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Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems☆

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Abstract

Virus-like particles have a wide range of applications, including vaccination, gene therapy, and even as nanomaterials. Their successful utilization depends on the availability of selective and scalable methods of product recovery and purification that integrate effectively with upstream operations. In this work, a strategy based on aqueous two phase system (ATPS) was developed for the recovery of double-layered rotavirus-like particles (dlRLP) produced by the insect cell-baculovirus expression system. Polyethylene glycol (PEG) molecular mass, PEG and salt concentrations, and volume ratio (Vr, volume of top phase/volume of bottom phase) were evaluated in order to determine the conditions where dlRLP and contaminants concentrated to opposite phases. Two-stage ATPS consisting of PEG 400-phosphate with a Vr of 13.0 and a tie-line length (TLL) of 35% (w/w) at pH 7.0 provided the best conditions for processing highly concentrated crude extract from disrupted cells (dlRLP concentration of 5 μg/mL). In such conditions intracellular dlRLP accumulated in the top phase (recovery of 90%), whereas cell debris remained in the interface. Furthermore, dlRLP from culture supernatants accumulated preferentially in the interface (recovery of 82%) using ATPS with a Vr of 1.0, pH of 7.0, PEG 3350 (10.1%, w/w) and phosphate (10.9%, w/w). The purity of dlRLP from culture supernatant increased up to 55 times after ATPS. The use of ATPS resulted in a recovery process that produced dlRLP with a purity between 6 and 11% and an overall product yield of 85% (w/w), considering purification from intracellular and extracellular dlRLP. Overall, the strategy proposed in this study is simpler than traditional methods for recovering dlRLP, and represents a scalable and economically viable alternative for production processes of vaccines against rotavirus infection with significant scope for generic commercial application.

Keywords: Aqueous two-phase systems; Rotavirus like-particles; Insect cells

1. Introduction

Virus-like particles are composed of the main structural proteins of a virus, but lack its genetic material. They are produced by the recombinant expression of the structural proteins that, in the absence of non-structural proteins and genetic material, assemble into structures identical to the native virus [1,2]. Virus-like particles (VLP) have a wide range of applications, including vaccination, gene therapy, biosensors, and nanomaterials [3,4]. A relevant model for study is the rotavirus. Annually, more than 500,000 children die as victims of acute gastroenteritis caused by rotavirus [5]. VLP are attractive candidates for a prophylactic vaccine against rotavirus infection, because many drawbacks of traditional viral vaccines are absent in VLP, such as infection by inefficiently inactivated viruses or by reversion of attenuated strains. Double-layered rotavirus-like particles (dlRLP) consist of two concentric protein layers. The inner most layer is comprised by 60 dimers (120 molecules) of VP2 and the second shell is formed by 260 trimers of VP6. Such dlRLP induce a good immune response when administered intranasally [4].

The structure of dlRLP makes their production and purification a unique challenge [2,6,7]. However, in order for dlRLP to

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be a viable alternative for immunization, economical production of complete particles of good quality at large scale needs to be achieved. An expression system that has been proven to be especially suitable for the production of VLP is the insect cell-baculovirus expression vector system (IC-BEVS) [8]. Although the production of dlRLP has been studied before, the existing reports addressed mainly the upstream part of the process [1,7,9,10]. In general, purification of VLP is a complex task. A purification process must preserve the best possible yield and purity of intact VLP obtained as final products, minimizes processing time and decrease the consumption of resources (reagents) in order to make the process economically viable. Difficult to scale and time-consuming operations, such as ultracentrifugation, are currently used even at industrial scale. Such protocols are characterized by low product recovery and high cost associated to the scaling up of the process [11,12]. Consequently, process problems associated with these protocols limit their commercial application. The lack of reports addressing the downstream stages of the process for the recovery of dlRLP is evident.

The development of efficient and scalable biotechnological processes is needed for the commercial implementation of recovery processes. An alternative practical approach, exploiting the use of aqueous two-phase systems [13] to develop a process for the recovery of dlRLP is examined in this work. Aqueous two-phase systems (ATPS), constituted by a mixture of polymers (e.g. polyethylene, PEG) and salts (e.g. phosphate or sulphate), result in two-phases for the extraction of biomolecules. This technology has several potential advantages, including bio-compatibility, ease of scale-up and low cost [14]. Andrews et al. [15] have reported the use of ATPS for the recovery of hepatitis B VLP from yeast cells. They evaluated qualitatively the potential of ATPS to separate VLPs from cell debris and contaminants. However, no quantitative information was provided regarding concentration, purity or yield of VLPs obtained after ATPS. Such findings raised the generic potential application of ATPS for the recovery of VLPs. In the present study, a practical approach, which exploits the known effect of system parameters such as PEG and salt concentration and the nominal molecular mass of PEG upon product partition, was used. Purified dlRLP were initially used as a model system for evaluating their partition behaviour. Subsequently, the real complex system, consisting of cellular homogenate and supernatant from insect cell cultures was studied to obtain different conditions where the target product and the contaminants (e.g. cell debris) partitioned preferentially to opposite phases. The results of this work provide a strategy that greatly improves the traditional way in which dlRLP are recovered, with significant scope for generic commercial application.

2. Materials and methods

2.1. Production of dlRLP

dlRLP were produced in High Five insect cells (Invitrogen, Carlsbad, CA, USA), as described in Mena et al. [2]. Briefly, cells were cultivated in suspension in SF900-II medium (Invitrogen, Carlsbad, CA, USA) in shaker flasks. Cultures were simultaneously infected at a cell concentration of 0.5×10^6 cell/mL with a baculovirus coding for the fusion protein EGFPVP2 (kindly provided by Professor Jean Cohen, INRA, France) [16] and another coding for VP6 from strain SA11 (kindly provided by Dr. Susana López, IBT-UNAM, México) at a multiplicity of infection of five plaque forming units/mL of each virus. Viral titters were determined as described by Mena et al. [17]. Cells were harvested by centrifugation at 48 h post-infection at 10,000 rpm for 10 min (Beckman, UK). The supernatant was identified in this study as extra-cellular dlRLP source (cell debris-free extract). After cell harvesting, the biomass obtained from 200 mL of culture was re-suspended in 6 mL of Tris EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, 2% of sodium deoxicholate). Cell rupture was achieved by sonication for 2 min (periods of 10 s, separated by pauses of 5 s). The resulting homogenate was identified in this study as intracellular dlRLP source (crude extract). Material for the model systems was obtained by purifying dlRLP by the traditional method of ultracentrifugation [1]. Briefly, dlRLP from culture supernatants were concentrated by a 35% sucrose cushion by ultracentrifugation. dlRLP were obtained by ultracentrifugation in an isopicnic CsCl gradient at $148{,}000 \times g$ for 18 h. The band corresponding to dlRLP was recovered and ultrafiltrated through a 300 kDa MW cut-off membrane.

2.2. Analytical procedures

The concentration and purity of dlRLP was determined by gel exclusion HPLC, as described in Mena et al. [2]. The chromatographic system consisted in a controller, pumps, and a photodiodes array and a fluorescence detectors connected in series (Waters, Massachusetts, USA). The mode of operation of the chromatography was isocratic, with a Tris-EDTA pH 8.0 buffer mobile phase at a flow rate of 0.9 mL/min using an Ultrahydrogel 2000 size-exclusion column, or an Ultrahydrogel 2000 and an Ultrahydrogel 500 columns in series (Waters, Massachusetts, USA). Prior to injection into the HPLC equipment, samples were ultrafiltered (10 and 300 kDa MW cut-off, Nanosep, Pall Life Sciences, Ann Arbor, MI, USA) and centrifuged at $10{,}000 \times g$ for 15 min. EGFP fluorescence was determined with the fluorescence detector, and concentration of EGFPVP2 was determined by comparison with a standard curve of pure EGFP concentration versus fluorescence (excitation and emission wavelengths of 484 and 510 nm, respectively). Charpilienne et al. [16] have determined that the molar extinction coefficient of EGFPVP2 is that of EGFP. Thus, dlRLP concentration was determined from its content of EGFP. Namely, the molecular masses of the dlRLP and EGFP are 49.5×10^6 and 27×10^3, respectively. Therefore, 1 g of EGFP is contained in 15.27 g of dlRLP. The purity of dlRLP relative to contaminant proteins was estimated by absorbance at 280 nm using the photodiodes array detector (Waters, Massachusetts, USA). Transmission electron microscopy was performed as described by Mena et al. [2]. Briefly, dlRLP negatively stained with uranile acetate after fixation in a 200 mesh grid coated with Formvar-carbon (Structure Probe Inc., West Chester, PA, USA) were observed with an electron microscope Jeol 1200EXII (Jeol, Peabody, MA, USA).

2.3. Characterisation of aqueous two-phase systems

The binodal curves were estimated by the cloud point method [18] using poly(ethylene glycol) (PEG, Sigma Chemicals, St. Louis, MO, USA) of nominal molecular mass of 400, 600, 1000, 1450, 3350 and 8000 g/mol (50% (w/w) stock solution) and ammonium sulphate or di-potassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate (Sigma) (30%, w/w). Fine adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide.

2.4. Influence of system parameters upon partition behaviour of dlRLP from insect cells in PEG-salt systems

All experimental systems used to establish the operating conditions for the ATPS process were prepared for convenience on a fixed mass basis. Predetermined quantities of stock solutions of PEG, ammonium sulphate or potassium phosphate were mixed with either a single model system (containing purified dlRLP) or a complex system (containing 10% (w/v) supernatant or cell homogenate from insect cell cultures; referred above as extracellular dlRLP or crude extract (intracellular dlRLP), respectively), to give a final weight of 1.0 and 10 g for model and complex systems, respectively. The stock solutions (PEG or salts) were mixed and phases dispersed by gentle mixing for 30 min at 25 °C. Adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide. Complete phase separation was achieved by low speed batch centrifugation at 1500 × *g* for 20 min at 25 °C. Estimates of the volumes of top and bottom phases and solids, utilizing a calibrated syringe with a total volume of 100 μL or with a micropipette in the case of model systems, or 15 mL graduated centrifuge tubes in complex systems. The volumes of the phases were used to estimate the volume ratio (volume of the top phase/volume of the bottom phase, Vr). Samples were carefully extracted from the phases (top and bottom phase) and analyzed. In the particular case of sample from interface, top and bottom phases were withdraw with care to avoid interface perturbation. The remaining material identified as interface was then re-suspended in Tris-EDTA buffer and consequently diluted for biochemical analysis. The systems tie-line length (TLL), which represents the length of the line that connects the composition of the top and bottom phase of a defined ATPS, was estimated using the relation in which TLL is equal to $[(\Delta PEG)^2 + (\Delta salt)^2]^{1/2}$; where ΔPEG and Δsalt are the differences in concentration between top and bottom phase of PEG and salt, obtained from the interception of the tie-line of the ATPS with a defined Vr with the phase diagram as described by Albertsson [19]. The top phase and interface dlRLP recovery was estimated as the amount of dlRLP present in the upper phase or interface and expressed relative to the original amount loaded into the system. Bottom phase recovery was not estimated because the amount of dlRLP present in such phase was undetectable. Results reported are the average of three independent experiments and errors were estimated to be a maximum of ±5% of the mean value.

3. Results and discussion

3.1. Partition behaviour of purified dlRLP from insect cells in PEG-salt aqueous two-phase systems

The unknown mechanism governing the behaviour of dlRLP in ATPS limited the predictive design of extraction processes using ATPS. In this study, the influence of system parameters on the partition behaviour of dlRLP was studied using single model systems before designing the aqueous two-phase process. Such systems were characterised by the presence of purified dlRLP (in a concentration of 1000 ng/mL in the systems). These systems do not account for the influence of the whole range of proteins, contaminants, and cell debris which may be present in the crude extract of insect cells upon the performance of ATPS.

It has been established [13] that, the extent of the empirical experiments necessary to determine the process conditions of an ATPS extraction can be reduced by using a practical approach which exploits the known effect of system parameters such as tie-line length (TLL), phase volume ratio (Vr), system pH and molecular mass of PEG on the protein partition behaviour. In the present work, it was decided to use the practical approach exploited for proteins to examine the partition behaviour of purified dlRLP. Initially, the effect of increasing TLL upon partition behaviour of dlRLP was evaluated. Changes in the TLL affect the free volume available for a defined solute to accommodate in the phase and, as a consequence, the partition behaviour of such solute in the ATPS [20].

Table 1 illustrates the impact of increasing TLL upon recovery of dlRLP from model ATPS, when PEG of six different molecular mass (from 400 to 8000 g/mol) were used. For all these systems, volume ratio and pH were kept constant at 1.0 and 7.0, respectively. In the partition experiments that used purified dlRLP, these could not be detected in the bottom phase. It is possible that the majority of the dlRLP concentrated in the top phase or interface, or that dlRLP were destabilized in the bottom phase due to its high ionic strength. To test this second hypothesis, dlRLP were submitted to 500 mM of ammonium phosphate for 30 min, treated as the samples from ATPS, and analyzed by HPLC. As can be seen in Fig. 1, such a treatment completely eliminated fluorescence from the chromatograms. Exposure of dlRLP for 30 min to 500 mM of ammonuim phosphate completely abated fluorescence. The decrease in fluorescence can only be a consequence of the loss of structure of EGFP, since quenching can be discarded as salts were eliminated prior to injection to the HPLC system. As the EGFP portion of EGFPVP2 is inside dlRLP, it is most likely that the lack of fluorescence was preceded by a destabilization of the particle. A similar effect can be expected from the exposure of dlRLP to the bottom phase of ATPS, which has an ammonium phosphate concentration superior to 1.0 M.

The absence of a detectable amount of dlRLP in the bottom phase impeded, as for the case of soluble material, the estimation of the partition coefficient (K = concentration of dlRLP in the top phase/concentration of dlRLP in the bottom phase) in all the systems studied. Alternative, partition ratio of the systems could be estimated and used to evaluate the impact of system

Table 1
Influence of increasing tie-line length (TLL) upon the recovery of double-layer rotavirus-like particles (dlRLP) from PEG/phosphate ATPS

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Top phase recovery of dlRLP (%)	Interface recovery of dlRLP (%)
1	400	19.4	16.0	35	60 ± 3	24 ± 1.2
2		25.1	17.9	52	57 ± 2.9	26 ± 1.2
3	600	14.0	15.5	22	55 ± 2.8	29 ± 1.5
4		18.3	17.4	42	49 ± 2.5	32 ± 1.6
5	1000	18.2	15.0	35	ND	84 ± 4.2
6		18.9	16.0	40	ND	88 ± 4.4
7		22.2	19.0	48	ND	83 ± 4.2
8		24.1	20.1	57	ND	95 ± 4.1
9	1450	15.1	13.0	27	ND	80 ± 4.0
10		17.5	14.3	37	ND	74 ± 3.7
11		21.9	18.0	53	ND	97 ± 3.0
12		23.0	19.8	59	ND	77 ± 3.9
13	3350	10.1	10.9	17	ND	98 ± 2.1
14		11.0	11.4	23	ND	85 ± 4.3
15		12.2	11.8	27	ND	96 ± 3.1
16		13.7	12.3	31	ND	96 ± 3.1
17	8000	12.2	9.7	22	ND	90 ± 4.5
18		13.2	10.3	26	ND	80 ± 4.0

The tie line lengths (TLL) of the systems were estimated from the composition of PEG and phosphate as described in Section 2. The top phase and interface recovery is expressed relative to the original amount of purified dlRLP loaded into the systems. Concentration of dlRLP in the ATPS was 1000 ng/mL. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively. ND, not detected.

parameters upon the partition behaviour of purified dlRLP by monitoring the partition ratio. However, to address the primary recovery of the product, it was decided to use the dlRLP practical phase recovery (expressed as the amount of dlRLP in the phase relative to the total amount loaded into the ATPS) from the top PEG-rich phase or interface as the response variables to evaluate the effect of system parameters on the behaviour of the dlRLP in ATPS. In some cases, not all the purified dlRLP introduced into the ATPS could be detected in any of the phases or the interface.

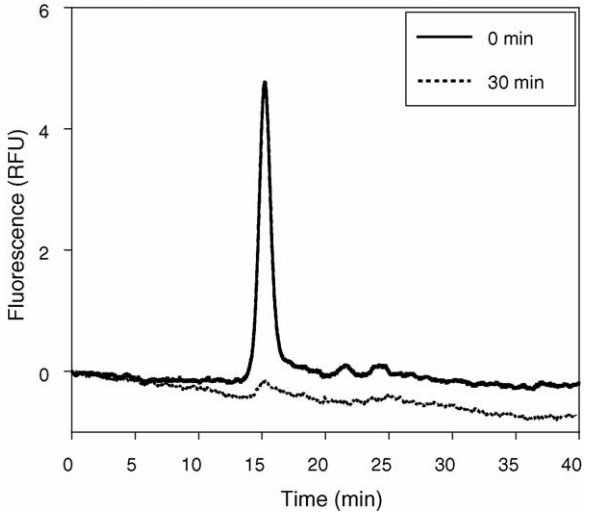

Fig. 1. Effect of high salt concentration on dlRLP stability. Seven hundred nanograms of dlRLP were subjected to 500 mM of ammonium phosphate for 30 min, treated as dlRLP recovered from ATPS, and analyzed by HPLC. Zero minute refers to the sample without treatment and 30 min to the treated sample.

Again such discrepancy may be caused by particle instability and loss of structural integrity (as measured by loss of fluorescence) of EGFP in ATPS (see Fig. 1). The results of Table 1 showed that, increasing TLL caused no significant effect upon the recovery of dlRLP from the top PEG-rich phase or the interface for model systems (with purified dlRLP) of each molecular mass of PEG used. In systems with low molecular mass of PEG (i.e. 400 and 600 g/mol) approximately 50–60% of the purified dlRLP can be recovered from the top phase. However, an increase in the molecular mass of PEG of the systems caused a change in the accumulation of dlRLP from the top phase to the interface. The effect of increasing molecular mass of PEG upon solute partition behaviour has been explained on the basis of protein hydrophobicity [21,22] and phase excluded volume [23,24]. In the case of dlRLP, the decrease in free volume available in the top phase for solute dissolution with the increase in the molecular mass of PEG, may explain the potential migration of dlRLP from the top phase to the interface. ATPS with low molecular mass of PEG (i.e. PEG 400 and PEG 600) exhibited the best conditions for dlRLP top phase recovery. ATPS using PEG of higher molecular mass (greater than 600 g/mol) can be considered for interface dlRLP recovery. In this case the majority of dlRLP (more than 95%; see Table 1) can be potentially recovered from the interface. Once the impact of increasing TLL upon the recovery of dlRLP was evaluated, the effect of system pH upon dlRLP partition behaviour was investigated using a model system.

The influence of system pH on protein partition behaviour has been discussed before [25,26]. In general, these reports concluded that increasing the pH (e.g. from 6.5 to 9.0) caused changes in the partition behaviour of proteins attributed to free-

Table 2
Influence of double-layer rotavirus-like particles (dlRLP) concentration upon product recovery from PEG/phosphate ATPS

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	dlRLP concentration (ng/mL)	Top phase recovery of dlRLP (%)	Interface recovery of dlRLP (%)
a	400	19.4	16.0	1000	60 ± 3	24 ± 1.2
				5000	32 ± 2	13 ± 1.0
b	3350	10.1	10.9	1000	ND	98 ± 2.1
				5000	ND	32 ± 1.6

The top phase and interface recovery is expressed relative to the original amount of purified dlRLP loaded into the systems. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively. ND, not detected.

volume effects [26], to speciation of the phosphate salts over the pH range, and to conformational changes in the structural integrity of proteins [27]. However, for all the ATPS studied, no significant influence of the system pH (from 7.0 to 9.0) upon the recovery of dlRLP was observed (data not shown). Since potential recovery of dlRLP from the interface in selected ATPS (e.g. PEG 3350) exceeded 90% of the total amount loaded into the systems (see Table 1), further evaluation of additional systems parameters (i.e. volume ratio) was not pursued at this stage.

In an attempt to improve the recovery of dlRLP using an ATPS process (by increasing the amount of dlRLP that can be processed by the systems), the effect of an increase in the dlRLP concentration on the performance of ATPS was evaluated. It was decided to examine the impact of increasing dlRLP concentration (from 1000 to 5000 ng/mL) on product recovery by using selected ATPS with PEG with molecular mass of 400 and 3350 g/gmol. The ATPS used in this part of the study (i.e. systems "a" and "b" in Table 2; equivalent to systems 1 and 13 in Table 1) were selected on the basis of product recovery (from the top phase and interface) from the previous experiments (see Table 1). Systems 1 and 13 exhibited the best top phase and interface dlRLP recovery, respectively, from the ATPS studied. Table 2 illustrates the effect of dlRLP concentration in the systems on product recovery. It is clear that interface and top phase dlRLP recovery decreased for both ATPS when the concentration of product of interest (dlRLP) increased. It is probable that the dlRLP migrated to the bottom phase once the free-space of top phase and interface is saturated. However, dlRLP were not detected in the lower phase, possibly because of a negative effect of the high salt concentration environment on dlRLP structural integrity. It is clear that an attempt to improve the recovery systems (using purified dlRLP) by increasing the amount of biological material that can be processed (simulated by an increase in dlRLP concentration in the ATPS), resulted in a decrease of the percentage of product recovered either from the top phase or interface (Table 2).

In general, from the ATPS studied with purified dlRLP, the systems with Vr = 1.0, PEG 400 19.4% (w/w), phosphate 16.0% (w/w) at pH 7.0 provided the required conditions to concentrate dlRLP from insect cells in the top phase (i.e. top phase recovery of 60%). In contrast, systems with Vr = 1.0, PEG 3350 10.1% (w/w), phosphate 10.9% (w/w) at pH 7.0 facilitated the accumulation of dlRLP in the interface (i.e. interface recovery of 98%). It is clear that such findings will facilitate the potential generic application of ATPS process to recover dlRLP from complex systems (i.e. crude extract and cell debris free-supernatant).

3.2. *Recovery of intracellular dlRLP from insect cells in PEG-salt aqueous two-phase systems*

Once the feasibility of dlRLP recovery in ATPS was established using model systems, the potential recovery of dlRLP from complex systems in ATPS was investigated. Initially, the processing of the homogenate of insect cell culture (crude extract) for the recovery of intracellular dlRLP in ATPS was attempted. This complex system was characterized by the presence of intracellular dlRLP, contaminant proteins and particularly cell debris derived from the cell disruption stage. In order to evaluate the potential concentration of cell debris and dlRLP in opposite phases, cell debris partition behaviour in selected ATPS was studied by visual observation. Table 3 illustrates the partition behaviour of cell debris from insect cell culture in PEG/phosphate ATPS. According to the results with model sys-

Table 3
Partition behaviour of cell debris from insect cell culture in PEG/phosphate ATPS

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Cell debris phase preference
I	400	19.4	16.0	35	I
II	400	25.1	17.9	52	I
III	1000	18.2	15.0	35	I
IV	1000	24.1	20.1	57	I
V	3350	10.1	10.9	17	B–I
VI	3350	13.7	12.3	32	I

The tie line lengths (TLL) of the systems were estimated from the composition of PEG and phosphate as described in Section 2. Cell debris preference was estimated by visual observation. I and B denote interface and bottom phase preference for cell debris, respectively. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively.

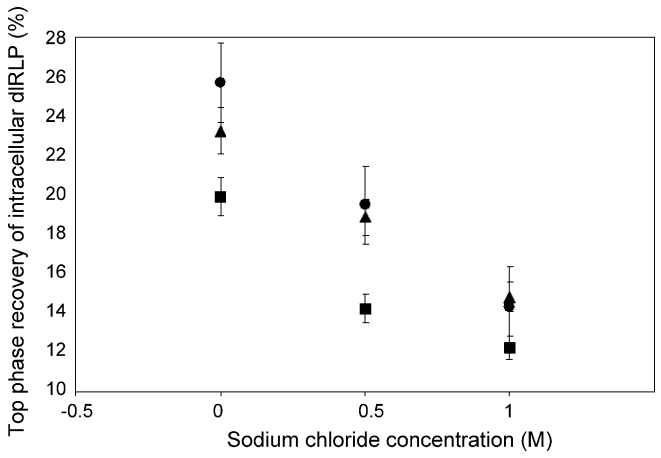

Fig. 2. Influence of sodium chloride concentration upon the top phase recovery of intracellular double-layer rotavirus-like particles (dlRLP) from PEG/salt ATPS. The top phase recovery is expressed relative to the original amount of intracellular dlRLP from crude extract loaded into the systems and it is reported relative to the total concentration of sodium chloride in the ATPS. The selected systems were; 19.4% (w/w) PEG 400 and 16.0% (w/w) phosphate (triangle symbols), 14.0% PEG 600 and 15.5% (w/w) phosphate (square symbols) and 20.0% (w/w) PEG 600 and 13.5% (w/w) ammonium sulphate (circle symbols). For all systems, pH was kept constant at 7.0.

tems (see Tables 1 and 2), ATPS of low and high molecular mass of PEG (i.e. 400 and 3350 g/mol) were selected, together with a system of PEG molecular mass of 1000 g/mol. Cell debris from the crude extract of insect cells exhibited an interface preference. In system of high molecular mass of PEG and short TLL (i.e. system V in Table 3) cell debris exhibited both, interface and bottom phase preferences. Although a clear explanation for the behaviour of the cell debris from insect cell homogenate is not currently available, the results obtained facilitate the selection of operating conditions to favour cell debris separation from the intracellular dlRLP. Such situation can avoid the addition of a cell debris removal process step. It is clear that in order to fractionate insect cell homogenate to recover intracellular dlRLP in ATPS, low molecular mass of PEG (i.e. 400 and 600 g/mol) need to be used. In these systems the majority of purified dlRLP were concentrated on the top phase. Consequently, by using such systems cell debris and intracellular dlRLP can be accumulated at the interface and top phase, respectively.

Processing of intracellular dlRLP in ATPS was attempted using systems of low molecular mass of PEG. In was decided to evaluate the influence of molecular mass of PEG (i.e. 400 and 600 g/mol) and the type of salt forming phase (i.e. phosphate and sulphate) on the recovery of intracellular dlRLP. Furthermore, as suggested by Andrews et al. [15] for the purification in ATPS of VLP of hepatitis B virus, the effect of molar concentration of sodium chloride in the ATPS upon the recovery of dlRLP was also studied. Such strategy was followed in an attempt to increase the top phase dlRLP recovery. Fig. 2 illustrates the influence of sodium chloride concentration upon the top phase recovery of intracellular dlRLP from PEG 400 and 600/salt ATPS when two salts forming phase (i.e. phosphate and sulphate) were used. It is clear that an addition of sodium chloride to the systems resulted in a negative effect on the top phase recovery of the dlRLP, regardless of the use of phosphate or sulphate salts (see Fig. 2). Such behaviour may be explained by the migration of dlRLP to the interface caused by the saturation of the free-volume of the top phase by sodium chloride addition. The observed turbidity of the top PEG-rich phase when sodium chloride was added may suggest phase saturation. An additional explanation may involve the possible degradation of dlRLP due to the excessive ionic strength generated by addition of sodium chloride [28]. Therefore, regardless of the mechanism behind the decrease of recovery of dlRLP, sodium chloride in the concentrations tested in this work should not be added to ATPS for the purification of dlRLP. The differences observed in the recovery of dlRLP from the top PEG-rich phases from the model and complex systems (see Table 1 and Fig. 2) are explained by the nature of the experimental vehicle (purified dlRLP and insect cell homogenate). In the case of model systems, the sole presence of the target product resulted in a top phase dlRLP recovery of 49–60% (Table 1). In contrast, for the complex systems, the presence of contaminants (particularly cell debris) from the insect cell homogenate affected the partition behaviour and top phase recovery of dlRLP (20–26%; Fig. 2). Apparently, the presence of a variety of contaminants from the insect cell homogenate contributed to the top PEG-rich phase saturation. Consequently, the free-volume available for dlRLP allocation was greatly reduced and the top phase dlRLP recovery was negatively affected. Such situation can be addressed by decreasing the amount of contaminants in the homogenate or by increasing the volume of the top phase of the ATPS. A decrease in the amount of contaminants from the homogenate can be achieved by a dilution strategy. However, a dilution strategy implies processing greater volumes which will necessarily affect the potential implementation of the resulting process.

In an attempt to increase the top phase recovery of dlRLP, ATPS with a larger top PEG-rich phase (Vr greater than 1) were selected. In these systems it was expected that the free-space available for dlRLP and contaminants accumulation was favoured and as a result top phase dlRLP recovery can be increased. Table 4 illustrates the effect of system Vr and dlRLP concentration upon product recovery in PEG/salt systems. It is clear that an increase in the system Vr benefited the top PEG-rich dlRLP recovery from the ATPS of 400 and 600 molecular mass of PEG. Furthermore, an increment in the concentration of the crude extract (as represented by the increase in the dlRLP concentration from 2000 to 5000 ng/mL) did not negatively affect the top phase product recovery in ATPS characterized by Vr greater than 1. The increase in the top phase product recovery observed with increasing Vr in ATPS was similar regardless the salt forming phase (i.e. phosphate or sulphate) of the ATPS used. However, a slight advantage was obtained when ATPS of low molecular mass of PEG (i.e. 400 g/mol) was used. In general, from the ATPS evaluated, the system comprising Vr = 13.0, PEG 400/phosphate, TLL 35% (w/w) at pH 7.0 provided the best conditions to process high concentrated crude extract (dlRLP concentration of 5000 ng/mL) and accumulate intracellular dlRLP in the top phase (i.e. top phase dlRLP recovery of 47%) and cell debris in the interface. In order to further increase the process recovery, a subsequent ATPS extraction stage was used. In

Table 4
Influence of system volume ratio (Vr) and crude extract concentration upon the recovery of intracellular double-layer rotavirus-like particles (dlRLP) from PEG/salt ATPS

System	Molecular mass of PEG (g/mol)	TLL (%, w/w)	System volume ratio (Vr)	dlRLP concentration (ng/mL)	Top phase recovery of dlRLP (%)
i	400	35	1.0	2000	23 ± 2.0
			11.0	2000	50 ± 3.4
			13.0	5000	47 ± 1.0
ii	600	27	1.0	2000	26 ± 2.0
			5.0	2000	31 ± 1.2
			6.0	5000	42 ± 3.0

The tie line lengths (TLL) of the systems were estimated from the composition of PEG and salt as described in Section 2. Systems i and ii comprise PEG400/phosphate and PEG600/sulphate ATPS, respectively. System volume ratio (Vr) estimated from non-biological experimental systems after phase separation in graduated tubes. The top phase and interface recovery is expressed relative to the original amount of intracellular dlRLP from crude extract loaded into the systems. For all systems, pH was kept constant at 7.0.

this additional extraction stage, the interface (where the unrecovered dlRLP together with cell debris was present) from the previous ATPS, was further processed by the addition of fresh PEG and phosphate. The process conditions for the subsequent ATPS extraction stage were kept constant and equal to those used for the first ATPS extraction. The second extraction had a high efficiency of dlRLP recovery, most likely due to the lower amount of dlRLP and contaminants present in the crude extract. Such result agrees with the effect of dlRLP load in model systems (see Table 2). The use of a consecutive aqueous two-phase systems resulted in a two-stage ATPS process with a 90% overall dlRLP top phase recovery (i.e. 47 and 43% from the first and second ATPS extraction stage, respectively and relative to the original amount of dlRLP loaded to the first extraction stage). It is clear that such strategy proved to be effective to increase the dlRLP recovery (from the top PEG-rich phase) by further removing (in the interface and bottom phase) the majority of cell debris and protein contaminants.

3.3. Recovery of extracellular dlRLP in PEG-salt aqueous two-phase systems

In this study, extracellular dlRLP were defined as those obtained from the insect cell culture supernatant. It has been determined that approximately 60% of the total dlRLP from the insect cell culture are contained in the supernatant and the remaining 40% are intracellular [2]. Therefore, it is relevant (in order to increase process yield) to also pursue the potential recovery of dlRLP from the supernatant. In this particular case, the problem of cell debris accumulation at the interface is not longer present. Thus, it is beneficial to process a cell debris-free extract in ATPS for the recovery of dlRLP. Consequently, ATPS in which dlRLP can be accumulated at the interface are recommended for the recovery of extracellular dlRLP. From the studies of the influence of system parameters upon dlRLP recovery in ATPS using model systems (see Table 1), ATPS of low and high molecular mass of PEG (i.e. 400, 1000 and 3350 g/mol) were selected to process the supernatant from insect cell culture. Such systems were evaluated to establish the conditions for an ATPS extraction stage of extracellular dlRLP. Table 5 illustrates the effect of molecular mass of PEG upon the recovery of extracellular dlRLP from PEG/phosphate ATPS. Approximately 50% of the dlRLP were accumulated at the interface of ATPS of low molecular mass (i.e. 600 and 1000 g/mol). Nonetheless, it is evident that the use of ATPS (comprised of PEG 10.1% (w/w), phosphate 10.9% (w/w), Vr of 1.0 at pH of 7.0) with PEG of high molecular mass (i.e. 3350 g/gmol) was more favourable for the accumulation of dlRLP at the interface (i.e. interface recovery of dlRLP of 82%; see Table 5).

The study presented here resulted in a process strategy that produced intracellular and extracellular dlRLP recovery of 90 and 82%, respectively (Table 6). By considering that 40% of the dlRLP are intracellular and 60% of dlRLP are extracellular [2], the overall recovery of dlRLP from insect cell culture in ATPS process was approximately 85%. The purity of the resulting product increased from 0.2% in the culture supernatant [2] to 6–11% (see Table 6), which represented an increase of 30–55 times. The outline of the new proposed process is summarized in Fig. 3. In the current study centrifugation was used to obtain cell debris-free supernatant containing the extracellular dlRLP and the biomass for the cell disruption stage. Cell disruption was archived by sonication. This would, of course, be impractical at

Table 5
Influence of molecular mass of PEG upon the recovery of extra-cellular double-layer rotavirus-like particles (dlRLP) from PEG/phosphate ATPS

System	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Molecular mass of PEG (g/mol)	Interface recovery of dlRLP (%)
A	14.0	15.5	22	600	44 ± 1.1
B	24.1	20.1	57	1000	48 ± 6.0
C	10.1	10.9	17	3350	82 ± 2.3

The interface recovery is expressed relative to the original amount of extra-cellular dlRLP from cell debris-free supernatant loaded into the systems. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively.

Table 6
Potential aqueous two-phase systems for the primary recovery of double-layer rotavirus-like particles (dlRLP) from insect cells

Type of dlRLP (source)	Molecular mass of PEG (g/mol)	TLL (%, w/w)	System volume ratio (Vr)	Top phase recovery of dlRLP (%)	Interface recovery of dlRLP (%)	Purity of dlRLP (%)
Intracellular (crude extract)	400	35	13.0	90 ± 5.0	ND	11.0
Extra-cellular (cell debris-free extract)	3350	17	1.0	ND	82 ± 2.3	6.4

The top phase recovery of intracellular dlRLP represents the sum of the top phase product recovery from the first and second ATPS extractions and is expressed relative to the original amount of intracellular dlRLP from crude extract loaded to the first extraction system. Interface dlRLP recovery is expressed relative the original amount of extra-cellular dlRLP from cell debris-free supernatant, loaded into the system. Purity of dlRLP was estimated as described in Section 2. ND, not detected.

process scale but can be easily substituted by a mechanical cell disruption (e.g. bead mill or homogenization). ATPS extraction was then applied for the processing of both intracellular and extracellular dlRLP at conditions defined in Table 6. An ultrafiltration stage can then be implemented for polymer removal to obtain a potential process recovery of 85% (w/w). This novel process greatly reduces the processing time and the consumption of reagents and simplifies the traditional way in which dlRLP expressed in insect cell-baculovirus system can be recovered, with significant scope for generic commercial application. It is clear that, for dlRLP, this bioengineering strategy opens the way to further bioprocess improvement. Particularly, products, such as VLP, in which their potential production using conventional processes is not economically feasible.

3.4. Direct comparison of the primary recovery of dlRLP with PEG-salt aqueous two-phase systems and zonal centrifugation

To evaluate the primary recovery of dlRLP with other purification methods, dlRLP from culture supernatants were purified by CsCl gradients or recovered by ATPS, and compared. dlRLP obtained by both methods were observed by electron microscopy. Representative micrographs are shown in Fig. 4. It can be seen that, in both cases, dlRLP were intact and had a similar morphology to that previously reported [2]. Analysis by gel-permeation HPLC (Fig. 5) showed that particles recovered using both methodologies had the same retention time, meaning that they had a similar size distribution (Fig. 5, peak a). The chromatograms in Fig. 5 also show that the purity of dlRLP recovered by ATPS was higher to that obtained after zonal centrifugation. The purity of particles obtained by cesium chloride gradients, as determined from integrating the peaks, was of 2.3%, with a yield of 1.8%. Such values contrast with the performance of ATPS, with yields of 85% and a purity of 6–11%. It should be noted that in the case of dlRLP purified by CsCl gradients, a large peak (b) was detected by fluorescence. It has been previously shown that such a peak corresponds to EGFP, which is the product of the cleavage of the fusion protein GFP-VP2. The appearance of non-fused EGFP indicates that dlRLP were destabilized and EGFP-VP2 was afterwards cleaved [2]. The large area of the EGFP peak in the sample purified by CsCl gradients underlines the extreme conditions to which particles are exposed during zonal centrifugation. In contrast, degradation of dlRLP recovered by ATPS was much lower (Fig. 5B). It can be concluded that the primary recovery of dlRLP by ATPS results in higher yields, low dlRLP destabilization, and a higher ratio between assembled and disassembled proteins.

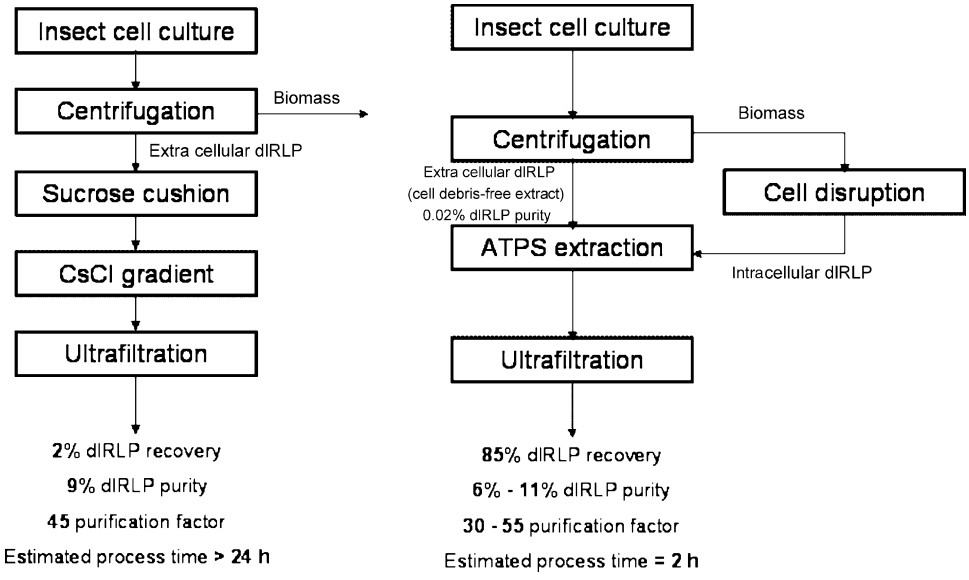

Fig. 3. Simplified representation of the current protocol for the primary recovery of double-layer rotavirus-like particles dlRLP produced by insect cells and the new proposed strategy using aqueous two-phase systems

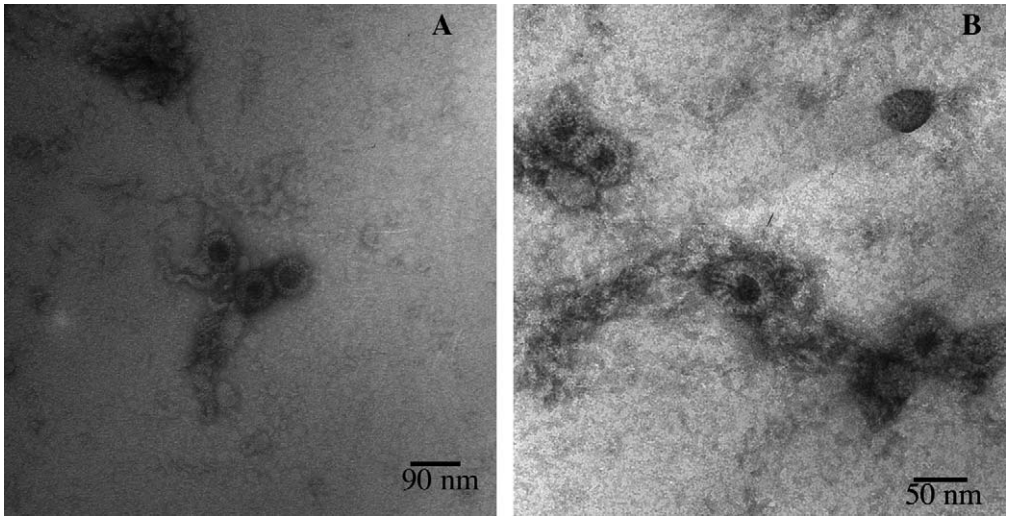

Fig. 4. Electron microscopy of dlRLP recovered by cesium chloride gradients (A) or ATPS (B). Magnification of (A) 85,000× and (B) 140,000×.

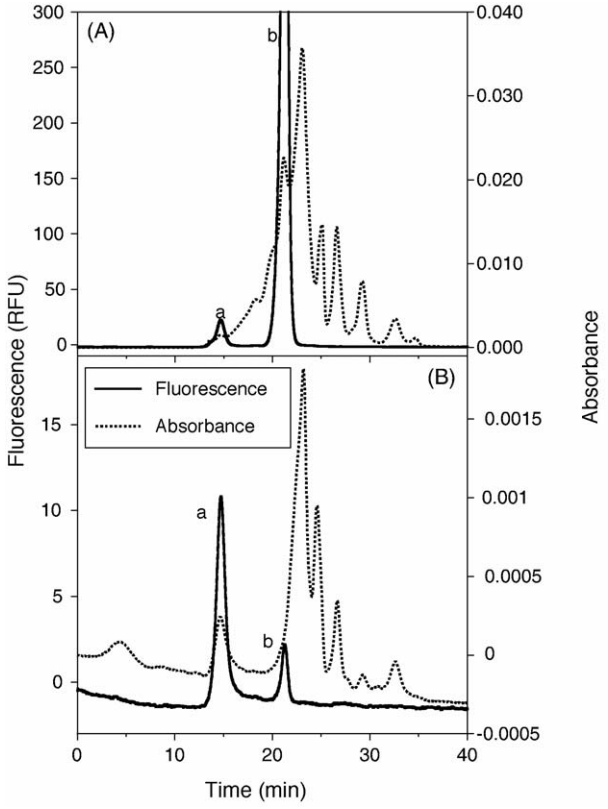

Fig. 5. Gel-permeation chromatograms obtained from dlRLP recovered by (A) cesium chloride gradients or (B) ATPS. An Ultrahydrogel 500 and a Ultrahydrogel 2000 columns were connected in series. Peak (a) has been identified as dlRLP, and peak (b) as EGFP.

4. Conclusions

This study reports the fractionation of cell homogenate and supernatant of insect cell-baculovirus expression system in aqueous two-phase systems for the development of a process for the primary recovery and potential purification of dlRLP. It was shown that molecular mass of PEG influenced the partition behaviour of purified dlRLP when model systems were used. dlRLP accumulated at the top PEG-rich phase when ATPS of PEG molecular mass of 400 and 600 g/mol were used and at the interface in ATPS comprising molecular mass of PEG1000 g/mol and higher. Increasing dlRLP concentration (>1000 ng/mL) in the ATPS resulted in reduced product yield. Cell debris from the insect cell homogenate accumulated at the interface which compromises the potential recovery of intracellular dlRLP from that phase. Addition of sodium chloride to the ATPS proved to be unsuitable to increase the recovery of dlRLP since a reduction of product yield from the top phase was observed. The operating conditions established for the PEG400 and PEG3350-phosphate ATPS extraction resulted in a process for the potential recovery of dlRLP from insect cell culture. These conditions accumulated the intracellular dlRLP preferentially to the top phase and the extracellular dlRLP to the interface. Overall, the results reported here demonstrate the potential application of ATPS for the recovery of structural VP2/VP6 proteins of dlRLP as a first step in a process simple to scale-up.

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