frac{V_T}{V_B} \tag{1}$$

$$K = \frac{C_T}{C_B} \tag{2}$$

2.5. Protein determination

The protein concentration was determined with the BCA assay [28]. The BCA assay kit was obtained from Perbio Science Ltd., UK. In a 96 well plate, samples of 25 μl were mixed with 200 μl of working reagent. After incubation at 37 °C for 30 min, the absorbance at 540 nm was measured using a plate reader (Spectra III, SLT Instruments, Austria). Protein concentration was calculated by using a calibration curve constructed with pure BSAs (Perbio Science Ltd., UK) at defined concentrations ranging from 25 μg/ml to 2000 μg/ml. Experimental samples were expressed in terms of BSA equivalents.

2.6. Effect of ATPS chemical components upon bacteriophage T4

For gene therapy vectors, it is fundamental to preserve infectivity during the process. This characteristic can be affected by the ATPS components. For this reason, the maximum possible concentrations of the PEG 8000-phosphate and PEG 600-sulphate ATPS components present in the system studied were tested separately on the bacteriophage T4 infectivity.

The same amount of bacteriophage T4 sock solution (1 ml) was loaded in 9 g of the sterile stock solution of the ATPS phase forming chemicals prepared in phage buffer. The systems

Table 1
Measured volume ratios in PEG 8000-phosphate ATPS at different scales in the presence and absence of BSA

Scale	System	Top phase (ml)	Bottom phase (ml)	Total volume (ml)	V_R	Average V_R
10 g	Blank	4.623	4.498	9.121	1.028	1.028 ± 0.000
		4.618	4.491	9.109	1.028	
	BSA	4.615	4.485	9.101	1.029	1.032 ± 0.005
		4.629	4.469	9.098	1.036	
2 g	Blank	0.979	0.959	1.938	1.020	1.024 ± 0.005
		0.981	0.955	1.936	1.028	
	BSA	0.987	0.971	1.958	1.017	1.009 ± 0.011
		0.980	0.979	1.959	1.001	
300 μl	Blank	0.106	0.112	0.217	0.945	0.923 ± 0.032
		0.099	0.110	0.210	0.900	
	BSA	0.098	0.101	0.199	0.971	0.931 ± 0.057
		0.097	0.109	0.206	0.891	

This table shows the V_R of PEG 8000-phosphate ATPS (10% (w/w) PEG 8000, 10% (w/w) K_2HPO_4–KH_2PO_4, ratio 18:7, pH 7.5). These values were obtained by weighing distilled water corresponding to the volume occupied by the phases in the container. The ATPS were constructed according to Section 2.4 and BSA was added to the systems at final concentration of 600 μg/ml. The V_R was calculated according to Eq. (1).

formed were composed of 10% (w/w) PEG 8000, 10% (w/w) K_2HPO_4–KH_2PO_4 (ratio 18:7), 20% (w/w) PEG 600 and 20% (w/w) $(NH_4)_2SO_4$. Samples were taken from each system and analyzed for infectivity. A system prepared with distilled water was also analyzed since this was a standard component in all ATPS. Furthermore, the effect of the McIlvaine buffer was investigated. Triplicates of three experiments were analyzed and the mean infectivity obtained in each system was compared to that obtained in the phage buffer, which was the control in this study since this buffer provides the optimal conditions for maintaining the infectivity of the bacteriophage T4.

3. Results and discussion

3.1. Miniaturization of ATPS

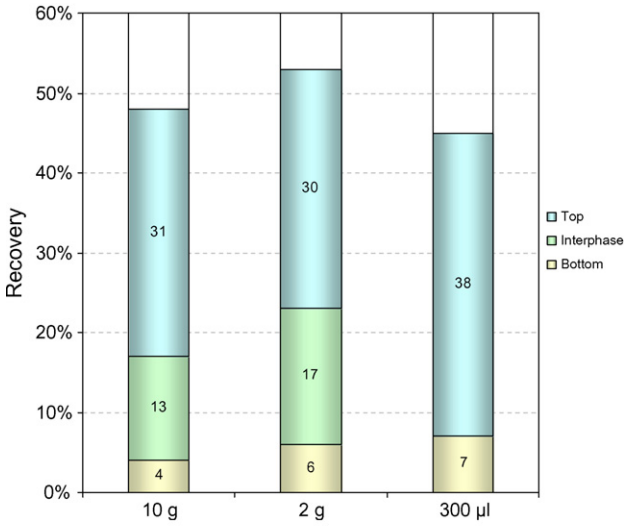
The potential miniaturization of ATPS was investigated with pure BSA by comparing the partition behavior at three different scales including 10 g, 2 g and 300 μl systems in PEG 8000-phosphate ATPS. Partitioning studies of BSA were not handled under sterile conditions. For the 10 g, 2 g and 300 μl systems 1 ml, 200 μl and 30 μl of BSA stock (6 mg/ml) was added, respectively, in order to have a final concentration of 600 μg/ml BSA. The V_R and K were calculated according to Eqs. (1) and (2), respectively. The results in Table 1 show that the volume ratio in PEG 8000-phosphate was reproducible not only in the blank systems but also in the systems loaded with BSA. Additionally, V_R was not altered substantially by the addition of the BSA in 10 g, 2 g and 300 μl systems. However, the V_R of the 300 μl system was slightly different (less than 10% variation) when compared with V_R values of higher-scale systems. Such difference could be attributed to the difficulties experienced during the measurement of the volumes in the 300 μl systems due to the meniscus formation in the containing well as result of the geometry of the container. The linearity of V_R during the scaling-up of ATPS including 10 ml, 1 l and 30 l has been reported previously [29]. According to the results obtained in the present study, such linearity in V_R was maintained when ATPS were scale-down 33 times. This finding demonstrated and confirmed the possibility of miniaturization of ATPS.

In order to further study the potential miniaturization of ATPS, the K value for BSA in selected PEG 800-phosphate ATPS was evaluated. The final concentration of BSA in all systems was 600 μg/ml. The phases from each system were harvested and BSA concentrations in each phase were determined. The percentage of recovery was calculated considering the concentration of the BSAs in each phase and the total mass in the total system. Results for the partition of BSAs are summarised in Table 2. As indicated in Table 2 for PEG 8000-phosphate systems, the K value of BSA was similar for all scales with less than 15% variation. The percentage recovery of BSA in each phase was also similar in all the scales studied. These results also provided evidence for the possible miniaturization of ATPS. According to the linearity obtained for V_R and K in the scales analyzed the miniaturization of the PEG 8000-phosphate system was possible. In order to extend the miniaturization studies to bio-nanoparticle products, the partition behavior of bacteriophage T4 was evaluated in the same PEG 8000-phosphate systems. Bacteriophage T4 partition studies were also extended

Table 2
Partition behavior of BSAs in PEG 8000-phosphate ATPS at different scales

Scale	Phase	Average BSAs concentration (μg/ml)	Recovery (%)	K
10 g	Top	60.6	4.7	0.047 ± 0.002
	Bottom	1295.3	96.7	
2 g	Top	62.3	5.1	0.054 ± 0.010
	Bottom	1160.6	94.3	
300 μl	Top	76.0	4.1	0.054 ± 0.058
	Bottom	1399.8	81.7	

This table shows the partition behavior of BSAs in PEG 8000-phosphate (10% (w/w) PEG 8000, 10% (w/w) K_2HPO_4–KH_2PO_4, ratio 18:7, pH 7.5) ATPS at different scales including 10 g, 2 g and 300 μl. BSAs was added to the systems at final concentration of 600 μg/ml. The V_R estimated for these systems is shown in Table 1.



System	Phase	Volume (ml)	V_R	Infectivity (pfu/ml)	Infective particles (pfu)	K
10 g	Top	4.6	1.0	1.4×10^8	6.44×10^8	8.23
	Interphase	0.5		5.4×10^8	2.70×10^8	
	Bottom	4.6		1.7×10^7	7.82×10^7	
2 g	Top	0.9	1.0	1.4×10^8	1.26×10^8	5.18
	Interphase	0.2		3.5×10^8	7.00×10^7	
	Bottom	0.9		2.7×10^7	2.43×10^7	
300 µl	Top	0.1	1.0	2.4×10^8	2.40×10^7	5.71
	Bottom	0.1		4.2×10^7	4.20×10^6	

Fig. 1. Partition behavior of bacteriophage T4 in PEG-phosphate ATPS. This figure shows the partition behavior of bacteriophage T4 in PEG 8000-phosphate (10% (w/w) PEG 8000, 10% (w/w) K_2HPO_4–KH_2PO_4, ratio 18:7, pH 7.5) systems at different scales. The ATPS were constructed according to Section 2.4. The volumes of bacteriophage T4 stock (2.1×10^9 pfu/ml) added to the 10 g, 2 g and 300 µl systems were 1 ml, 200 µl and 30 µl, respectively. Infectivity was determined by plaque assay. The V_R and the K were calculated according to Eqs. (1) and (2), respectively.

to PEG 600-sulphate system, which had been evaluated previously for the recovery of adenoviral vectors [9]. The results generated in this study provided extra valuable information for the characterisation of such systems for the generic recovery of nanoparticulate bioproducts.

3.2. Recovery of bacteriophage T4 in PEG 8000-phosphate ATPS

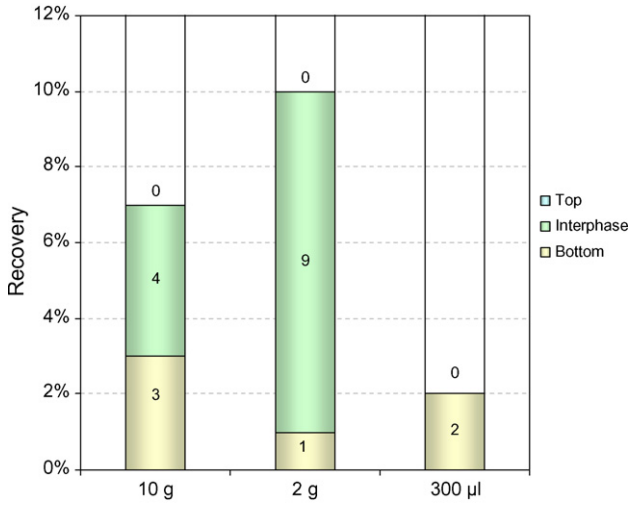
The partition behavior of the bacteriophage T4 was determined in PEG 8000-phosphate ATPS at different scales including 10 g, 2 g and 300 µl. A bacteriophage stock solution with a titer of 2.1×10^9 pfu/ml was used for systems at all scales. The volume of bacteriophage T4 stock solution added to complete ATPS was 1 ml for the 10 g system, 200 µl for the 2 g system and 30 µl for the 300 µl system. The V_R was determined using Eq. (1) and the bacteriophage T4 infectivity in each phase, given in pfu/ml, was determined by phage assay. Fig. 1 summarizes the partition behavior of bacteriophage T4 in PEG 8000-phosphate ATPS. The recovery of bacteriophage T4 in all systems was approximately 50% of the infective input. These values differed from those reported previously for the recovery of adenoviral vectors in same composition of ATPS, where recoveries higher than 90% were obtained [9]. This could be due to the negative effect of the ATPS phase forming chemicals upon the infective bacteriophage T4. This result suggested that bacteriophage T4 was more sensitive to the ATPS environment than adenoviral vectors.

As indicated in Fig. 1, the bacteriophage T4 was concentrated in the top phase at all scales analyzed. These results differed from those reported for adenoviral vectors in the same system [9] where the infective particles were concentrated in the interphase. The divergence in the partition behavior may be attributed to the different number of particles loaded in the system. The total number of infective adenoviral vectors loaded into the system in the previous studies in 10 g systems was 1.65×10^{11} [9], whilst in the case of bacteriophage T4, the value was 2.1×10^9. Additionally, the adenoviral vectors were purified by density gradient centrifugation prior to the partition in ATPS. Such processes eliminated contaminant materials present in the crude virus lysate (CVL), such as empty capsids, debris and free components of the adenoviral vectors. The bacteriophage T4 utilized for the partition studies was partially clarified by filtration of the lysate using a 0.22 µm pore diameter filter. The partition behavior of bioproducts previously purified differs from that when such systems are challenged with crude or partially purified feedstock. This effect has been reported previously where the partition of biological suspensions had an impact upon the performance of the ATPS [23,30].

The K of bacteriophage T4 in 10 g PEG 8000-phosphate systems (Fig. 1) differed from the 2 g and 300 µl systems. This variation was attributed to the imprecision of the phage assay, which is a biological assay dependent on many factors including the state of the host cells (age of culture, number of subcultures), presence of cell debris that interfere during the infection, small variations in time of contact during the infection when large number of samples are processed and visualization and counting of plaques. The differences observed in the K of bacteriophage T4 in PEG 8000-phosphate systems showed the complexity of ATPS miniaturization for the recovery of nanoparticles with biological assays-dependent for their characterization.

3.3. Recovery of bacteriophage T4 in PEG 600-sulphate ATPS

The recovery of adenoviral vectors in PEG 600-sulphate ATPS constructed with McIlvaine buffer has been reported previously [9]. In the present study, the same system was investigated for the recovery of bacteriophage T4 in order to compare its partition behavior with that obtained for adenoviral vectors. PEG 600-sulphate ATPS at different scales including 10 g, 2 g and 300 µl were constructed under sterile conditions. A bacteriophage stock solution with a titer of 2.1×10^9 pfu/ml was used for systems at all scales. The volume of bacteriophage T4 stock solution added to complete ATPS was 1 ml for the 10 g system, 200 µl for the 2 g system and 30 µl for the 300 µl system. The V_R was determined using Eq. (1) and the bacteriophage T4 infectivity in each phase given in pfu/ml was determined by phage assay.



System	Phase	Volume (ml)	V_R	Infectivity (pfu/ml)	Infective Particles (pfu)	K
10 g	Top	4.0	0.8	5.2×10^5	2.08×10^6	0.05
	Interphase	0.5		1.7×10^8	8.50×10^7	
	Bottom	5.0		1.1×10^7	5.50×10^7	
2 g	Top	0.8	0.8	4.6×10^5	3.68×10^5	0.11
	Interphase	0.2		1.9×10^8	3.80×10^7	
	Bottom	1.0		4.2×10^6	4.20×10^6	
300 µl	Top	0.08	0.8	3.0×10^6	2.40×10^5	0.21
	Bottom	0.10		1.4×10^7	1.40×10^6	

Fig. 2. Partition behavior of bacteriophage T4 in PEG 600-sulphate ATPS. This figure shows the partition behavior of bacteriophage T4 in PEG 600-sulphate (20% (w/w) PEG 600, 20% $(NH_4)_2SO_4$, pH 6.8) systems at different scales. The accompanying table displays the volumes of the phases used in estimating the distribution of infectivity. The V_R and the K were calculated according to Eqs. (1) and (2) respectively.

Fig. 2 indicated the maximum total recovery of bacteriophage T4 in PEG 600-sulphate ATPS was 10% of the infective particles loaded in to the system. This recovery was lower than that obtained from the PEG 8000-phosphate systems. This may be due to the negative effect of the ATPS environment on the bacteriophage T4 infectivity. It can be also noted from Fig. 2 that the infective bacteriophage T4 was concentrated in the interphase in all systems. This result agrees with that reported previously for the intensified ATPS procedure for the recovery of adenoviral vectors in the same system where 60% of the total infective virus loaded (2.07×10^{11}) was concentrated in the interphase [9], despite the differences in purity and number of infective particles loaded into the system discussed earlier. These results showed the robustness of the PEG 600-sulphate ATPS and proved its generic application for the recovery of different nanoparticulate bioproducts. The K of bacteriophage T4 in PEG 600-sulphate systems varied slightly among the scales evaluated. This may be due to the imprecision of the phage assay during the determination of infective particles in the ATPS phases as discussed earlier. Another important aspect to be considered is the geometry of the devices utilized. The ratio height/diameter was not constant among the three containers used. However, the V_R in all of them was similar. In order to determine the causes of the total low recovery of infective bacteriophage T4 in PEG 8000-phosphate and PEG 600-sulphate, the effect of the ATPS chemical components of such systems was evaluated upon the infective particles.

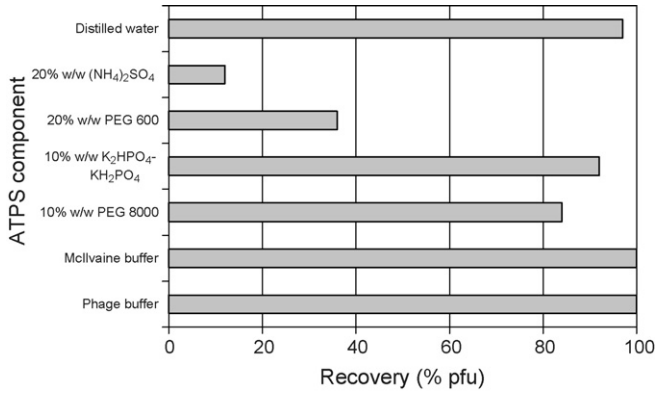


Fig. 3. Effect of ATPS chemical components upon bacteriophage T4. This figure shows the effect of the ATPS chemical components of the PEG 8000-phosphate and PEG 600-sulphate systems upon the infective bacteriophage T4. Same titer of bacteriophage T4 was loaded in each system (2.1×10^9 pfu/ml). Percentages of recovery are expressed relative to the infective particles determined in phage buffer.

3.4. Effect of the ATPS chemical components upon bacteriophage T4

The chemical composition of an ATPS is frequently represented by the total concentration of the phase forming chemicals. However, the chemical composition of top and bottom phase can vary dramatically, being usually enriched in one of the chemical components [9,24]. To determine the effect of the ATPS chemical components upon the infectivity of the bacteriophage T4, the maximum possible concentrations of the PEG 8000-phosphate and PEG 600-sulphate ATPS components present in the system were tested separately on the bacteriophage T4 infectivity. The data in Fig. 3 illustrates the infectivity of the bacteriophage T4 determined in the systems constructed with each one of the chemical components of the ATPS. As can be seen in Fig. 3 the infectivity obtained with McIlvaine buffer was equivalent to that obtained in the control (phage buffer). This confirmed the generic application of the McIlvaine buffer for maintaining the integrity of viral particles such as adenoviral vectors. Other systems that showed similar values of infectivity included distilled water and 10% K_2HPO_4–KH_2PO_4 mixture (ratio 18:7). This demonstrated the friendly environment that can be provided to bioproducts by the aqueous environment and ATPS components as reported previously [1]. Conversely, very low infectivity was obtained from the systems composed of 20% (w/w) PEG 600 and 20% (w/w) $(NH_4)_2SO_4$. The low infectivity obtained with PEG 600 may be correlated with that reported previously [31] where same concentration of PEG 600 was utilized to sediment the bacteriophage T4 and only yields up to 30% were obtained. In the case of the $(NH_4)_2SO_4$ the low infectivity recovered was attributed to the osmotic shock produced by the salt concentration used. The osmotic shock sensitivity of the bacteriophage T4 has been reported previously [32]. These results explained the very low recovery of the infective bacteriophage T4 particles in the recovery studies using the PEG 600-sulphate system.

4. Conclusions

In this study, the potential miniaturization of ATPS was investigated using PEG 8000-phosphate and PEG 600-phosphate systems in order to develop a protocol to carry out a large number of experiments with a minimum amount of valuable bioproducts. The results obtained showed that the V_R and K for BSA were similar at different scales (10 g, 2 g and 300 μl) for the same ATPS. These results demonstrated the reliability of the miniaturization of ATPS. When the miniaturized PEG 8000-phosphate and PEG 600-sulphate systems were extended for the recovery studies of the bacteriophage T4, some discrepancies among the different scales were observed, particularly in the K value. This was probably due to the inaccuracy in the determination of the number of infective particles by the biological-based assay denominated phage assay. In addition to the error from the analysis, the experimental error also has to be taken into account especially when complex feedstocks are studied. Furthermore, in the present paper the partition behavior of bacteriophage T4 in the PEG 8000-phosphate and PEG 600-sulphate ATPS was compared to that reported previously for the recovery of adenoviral vectors in the same systems. According to the results obtained, the partition behavior of the bacteriophage T4 was different from that obtained for adenoviral vectors in the same PEG 8000-phosphate ATPS. This may be due to the differences in feedstock purity and the number of infective particles loaded. In studies reported previously for the recovery of adenoviral vectors the experiments were carried out with pure particles loaded at total values of 10^{11} [9]. In the present study, the bacteriophage T4 was not purified, and was loaded at values of 10^9 particles.

In the case of the PEG 600-sulphate ATPS, despite the differences mentioned above, the partition behavior the bacteriophage T4 was comparable to that observed for adenoviral vectors in same system. This showed the robustness of the system by partitioning similarly two different nanoparticulate bioproducts, even from partially purified feedstock. Unfortunately, the chemical components of this particular system had a negative impact upon the infective bacteriophage T4 infectivity, making this system unexploitable for recovery.

Acknowledgement

The authors gratefully acknowledge the financial support from the National Council of Science and Technology-Mexico (CONACYT).

References

[1] P.A. Albertson, Partition of Cell Particles and Macromolecules, first ed., Wiley, NY, 1986.
[2] B.Y. Zaslavsky, Aqueous Two-phase Partitioning: Physical Chemistry and Bioanalytical Applications, Marcel Dekker, NY, 1995.
[3] D. Platis, N.E. Labrou, J. Chromatogr. A 1128 (2006) 114.
[4] A.I. García-Pérez, P. Sancho, M. Pinilla, J. Chromatogr. B 711 (1998) 301.
[5] J. Benavides, J.A. Mena, M. C-Ruiz, O.T. Ramirez, L.A. Palomares, M. Rito-Palomares, J. Chromatogr. B 842 (2006) 48.
[6] S.G. Walker, A. Lyddiatt, J. Chem. Technol. Biotechnol. 74 (1999) 250.
[7] S.C. Ribeiro, G.A. Monteiro, J.M. Cabral, D.M. Prazeres, Biotechnol. Bioeng. 78 (2002) 376.
[8] I.P. Trindade, M.M. Diogo, D.M.F. Prazeres, J.C. Marcos, J. Chromatogr. A 1082 (2005) 176.
[9] G.M.F. Braas, S.G. Walker, A. Lyddiatt, J. Chromatogr. B 743 (2000) 409.
[10] M. Rito-Palomares, A. Negrete, L. Miranda, C. Flores, E. Galindo, L. Serrano-Carreon, Enzyme Microb. Technol. 28 (2001) 625.
[11] T.D. Przybycien, N.S. Pujar, L.M. Steele, Curr. Opin. Biotechnol. 15 (2004) 469.
[12] M. Rito-Palomares, A. Lyddiatt, Chem. Eng. J. 87 (2002) 313.
[13] K. Schuegerl, J. Hubbuch, Curr. Opin. Microbiol. 8 (2005) 294.
[14] O. Aguilar, V. Albiter, L. Serrano-Carreon, M. Rito-Palomares, J. Chromatogr. B 835 (2006) 77.
[15] L. Simon, S. Gautam, J. Chromatogr. A 1043 (2004) 135.
[16] H. Cabezas Jr, J. Chromatogr. B 680 (1996) 3.
[17] J. Huddleston, A. Veide, K. Köhler, J. Flanagan, S.O. Enfors, A. Lyddiatt, TIBTECH 9 (1991) 381.
[18] U. Gündüz, K. Korkmaz, J. Chromatogr. B 743 (2000) 255.
[19] M.C. Tejedor, C. Delgado, M. Grupeli, J. Luque, J. Chromatogr. 589 (1992) 127.
[20] J. Persson, L. Nystrom, H. Ageland, F. Tjerneld, J. Chromatogr. B 711 (1998) 97.
[21] S. de Belval, B. le Breton, J. Huddleston, A. Lyddiatt, J. Chromatogr. B 711 (1998) 19.
[22] B. Lebreton, A. Lyddiatt, J. Chromatogr. B 743 (2000) 263.
[23] M. Rito-Palomares, L. Cueto, J. Chromatogr. B 743 (2000) 5.
[24] G.M.F. Braas, S.G. Walker, Z. Zhang, A. Lyddiatt, Trans. IChemE 78 (2000) 11.
[25] P.A. Barrow, J.S. Soothill, Trends Microbiol. 5 (1997) 268.
[26] P. Ildes, D. Kay, Br. J. Exp. Pathol. 38 (1957) 563.
[27] J. Douglas, Bacteriophages, Chapman and Hall, London, UK, 1975.
[28] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[29] M.J. Costa, M.T. Cunha, J.M. Cabral, M.R. Aires-Barros, Scale-up of recombinant cutinase recovery by whole broth extraction with PEG-phosphate aqueous two-phase, Bioseparation 9 (2000) 231–238.
[30] S.G. Walker, C.J. Dale, A. Lyddiatt, J. Chromatogr. B 680 (1996) 91.
[31] K.R. Yamamoto, B.M. Alberts, R. Benzinger, L. Lawhorne, G. Treiber, Virology 40 (1970) 734.
[32] T.F. Anderson, C. Rappaport, N.A. Muscatine, Ann. Inst. Pasteur 84 (1953) 5.