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351

ATPS as an Efficient Method for Separation of Bionanoparticles: Investigation and Optimization of Partition Behavior of pDNA

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In this paper, the efficiency of an aqueous two-phase system (ATPS) for purification of nanometer-sized bioparticles, plasmid DNA (pDNA), was studied. Polymer-salt ATPS consisting of polyethylenglycol (PEG)-K_2HPO_4 was used for the purification of 7 kb and 14 kb plasmid vectors. PEG-300 and PEG-1450 were applied to investigate the influence of different molecular mass of PEG on partitioning behavior of pDNA. The Taguchi design of experiments has been applied in order to optimize the significant system characteristics including PEG/salt ratio, temperature, lysate mass fraction and size of plasmid for pDNA separation by using ATPS. The results indicated that PEG/salt ratio has a considerable contribution on pDNA recovery both in the presence of PEG-300 and PEG-1450. It is also obtained that the size of pDNA in the range of 7 kb to 14 kb is not a significant factor on its partitioning. Furthermore, pDNA is easily partitioned to polymer-rich top phase in PEG300/salt system; and in salt-rich bottom phase in PEG1450/salt system. Under optimum conditions, pDNA was extracted in top phase of PEG-300/K_2HPO_4 with mass percent of 26 : 17 at 25 °C with a recovery percent of 85.

Key words:
Aqueous two-phase system, Plasmid DNA, Taguchi, Design of experiments, nanobioparticles

Introduction

Gene therapy categorized in two viral and non-viral systems can be applied to the treatment or prevention of genetic or acquired diseases. Plasmid DNA belongs to the category of non-viral systems which is considered more scalable in production, easier in controlling, monitoring and quality control testing than those for viral vectors.

The development of molecular therapies such as non-viral gene therapy and DNA vaccination has increased the need for high quantities of highly purified plasmid DNA.[1,2] A problem that is associated with the large size of nanobioparticles such as pDNA is their greatly reduced rate of diffusion in comparison with smaller biomolecules. Such reduced diffusion rates can cause mass transfer limitation during conventional chromatographic adsorption process.[3,4] It may be concluded that the above problems led to an increase in research directed towards the development of alternative methods for the downstream processing of these nanobioparticles.

An ATPS is formed when low concentrations of two incompatible polymers or of one polymer and an inorganic salt are mixed so that two immiscible phases coexist.[5] These systems are high in water fraction (w = 70–90 %), have low interfacial tension, and are safe, non-flammable, and relatively environmentally benign as extraction or reaction media. The other well-known advantages of ATPS are volume reduction, high capacity, easy scale-up and suitability for continuous large- scale operations.[6] This system is widely used in biochemistry and biotechnology for purification of proteins,[7,8] enzymes,[9,10] amino acids,[11] antibiotics,[12,13] aroma compounds[14] and lactic acid.[15] Application of ATPS has also been extended to paper pulp processing for delignification.[16–18] But the use of ATPS for the separation and purification of nanobioparticles is still a niche area.[19] Recently, a wide range of applications of the polymer-salt ATPS in the separation of organic molecules, metal ions, radiochemicals, and the recovery of nanoparticle matter and minerals have been demonstrated.[16,20–27] The driving force behind separation of enzymes, amino acids or antibiotics in a polymer-salt ATPS is not com-

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pletely understood, although phase separation may be due to water structuring and the partial dehydration of the polymer chain within the two phases.[28–30] The relative ability of various salts to salt-out PEG has been found to be dependent on ionic charge, hydration radius, ability to structure water, and specific interactions between the polymer and salt. Salting-out effects of ions can also be related to the ion's lyotropic number,[31] position in the Hofmeister series or the Gibbs free energy of hydration of the ions and their salting-out ability.[32]

Conventional optimization procedures involve altering of one parameter at a time keeping all other parameters constant, which enables assessment of the impact of those particular parameters on the process performance. These procedures are time-consuming, cumbersome, require more experimental data sets and cannot provide information about the mutual interactions of the parameters.[33] Therefore, new designs of experiments (DOE's) have been developed. Taguchi method has been used widely in industrial process design, principally in developmental trials. This technique is used to generate enough process information by using limited experiments to establish the screening and optimal conditions of parameters for a particular process. Taguchi method of DOE involves establishment of a large number of experimental situations described as orthogonal arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments. In the design of an OA, each column consists of a number of conditions depending on the levels assigned to each factor.[34]

In the present study, the polyethylene glycol (PEG)-salt (K_2HPO_4) ATPS is employed. The polymer-salt ATPS's have several advantages over polymer-polymer systems consisted of lower cost of phase-forming chemicals and their lower phase viscosities which make them easier to handle on a large scale.[35,36] Plasmid DNA, 7 kb and 14 kb in size, was used and the experiments were carried out on PEG with molecular mass of 300 and 1450. First, we considered a range of temperature and lysate mass fraction for investigating the partition behavior of pDNA in ATPS. Then, the effect of significant factors on pDNA separation by Taguchi DOE's was studied.

Materials and method

Chemicals

PEG 300 and 1450 was obtained from Sigma (St. Louis, MO, USA). Potassium acetate was purchased from Merck. All other reagents used were of analytical grade.

Plasmids production

The host-plasmid system used for pDNA extraction by ATPS carried on pGP1-2 plasmid, 7200 bp in size[6] and pDI121 (Novagen), 14000 bp in size with *Escherichia coli* DH5-α as the host. *E. coli* cells harboring plasmids were cultivated overnight in 1000 mL shake flasks containing 250 mL of LB (Luria Bertani) supplemented with 30 g mL^{-1} of kanamycin (Sigma) at 37 °C and $n = 180$ min^{-1}. *E. coli* cells were resuspended in one volume of resuspension buffer ($c = 50$ mmol L^{-1} Tris-HCl, 10 mmol L^{-1} EDTA, pH 8). Two volume of lysis buffer ($c = 155$ mmol L^{-1} NaOH, $w = 1$ % SDS) were added and mixed for 10 min at ambient temperature. One volume of neutralization buffer ($c = 3$ mol L^{-1} potassium acetate, 10 mmol L^{-1} EDTA, pH 5.5) was then added to the suspension, mixed and incubated for up to 1 h at ambient temperature. Finally, this prepared lysate is used for further processing with ATPS as described below.

Phase diagram and preparation of ATPS

ATPS was made in 100 mL glass bottles. Different mass mixtures of PEG and salt (K_2HPO_4) with or without lysate were prepared. The mixtures were placed in a water bath (memmert, Germany) to keep the defined temperature. For equilibrium and phase separation, the mixtures were centrifuged (Bechman, GPR centrifuge model) at $1000 \cdot g$ for 3 min. The each phase was transferred to a separate bottle for further analysis. ATPS may be characterized by a phase diagram constructed from the component compositions of PEG and salt required to induce bi-phase formation. The binodial curve separated the mono-phase from the bi-phase area. Volume ratio (Ψ) is the ratio of top phase volume (V_T in mL) to bottom phase volume (V_B in mL) that is measured in all the experiments as follows:

$$\Psi = \frac{V_T}{V_B} \qquad (1)$$

Any mixture of PEG and salt was assembled and described on a mass fraction basis to form two phase systems. Clean (blank) systems contained only PEG, salt and distilled water. Crude systems was also contained the lysate. The pH of ATPS containing cell lysate was adjusted to 8 with concentrated HCl. It was almost equal in all experiments.

Assay

The volumes of top and bottom phases were recorded for the calculation of volume ratios and mass balances. Volume ratio was measured by dividing top phase to bottom phase volumes (eq. 1). Material at the interface was harvested together

with small volumes of top and bottom phase next to the interface. The amount of pDNA in each phase (top, bottom an interface phases) was detected ($\mu g\ mL^{-1}$) by spectrophotometer (Beckman DU530, USA) at $\lambda = 260$ nm. The pH was measured with a pH meter (Beckman, USA). The total solubale protein mass concentration was estimated by Bradford protein assay[37] using bovine serum albumin as a standard. For determination of recovery, the amount of pDNA in each phase (top, bottom, interface) was read in spectrophotometer and then divided by the total amount of pDNA that initially entered. The results are presented in percent of pDNA in each phase.

Optimization methodology

Taguchi method was applied to determine the parameters, which significantly influence the partition behavior of plasmid DNA in ATPS. A multiple regression analysis of the data was done with the statistical package of Design-Expert, version 6.0.10 (Stat-Ease Inc., Minneapolis, MN, USA). Four major variables including PEG/salt ratio, temperature, lysate load and size of pDNA were considered as the important factors in partitioning behavior of pDNA in ATPS. We have attempted to find the effect of these factors on pDNA separation and purification. All variables were investigated in three levels except the latter one that considered in two levels. The volume ratio and recovery of pDNA in top, bottom and interface also were measured to determine the partition behavior of pDNA.

Results and discussion

Phase diagram for PEG-300/K_2HPO_4 and PEG-1450/K_2HPO_4

Many experiments were precisely carried out to determine the binodial curve for clean and crude systems. Fig. 1 shows a phase diagram for PEG-300 and K_2HPO_4. The binodial curve for clean compositions and for compositions containing $w = 40$ % lysate are shown. In the presence of crude components in the lysate, the two phase region widens, i.e. the crude binodial curve is shifted to the left of the clean binodial curve. This conformed to the general observation that crude components influence equilibrium conditions of an ATPS in a way that the two phases form at lower concentrations of phase-forming chemicals.[38,39] This trend was observed in phase diagram for PEG-1450 and K_2HPO_4 (Fig. 2). Any mixture of PEG/salt compositions to the right of binodial curve results in two phases. The comparison of two figures indicated that ATPS was formed at smaller
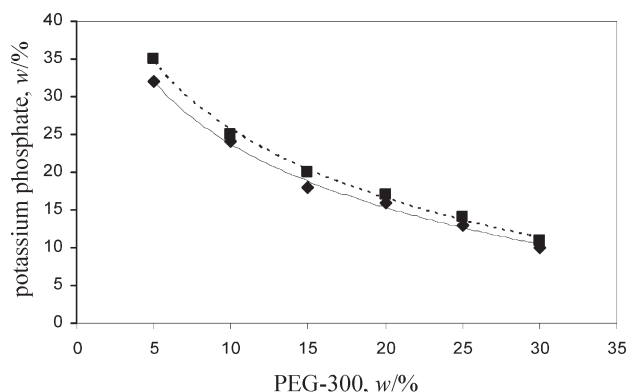

F i g . 1 – *Phase diagram for PEG-300/K_2HPO_4: (♦) crude binodial with w = 40 % lysate and (■) clean ATPS. Biphase regions are to the right of the binodial curve.*
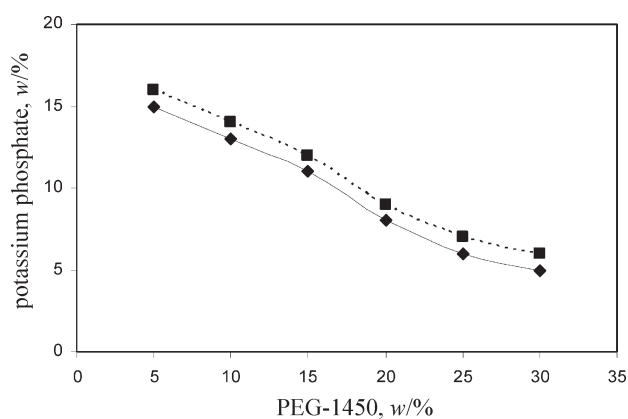

F i g . 2 – *Phase diagram for PEG-1450/K_2HPO_4: (♦) crude binodial with w = 40 % lysate and (■) clean ATPS*

amounts of salt when higher molecular mass of PEG was applied. This is due to the space available for other molecules called excluded volume, which decreases with the increase of PEG molecular mass.

The effect of temperature on pDNA partition behavior

An ATPS composed of PEG-300/K_2HPO_4 with $Z = 15:24$ and $w = 40$ % lysate was chosen to study the influence of temperature on partitioning of pDNA. The distribution profile at temperatures ranging from $\theta = 5$ to 45 °C is shown in Fig. 3. The results indicated that the experimental temperature greatly influenced the partition in a 15:22 ATPS. When temperature increases from $\theta = 5$ to 45 °C, the partition to the top phase was decreased, whereas partition to the bottom increased. At 40 °C, over 40 % of pDNA was recovered in the interface. The volume ratio gradually shifted down from 0.78 to 0.43 with increasing temperature from $\theta = 5$ to 45 °C, respectively. Even though there are some studies describing temperature effects in polymer-polymer systems,[40] the effect of temperature on
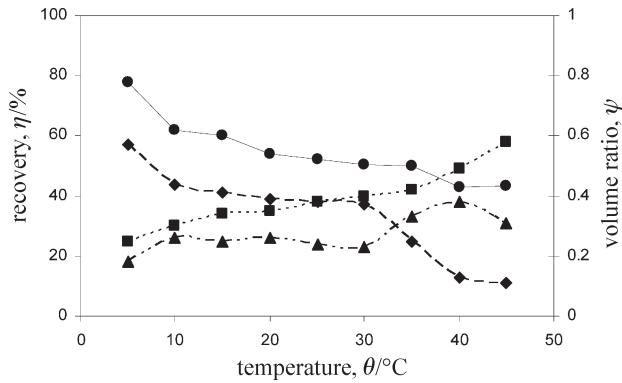
F i g . 3 – *Partition result for plasmid DNA in 15:22 ATPS (w = 15 % PEG-300, w = 22 % phosphate and w = 40 % lysate) with respect to system temperature: (◆) top phase, (■) bottom phase, (▲) interface, (●) volume ratio*

F i g . 4 – *Partition result for plasmid DNA in 15:22 ATPS (w = 15 % PEG-300, w = 22 % phosphate and w = 40 % lysate) with respect to lysate fraction at 20 °C: (◆) top phase, (■) bottom phase, (▲) interface, (●) volume ratio*

the partition of biomolecules in polymer-salt ATPS has hardly been reported in the literature. It has been suggested that the temperature influences the partition behavior of solutes indirectly by changing the chemical compositions of the two bulk phases.[5] It is demonstrated that an increase in the temperature of a polymer/salt system shifts the binodial curve to the left-hand side. Moreover, the volume ratio of an ATPS composed of PEG and salt decreases with temperature.[41] The partition behavior of pDNA can be described in terms of excluded volume effects in the top phase caused by the steric exclusion of pDNA by PEG molecules. Therefore, the fraction of PEG in the top phase increases by increasing the temperature and consequently pDNA is excluded from that phase. On the other hand, the fraction of salt in the bottom phase decreases. This situation results in better solubilization of pDNA in the salt phase. It may be concluded that salting-out effects in the bottom phase become less dominant at higher temperatures.

The influence of lysate load on pDNA partition behavior

It seems that the distribution of pDNA was caused not only by temperature but also by components in the lysate. Therefore, the effect of the amount of crude cell lysate added to a 15:22 ATPS was investigated at three different temperatures, $\theta = 20$, 25 and 30 °C. The plasmid DNA recoveries and the resulting volume ratios for lysate fraction from $w = 20$ to 50 % at 20 °C and 25 °C are shown in Figs. 4 and 5, respectively. At low lysate fraction, pDNA partitioned to the top phase regardless of temperature. With increasing lysate fraction from $w = 20$ to 50 %, pDNA partitioning to the top phase was decreased. The partitions in the top and bottom phases were almost the same in 40 % lysate fraction. It can be as a result of decreasing salting-out effects by increasing lysate fraction. There-
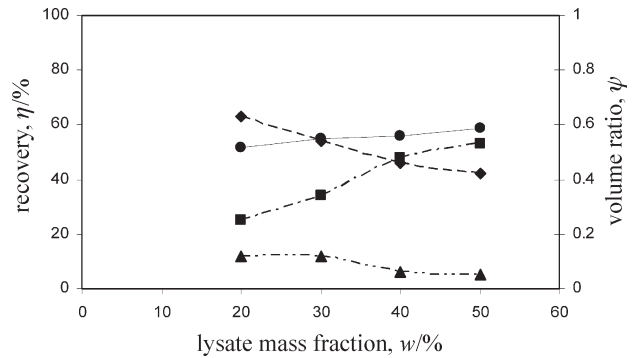

F i g . 5 – *Partition result for plasmid DNA in 15:22 ATPS (w = 15 % PEG-300, w = 22 % phosphate and w = 40 % lysate) with respect to lysate fraction at 25 °C: (◆) top phase, (■) bottom phase, (▲) interface, (●) volume ratio*
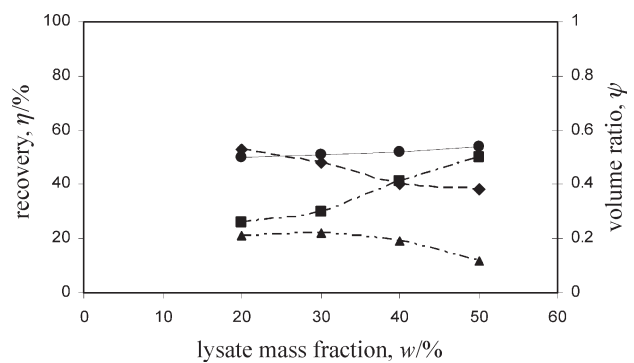
fore, pDNA tends to partition in the salt phase. For 30 °C, also with increasing lysate fraction partition to the bottom phase was increased and partition to the interface decreased for lysate mass fraction between $w = 10$ to 50 % (Fig. 6). For all the experiments, by increasing lysate fractions, the volume ratio was increased. As the lysate used was contaminated with chemical and biological components introduced during the alkaline lysis procedure, it
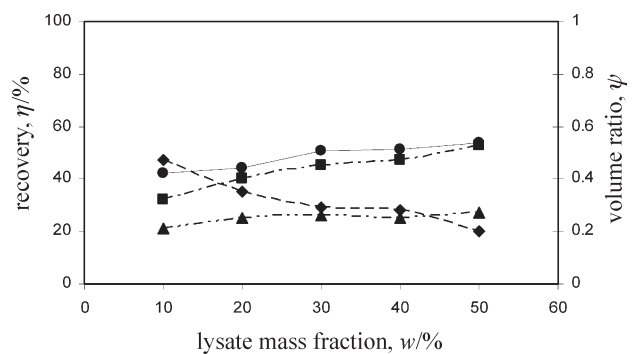

F i g . 6 – *Partition result for plasmid DNA in 15:22 ATPS (w = 15 % PEG-300, w = 22 % phosphate and w = 40 % lysate) with respect to lysate fraction at 30 °C: (◆) top phase, (■) bottom phase, (▲) interface, (●) volume ratio*

seems that they may change the properties of the ATPS and enhance the exclusion of pDNA from the top phase.

Optimization of pDNA recovery in ATPS of PEG300/salt with Taguchi method

The variables and their levels employed to evaluate the pDNA partition in the ATPS PEG-300/K$_2$HPO$_4$ are given in Table 1. These levels were selected based on previous data.

According to Taguchi's experimental design, the orthogonal array of L9 was considered and nine experiments performed as shown in Table 2. The last three columns show the recovery of pDNA in top, bottom and interface phases, respectively.

The ANOVA results indicated that $Z_{PEG/salt}$ ratio has a maximum contribution of 93 % for pDNA recovery in top phase. Plasmid DNA accumulated approximately 85 % in top phase when the $Z_{PEG/salt}$ ratio was 26 : 17 and temperature was increased to 25 °C. This behavior is the result of changes in the chemical composition and salting-out ability of the salt phase. The effect of pDNA size on pDNA recovery was insignificant. The maximum 86.3 % pDNA recovery in the top phase was predicted under optimum conditions consisting of: $Z_{PEG-300/salt}$ ratio of 26 : 17, lysate load 35 % at 25 °C. The sug-

gested conditions were repeated twice and the pDNA recovery of 85 % was achieved. Under optimal conditions, the amount of protein in top phase was decreased to $\gamma = 10$ μg mL^{-1} (Table 2).

Optimization of pDNA recovery in ATPS of PEG1450/salt with Taguchi method

The effect of four variables (Table 3) on pDNA purification in the ATPS of $Z_{PEG-1450/salt}$ was investigated. The orthogonal array of L9 was considered based on the number of factors and their levels and nine experiments designed, as shown in Table 4. The responses were the pDNA recoveries in top, bottom and interface phases.

The ANOVA results also indicated that PEG/salt ratio has maximum contribution of 97 % for pDNA recovery in bottom phase and other factors were not significant. Using higher molecular mass of PEG, pDNA was accumulated in bottom phase up to 73 %. It is obvious that the available space for other molecules decreases with the increase in molecular mass of the polymer. Protein was also partitioned to the bottom salty phase (Table 4).

However, it can be concluded that although two different molecular mass of PEG were used, the factors governing partition behavior of pDNA seemed to be the same.

T a b l e 1 – *Variables and their levels employed in ATPS consisting of PEG300/K$_2$HPO$_4$*

Factors	Level 1	Level 2	Level 3
A: ratio, $Z_{PEG/salt}$	26:17	15:25	10:30
B: temperature, θ/°C	25	30	35
C: lysate load, w/%	30	40	50
D: pDNA size (kb)	7	14	–

T a b l e 3 – *Variables and their levels employed in ATPS consisting of PEG-1450/K$_2$HPO$_4$*

Factors	Level 1	Level 2	Level 3
A: ratio, $Z_{PEG/salt}$	26:9	15:15	8:17
B: temperature, θ/°C	20	30	40
C: lysate load, w/%	20	30	40
D: pDNA size (kb)	7	14	–

T a b l e 2 – *L9 orthogonal array according to the Taguchi experimental design and corresponding recoveries in top, bottom and interface, and the protein quantification in top and bottom phases in ATPS consisted of PEG-300/K$_2$HPO$_4$*

Trial no.	A	B	C	D	Recovery in top, η/%	Recovery in bottom, η/%	Recovery in interface, η/%	Protein in top, γ/μg mL^{-1}	Protein in bottom, γ/μg mL^{-1}
1	26:17	25	30	7	84	10	6	10	120
2	26:17	30	40	14	80	10	10	10	120
3	26:17	35	50	7	75	13	12	20	130
4	15:25	25	40	7	43	16	41	10	80
5	15:25	30	50	7	43	42	15	30	80
6	15:25	35	30	14	50	33	17	90	275
7	10:30	25	50	14	36	46	18	80	280
8	10:30	30	30	7	28	50	22	80	290
9	10:30	35	40	7	45	14	41	85	285

T a b l e 4 – *L9 orthogonal array according to the Taguchi experimental design and the responses as recoveries in top, bottom and interface, and the protein quantification in top and bottom phases in ATPS consisted of PEG-1450/K_2HPO_4*

Trial no.	A	B	C	D	Recovery in top, η/%	Recovery in bottom, η/%	Recovery in interface, η/%	Protein in top, γ/μg mL^{-1}	Protein in bottom, γ/μg mL^{-1}
1	26:9	20	20	7	65	23	12	30	95
2	26:9	30	30	14	66	26	8	30	105
3	26:9	40	40	7	59	26	15	35	85
4	15:15	20	30	7	49	38	13	65	90
5	15:15	30	40	7	47	39	14	45	130
6	15:15	40	20	14	37	46	16	10	160
7	8:17	20	40	14	10	73	17	120	120
8	8:17	30	20	7	15	70	15	55	85
9	8:17	40	30	7	22	65	13	60	100

Conclusion

Aqueous two-phase systems (ATPS) show an interesting alternative and an integration process since several features of early processing steps can be combined in only one operation. The primary studies on the effect of temperature and lysate fraction, independently, on pDNA partition behavior indicated that they have great influence on plasmid DNA partitioning. The results showed that pDNA accumulated in the top phase at low lysate fractions. Furthermore, volume ratio increased for three different temperatures with increasing lysate fractions. It was also obtained that by increasing temperature with w = 40 % lysate fraction, volume ratio decreased gradually.

By using the Taguchi method in which the effect of four factors on pDNA partition were considered, it was concluded that the size of pDNA is not a significant factor in purification by ATPS. Moreover, pDNA could easily be separated and accumulated in top phase in ATPS of PEG-300/salt and in bottom phase of ATPS of PEG-1450/salt. These data describe that pDNA tends to be partition to enriched polymer phase when the molecular mass of PEG decreases. The results of protein assay in top and bottom phases of both ATPS conditions showed that protein was partitioned to the bottom salty phase either in high or low molecular mass of PEG. Therefore, it is preferred to use PEG-300 instead of PEG-1450 for purification of pDNA.

In summary, since the maximum pDNA recovery and purification was obtained in PEG-300/salt ATPS, this system was suggested for further experiments. The optimization results suggested that pDNA could be separated easily by ATPS of PEG-300/K_2HPO_4 with ratio of 26 : 17 at 25 °C in top phase with a recovery percent of 85.

It is quite clear that further studies are required using this system for purification of bionanoparticles and there is a need for a more fundamental understanding of the partitioning mechanism in this separation system.

List of symbols

n – rotational speed, min^{-1}
V – volume, mL, L
w – mass fraction, %
Z – mass fraction ratio, w_{PEG}/w_{salt}
γ – mass concentration, μg mL^{-1}
η – recovery, %
θ – temperature, °C
λ – wavelength, nm
Ψ – volume ratio
c – concentration, mmol L^{-1}, mol L^{-1}

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