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Dual affinity method for plasmid DNA purification in aqueous two-phase systems

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ABSTRACT

The DNA binding fusion protein, LacI–His_6–GFP, together with the conjugate PEG–IDA–Cu(II) (10 kDa) was evaluated as a dual affinity system for the pUC19 plasmid extraction from an alkaline bacterial cell lysate in poly(ethylene glycol) (PEG)/dextran (DEX) aqueous two-phase systems (ATPS). In a PEG 600–DEX 40 ATPS containing 0.273 nmol of LacI fusion protein and 0.14% (w/w) of the functionalised PEG–IDA–Cu(II), more than 72% of the plasmid DNA partitioned to the PEG phase, without RNA or genomic DNA contamination as evaluated by agarose gel electrophoresis. In a second extraction stage, the elution of pDNA from the LacI binding complex proved difficult using either dextran or phosphate buffer as second phase, though more than 75% of the overall protein was removed in both systems. A maximum recovery of approximately 27% of the pCU19 plasmid was achieved using the PEG–dextran system as a second extraction system, with 80–90% of pDNA partitioning to the bottom phase. This represents about 7.4 μg of pDNA extracted per 1 mL of pUC19 desalted lysate.

1. Introduction

Non-viral vectors, such as plasmid DNA, have been shown to be adequate to mediate both gene therapy and DNA vaccination. Expression of therapeutic proteins has been attained both *in vitro* and *in vivo* using these vectors [1]. Also, they are able to stimulate humoral and cellular immune responses to a specific antigen [1,2]. The increased number of clinical trials using naked/plasmid DNA (there are currently 281 ongoing trials) have brought a focus on the large-scale manufacturing of pDNA as high demand for these vectors is soon expected [3].

The most common methods for large-scale purification of plasmids are chromatographic [4]. However, other methods such as liquid-phase extraction in aqueous two-phase systems (ATPS) may be interesting alternatives since the problem of access and mass transfer within solid-phase chromatography matrices for large plasmids is avoided. Several studies show that ATPS extraction has several other advantages, such as; operational simplicity, easy scale-up and the possibility to combine several process steps in a single operation [5–7]. However, one of the main drawbacks of this technique is its low selectivity, which may be overcome by the use of affinity ligands [8,9]. The specificity and bio-recognition properties of affinity ligands for pDNA capture from a crude feedstock with large and structurally related impurities (such as, high molecular weight RNA, endotoxin and shear genomic DNA) may facilitate the preparation of pDNA products that fulfil the stringent specifications for market approval.

Although affinity based separations have been extensively applied for protein extraction in ATPS's [10–12], only two recent reports refer to the utilisation of pDNA ligands [8,9]. Duarte et al. [8] have used the cationic polymer poly(ethyleimine) (PEI) as non-specific ligand for the purification of pDNA from crude cell lysates in a two-step ATPS process. Although the authors report up to 100% pDNA recovery in the form of DNA/PEI polyplexes, with only minor protein impurities, they did not elute the pDNA from the polyplexes. Although it is not yet clear if the pDNA could be eluted and used in pure form, concerns remain about the use of PEI based vectors for gene therapy applications since the cationic polymers might present some toxicity issues [13]. In addition, the PEI/DNA interactions are mainly electrostatic, thus there is a potential risk for non-specific binding of the cationic polymer to other negative charged molecules, such as endotoxins, RNA and genomic DNA, which would also raise potential safety concerns.

Alternatively, Barbosa et al. [9] have described a protein-based ligand composed of glutathione-S-transferase protein (GST) fused to a Zinc Finger transcription factor (ZnF) designed as GST–ZnF. The GST–ZnF affinity protein was able to isolate pDNA bearing a ZnF recognition site from the bottom to the top phase of a PEG–dextran ATPS in either the native or a PEGylated form. GST–ZnF binds to a specific base sequence at the DNA recognition site, from which it

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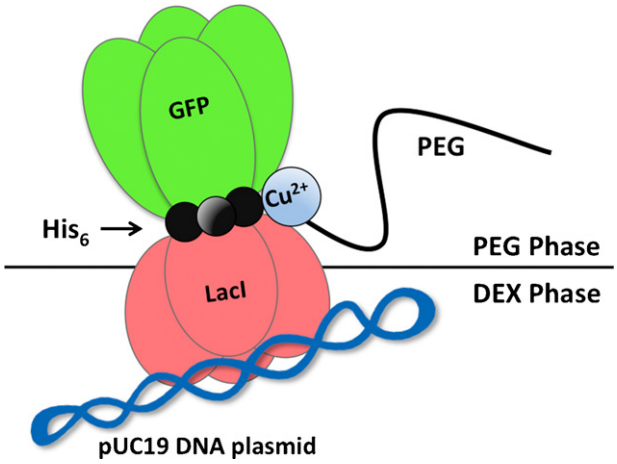

Fig. 1. Schematic representation of the dual affinity method developed for pDNA purification using PEG/dextran aqueous two-phase systems.

is not easily eluted. Thus, no attempts were made to extract pure pDNA from a complex mixture of a crude cell lysate.

In both approaches the ligand has to be covalently bound to poly(ethylene glycol) (PEG); through PEGylation [14,15]. In the PEG–dextran systems used, this PEGylation drives the ligand to the PEG phase where less contaminants usually accumulate. The presence of the ligand in the PEG phase may draw the pDNA to this phase by affinity binding, so separating it from the contaminants.

Though effective, this approach is limited by the need for PEGylation. This can be a complex and inefficient procedure and several products are often obtained with different degrees of PEGylation [9]. Such inhomogeneity in the PEGylated ligands may require further purification of the final DNA sample to obtain a homogeneous product and this in turn may lead to high production costs and to the increase in batch operation times. Additionally, because many PEGylations are inefficient, a stoichiometric excess of the reagent is needed, which can potentially make the entire process uneconomic for some ATPS purifications.

To circumvent these problems a modified PEG might be used that binds site specifically and *in situ* with the ligand. This approach has been already explored for the affinity purification of proteins bearing a his-tag in aqueous two-phase systems [16,17]. Thus, immobilized metal-ion affinity partitioning (IMAP) has been used as an alternative to immobilized metal-ion affinity chromatography (IMAC) [18]. IMAP exploits the grafting of a chelator, such as iminodiacetic acid (IDA), onto one the phase forming polymers, usually the PEG polymer. The IDA ligand chelates a transition metal ion (e.g., Cu^{2+} or Ni^{2+}), which binds preferentially to electron-rich amino acid residues such as histidines and cysteines that are accessible on the surface of proteins [19]. This enables the extraction of specific his-tagged proteins into the PEG phase while the bulk of contaminant proteins without a his-tag remain in the other phase [17].

The strategy set out above does not require the previous PEGylation of the protein and the binding to the his-tag occurs almost instantly. Here, the use of this highly selective method to enhance the partitioning of a DNA affinity protein to the PEG-rich phase of a PEG–dextran ATPS is exploited in order to steer the target pDNA to the same phase.

One of the ligands that is being investigated for the affinity chromatographic purification of plasmid DNA is the LacI repressor protein fused to both a His_6 tag and a Green Fluorescent Protein (GFP) moiety [20,21]. As shown in Fig. 1, the LacI–His_6–GFP fusion protein is a tetramer protein (two dimer units) composed of three different domains: (1) a LacI domain that binds sequence specifically the *Lac*O_3/*Lac*O_1 operator on the pUC19 plasmids; (2) the GFP domain that enables the specific detection of the protein in a mixture of other proteins; (3) The His_6 tag that bridges the two domains and enables the purification of the protein by IMAC. This protein has been immobilized onto an IMAC column for the purification of pDNA directly from crude bacterial cell lysates, yielding highly pure supercoiled pDNA with no RNA, genomic DNA or denatured pDNA [20,21].

Here, we report a dual affinity ATPS approach for the purification of plasmid DNA in which the heterofunctional DNA binding ligand LacI–His_6–GFP is combined with an immobilized metal-ion affinity PEG. The functionalised PEG binds to the his-tag of this protein, resulting in the IMAP of the LacI–His_6–GFP/pDNA complex to the PEG-rich top phase. The advantages of using this approach are that the covalent PEGylation of the DNA ligand is not necessary. The high pDNA sequence specificity of the DNA binding protein is effectively exploited and the binding of the functionalised PEG to the DNA binding protein can be performed in a crude cell extract that is then mixed with the pDNA lysate, thus potentially reducing operating costs and processing complexity.

2. Materials and methods

2.1. Reagents

PEG polymers, MW 10,000 and 600 Da were obtained from Sigma–Aldrich (St. Louis, MO, USA) and dextran MW 40 kDa was purchased from GE Healthcare (Chalfont St. Giles, UK). LB broth media, β-lactoglobulin protein, boron trifluoride ethyletherate (BF_3) and epichlorohydrin were purchased from Sigma–Aldrich.

2.2. Preparation of PEG–IDA–Cu(II)

PEG–IDA–Cu(II) was synthesised using the epichlorohydrin method, as similarly described in [22]. Briefly, 30 g of PEG 10,000 Da was dissolved in 100 mL toluene and 0.3 mL BF_3 (50%, w/w) and 0.4 mL epichlorohydrin were added dropwise. The reaction mixture was stored for 48 h and 2 mL NaOH (45%) was then added to the mixture, which was slowly stirred for 4 h at 37 °C. PEG was precipitated and the powder was then dissolved in 100 mL carbonate buffer pH 12.1 containing 0.35 M iminodiacetic acid (IDA) and the mixture was stirred at 70 °C overnight. After three extractions with chloroform, the product was crystallised twice in ice-cold diethyl ether (yielding 85–90% of total PEG). The Cu(II) complex with PEG–IDA was obtained by dissolving the PEG–IDA product in 50 mL sodium acetate buffer pH 4 containing 5 g of $CuSO_4$. The extraction was repeated yielding proximally 75% of the original PEG. The 0.88 μmol of Cu(II) per μmol PEG was determined by atomic absorption spectrometry using appropriate standards.

2.3. Expression and purification of LacI–His_6–GFP protein

LacI–His_6–GFP DNA construct [20] was transformed into *E. coli* BL21-gold cells (Stratagene, La Jolla, CA) and a single fresh colony was inoculated into 200 mL of LB media containing 50 μg/mL ampicillin and incubated overnight at 30 °C and 200 rpm. 1:1000 dilution of this culture was then prepared in 0.5 L of similar media and the LacI–His_6–GFP was expressed constitutively overnight at 200 rpm and 30 °C. Cells were then harvested by centrifugation and the pellet was stored at −80 °C until needed. Protein was purified using a 1 mL Histrap HP column (GE Healthcare). Briefly, a cell pellet from 0.5 L cell culture was re-suspended in 20 mL of 20 mM phosphate, 500 mM NaCl and 40 mM imidazole buffer pH 7.4 and lysed by 3 × 3 s pulse sonication at maximum power inside an ice bucket.

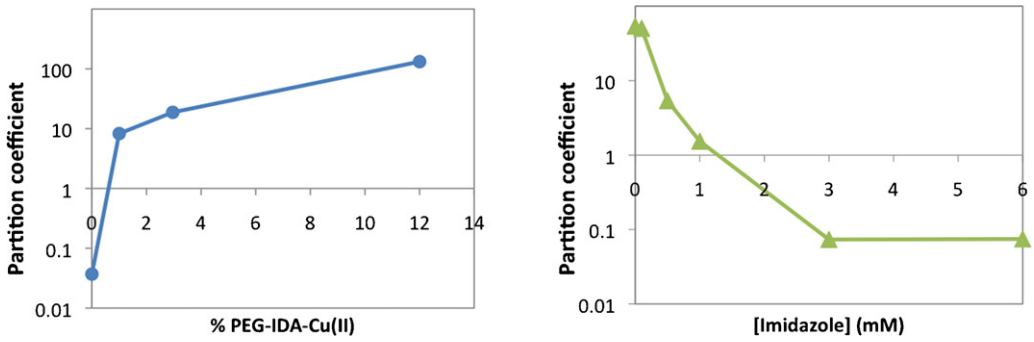

Fig. 2. Effect of modified PEG–IDA–Cu(II) and imidazole on the LacI–His_6–GFP partition coefficient (K_{LacI}) in systems composed by 18% (w/w) PEG 600–14% (w/w) DEX 40 in 50 mM Tris–HCl pH 7.4. (A) Variation of K_{LacI} with the addition of PEG–IDA–Cu(II). (B) Variation of K_{LacI} with the addition of imidazole in the presence of 10% PEG–IDA–Cu(II).

The resulting lysate was clarified by centrifugation at 19,000 rpm for 15 min at 4 °C and loaded into a Histrap column using a syringe pump. The column was extensively washed after the loading step with the re-suspended buffer until no absorbance was detected by UV 280 nm. Protein was eluted using 20 mM phosphate, 500 mM NaCl, 500 mM imidazole buffer pH 7.4. The resulting eluate containing LacI–His_6–GFP was dialysed at 7 °C against PBS buffer pH 7.4 to remove imidazole. The buffer was exchanged when necessary by using a PD10 desalting column (GE Healthcare). Protein concentration in solution was determined with the Bradford method (Pierce, Rockford, IL, USA) using BSA as standard.

2.4. Preparation of pure pUC19 plasmid and pUC19 alkaline lysate

Escherichia coli DH5α strain, harbouring the pUC19 plasmid was cultivated and the pUC19 plasmid was purified as previously described [9]. Preparation of the alkaline cell lysate was performed according to Duarte et al. [8]. The resulted clarified lysate was filtered using a 0.22 μm syringe filter and stored at −20 °C until needed. On the day of the experiment the buffer was exchanged to 20 mM phosphate buffer pH 7.4 using a PD10 column.

2.5. Affinity partitioning in aqueous two-phase systems

Aqueous two-phase systems composed of PEG 600–DEX 40 were prepared by weighing appropriate amounts of stock solutions (30–40%, w/w) of the phase forming polymers in 2 mL graduated tube. Immobilized metal-ion affinity partitioning experiments were performed by replacing part of the PEG with the modified PEG–IDA–Cu(II) (the amount of PEG derivative is given hereafter as a percentage of the total mass of PEG present in the systems). The two proteins under study (LacI–His_6–GFP and β-lactoglobulin) were then added into the systems and mixed by vortex shaking. The crude cell lysate containing pUC19 or the pure pUC19 solution was added and pH was adjusted to the desired pH using concentrate stock buffers. Finally, ultra pure water was added to make 1.5 g total system weight. The systems were well mixed by vortex shaking until all polymers were completely dissolved. Phase separation was accomplished by placing the tubes in a water bath at 30 °C for ~2 h to avoid phase turbidity. Each phase was collected and all further analyses were conducted at room temperature on the same day that the partitioning systems were prepared.

The second extraction stage was performed by collecting the desired top phase of a system (0.5 mL) and adding a fresh lower phase (DEX 40, 14%, w/w) or 0.4 mL potassium phosphate solution, K_2HPO_4–KH_2PO_4 (40%). IPTG or NaCl was added to the final concentrations described in Table 4.

2.6. Analytical methods

2.6.1. LacI–His_6–GFP and total protein partition analysis

The quantitative determination of the total protein in each phase was performed by the Bradford assay using BSA as standard. The specific concentration of LacI–His_6–GFP fusion protein in each phase was estimated by measuring the GFP fluorescence in a 96 well plate reader calibrated against pre-purified LacI–His_6–GFP as described previously [23]. All concentrations were calculated using the average of at least 3 independent measurements.

2.6.2. DNA partitioning analysis

Total DNA quantification was performed using a Picogreen assay (Invitrogen, Carlsbad, CA, USA) in a 96 well plate reader. Blanks for each system were prepared and a series of calibration curves using standard concentrations of purified pUC19 plasmid in the blank top or bottom phase of each system was used. To diminish any quenching effects of LacI–His_6–GFP bound to pUC19 plasmid all samples were treated with 1 μg/μL DNase-free proteinase K (Sigma–Aldrich) for 1.5 h prior to readings.

The partitioning of a component, x, in a two-phase system is described by the partition coefficient, K_x, and calculated as $K_x = C_{TP}/C_{BP}$, where C_{TP} and C_{BP} are the concentrations of the component in the top and bottom phases, respectively. For each component the amount recovered in each phase (i) was evaluated by the partition percentage (PP) calculated as:

$$\mathrm{PP} = \frac{[x]_i \times V_i}{x_{\mathrm{top}} + x_{\mathrm{bottom}}}$$

The amount of recovered DNA in relation to the total amount of pDNA added to the systems was calculated by the yield defined as

$$\mathrm{yield} = \frac{[x]_i \times V_i}{x_{\mathrm{added}}}$$

3. Results and discussion

3.1. LacI–His_6–GFP protein affinity partitioning

Whether the modified PEG–IDA–Cu(II) would increase the free LacI–His_6–GFP partitioning to the top phase of the 18% (w/w) PEG 600–14% (w/w) DEX 40 system was first examined. Several ATPSs were made with increasing concentration of functionalised PEG, but keeping the LacI–His_6–GFP protein concentration constant (Fig. 2A). In the absence of PEG–IDA–Cu(II) the LacI–His_6–GFP protein partitioned to the dextran-rich bottom phase (K_{LacI} = 0.037, 92.5%). The partitioning behaviour of LacI–His_6–GFP was significantly enhanced by increasing the concentration of PEG–IDA–Cu(II) and it accumulated to 99.5% in the PEG-rich top phase (K_{LacI} = 131) when 12% of total PEG was replaced by the functionalised PEG.

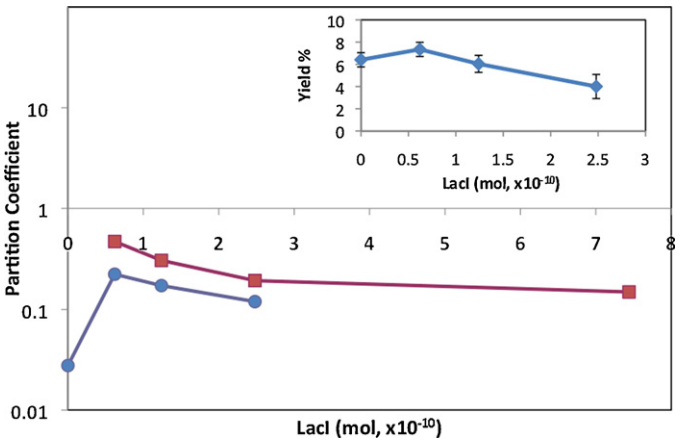

Fig. 3. pUC19 (circles) and LacI–His_6–GFP (squares) partition coefficient (K) in systems composed with increased LacI concentration and constant amount of PEG–IDA–Cu(II). Systems composed by 18.0% (w/w) PEG 600–13.4% (w/w) DEX 40 with 12% of the PEG replaced by PEG–IDA–Cu(II) in 50 mM Tris–HCl pH 7.4. Each system contained constant amount of pUC19 (7.9×10^{-12} mol, 14.36 μg). pUC19 accumulates on bottom phase only for systems with 7.4×10^{-10} mol of LacI–His_6–GFP. Inset shows the DNA yield on the top phase.

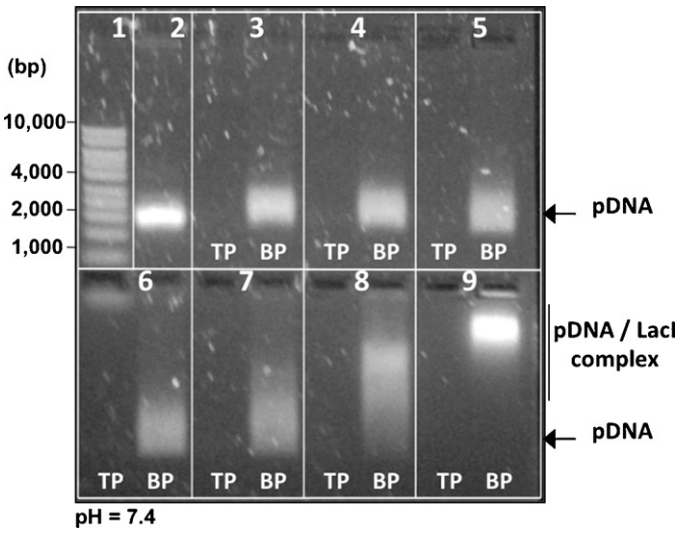

Fig. 4. DNA electrophoresis analysis of the top and bottom phase of systems composed with increased LacI–His_6–GFP concentration with constant amount of pUC19 plasmid (7.9×10^{-12} mol, 14.36 μg) and PEG–IDA–Cu(II) (12% of the total PEG). Systems composed by 18% (w/w) PEG 600–13.4% (w/w) DEX 40, 50 mM Tris–HCl pH 7.4. (1) DNA marker; (2) pUC19 plasmid; (3) top phase (TP) and bottom phase (BP) analysis of system with only pUC19 plasmid; (4) system with pUC19 plasmid and 12% PEG–IDA–Cu(II); (5–9) systems with pUC19 plasmid and 12% PEG–IDA–Cu(II) and 0.3, 0.6, 1.2, 2.4, 7.4×10^{-10} mol of LacI–His_6–GFP protein, respectively.

To confirm that the binding between protein/PEG was due to the coordination between the transition metal ion (Cu(II)) chelated by PEG–IDA and the imidazole rings of the histidines on the LacI–His_6–GFP surface, we added increased concentrations of exogeneous imidazole in solution. As seen in Fig. 2B, in systems where 10% of the PEG was replaced by PEG–IDA–Cu(II), increased concentration of exogeneous imidazole steered the LacI–His_6–GFP protein to the bottom phase. For 3 mM imidazole the protein partitioning coefficient was $K_{LacI} = 0.073$, which is close to that observed when no functionalised PEG was added ($K_{LacI} = 0.037$). This confirms that IMAP of the LacI fusion protein was achieved.

3.2. pUC19 plasmid affinity partition

Fig. 3 shows the effect of increased LacI fusion protein concentration on systems with constant amount of pUC19 (7.9 pmol) and PEG–IDA–Cu(II) (12% (w/w)). In systems with no LacI fusion protein present, the pre-purified pUC19 plasmid partitioned to the bottom phase (96.9%) whereas the his-tagged LacI fusion protein partitioned almost totally to the top phase in systems without the pDNA (see Fig. 2). When the LacI–His_6–GFP protein (0.062 nmol) was combined with pUC19 DNA in the same system there was a slight increase in pUC19 partitioning to the top phase (K_{pUC19} increased from 0.027 to 0.129). When the LacI–His_6–GFP concentration was increased further, a decrease in pUC19 partition coefficient was observed, eventually to such an extent that no DNA was detected in the top phase of these systems. The top phase recovery of pUC19 in all these systems was around 4–6% as shown in the inset to Fig. 3. Agarose gel electrophoresis analysis of the bottom and top phases of the systems was performed to determine if the pDNA was present in its free form or bound to the LacI fusion protein (Fig. 4). The pDNA was found to be bound to the affinity protein as an increase in the pDNA molecular weight was observed in the bottom phase of these systems, particularly for systems with higher protein concentration as 0.24 or 0.74 nmol (lanes 8 and 9 respectively). This initially suggested that the affinity between the DNA-protein was higher than that between protein and PEG–IDA–Cu(II), so compromising the use of the PEG–IDA–Cu(II) to enhance the partitioning of pDNA/protein complex to the top phase.

To further determine why the pDNA/LacI complex had partitioned to the bottom phase of these systems we also measured the decrease of the PEG–IDA–Cu(II) ligand in these systems, as shown in Fig. 5 and Table 1. Employing lower PEG–IDA–Cu(II) concentrations, while keeping pUC19 (2 pmol, 3.6 μg) and LacI (190 pmol, 54.2 μg) concentrations constant, enhanced the partitioning of both to the top phase (Fig. 5A). The percentage of LacI–His_6–GFP and pUC19 partitioned to the top phase in systems without PEG–IDA–Cu(II) were 27.2% and 11.4% respectively (Table 1). By contrast, with similar conditions but with 0.15% (w/w) of the total PEG replaced with the functionalised PEG, the LacI fusion protein partitioned to the top phase. Under these conditions, the LacI protein accumulated 85% in the top phase ($K_{LacI} = 5.09$) while pUC19 partitioned 89.4% ($K_{pUC19} = 7.29$) into the same phase. However, as the concentration of PEG–IDA–Cu(II) was increased, the partition coefficients of both protein and pDNA fell. Agarose gel analysis of the top and bottom phases of these systems (Fig. 5B) now showed that the pDNA was complexed with LacI protein in the top phase as a shift in the molecular weight of the supercoiled and open-circular bands of pDNA were observed (see for reference Fig. 5B, lane 1). It is speculated that the retention of the pDNA in the bottom phase for higher PEG–IDA–Cu(II) concentrations may have arisen because the functionalised PEG significantly changed the physical properties of the top phase, possibly due to the formation of dimers or multimeric PEG–IDA–Cu(II) chains. Since a 10 kDa PEG–IDA–Cu(II) was used, it is possible that a very high MW chain was formed, particularly at high concentrations of the func-

Table 1
Top phase partition percentage of pUC19 plasmid and LacI–His_6–GFP protein with increased PEG–IDA–Cu(II) concentration in systems composed by 16.0% (w/w) PEG 600–14.3% (w/w) DEX 40, 15 mM phosphate buffer pH 7.4. Each system contained constant amount of pUC19 (2×10^{-12} mol, 3.6 μg) and LacI–His_6–GFP (1.9×10^{-10} mol, 54.2 μg).

PEG–IDA–Cu(II) (%) (w/w)	Partition percentage (%mass)	
	LacI–His_6–GFP	pUC19
0	27.2 ± 2.5	11.4 ± 3.5
0.15	85.4 ± 0.7	89.4 ± 5.3
0.71	75.9 ± 0.5	75.3 ± 6.1
1.48	59.5 ± 0.6	49.9 ± 1.6
12.00	31.9 ± 0.9	21.6 ± 2.7

Table 2
Partition coefficient of pUC19 plasmid in the presence of β-lactoglobulin protein (negative control) in systems composed by 18.0% (w/w) PEG 600–13.4% (w/w) DEX 40 in 15 mM phosphate buffer pH 7.4.

System	β-Lactoglobulin (mol × 10^{-9}/μg)	pUC19 (mol × 10^{-12}/μg)	PEG–IDA–Cu(II) (% (w/w))	Partition coefficient		DNA partition percentage (%)[a]
				β-Lactoglobulin	pUC19	
1	–	3.62/6.3	–	–	0.024	3.954 ± 0.95
2	–	3.62/6.3	0.148	–	0.007	0.984 ± 0.05
3	3.5/65.3	3.62/6.3	0.148	2.0	0.015	2.46 ± 0.05
4	3.5/65.3	–	0.148	1.8	–	–
5	3.5/65.3	–	–	1.5	–	–

[a] DNA partition percentage to the top phase of each system.

tionalised polymer. Thus, excluding from that PEG-rich phase the pDNA/protein complex. This behaviour, an excluded volume effect, has been observed for several other proteins in ATPS with increase of the molecular weight of one of the phase forming polymers [24,25].

The selective partitioning of the his-tagged LacI protein was confirmed by replacing it by β-lactoglobulin (Table 2). In the same polymer–polymer systems in phosphate buffer pH 7.4 the β-lactoglobulin protein partitioned to the top phase, $K_{\beta\text{-lacto}} = 1.5$, and a slight increase of its partition value $K_{\beta\text{-lacto}} = 1.8$ was observed when 0.148% of the modified PEG–IDA–Cu(II) was added. When both β-lactoglobolin and pUC19 were added in the same systems there was no significant shift of pDNA partitioning to the top phase ($K_{pUC19} = 0.015$) similar to systems where no protein was added ($K_{pUC19} = 0.024$).

3.3. Extraction of pUC19 plasmid from crude cell lysate

Affinity capture of pUC19 directly from crude cell lysate was performed by adding the desalted lysate (14% load, w/w) into systems with 0.148% of the modified PEG. Preliminary experiments with the original pUC19 alkaline lysates had shown that no pDNA/LacI interaction was observed (data not shown). However, it was observed that exchanging the buffer prior to extraction the binding between the pDNA/LacI occurred. Therefore, in all further experiments, the lysate was desalted (i.e., buffer exchanged to phosphate buffer pH 7.4) prior to extraction. This observation may have been due to the possible excess of sodium dodecyl sulfate (SDS) detergent used in the cell lysis and to the low pH ≈5.6 of the lysate. SDS is a known protein denaturant and the optimal pH for LacI/pDNA binding occurs at pH 7.4 [20,21].

Although no genomic DNA (gDNA) is thought to be present on the lysates (see lane 3, Fig. 6A or B), we will report to total DNA partition (K_{DNA}) instead of pUC19 partitioning when lysates are used since the quantification method used here (Picogreen) does not differentiate between both forms of DNA.

As shown in Table 3, without LacI–His_6–GFP or PEG–IDA–Cu(II) ligands total protein ($K_{protein} = 0.78$) and DNA ($K_{DNA} = 0.022$) from the desalted lysate partition to the bottom phase of these systems in phosphate buffer pH 7.4. When 0.148% (w/w) of immobilized metal–PEG was added to the system an increase of total protein partitioning from the pUC19 lysate (to $K_{protein} = 0.96$) was observed, showing that some non-specific binding might occur. The competitive binding of the modified PEG to other proteins is expected to be low in ATPSs that have low concentrations of a binding competitor added, such as imidazole.

Increase of the his-tagged LacI concentration in these systems resulted in a concomitant enhancement of the DNA partitioning to the top phase. For example, in systems with 0.27 nmol of LacI–His_6–GFP, the DNA partition coefficient increased to $K_{DNA} = 1.54$ representing 72.8% top phase partition percentage whereas, in systems without LacI protein and PEG–IDA–Cu(II) the DNA partitioned only 3.6% to this phase ($K_{DNA} = 0.022$).

The top phase recovery (yield) achieved with the above system composition was about 49.2% of total DNA added. Given the crude nature of cell lysates it is significant that neither RNA nor genomic DNA is co-extracted to the top phase of these systems (lane 7-TB, Fig. 6A). Fig. 6B shows the binding of the LacI protein to the pUC19

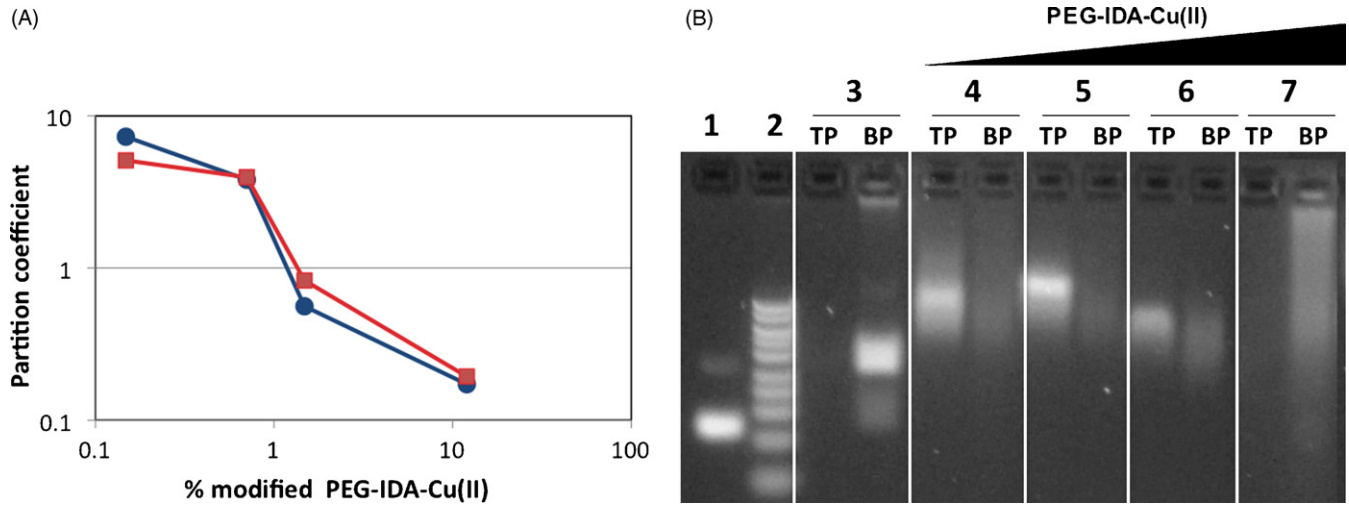

Fig. 5. Effect of PEG–IDA–Cu(II) on the pUC19 plasmid and LacI–His_6–GFP protein partition in systems composed by 16.0% (w/w) PEG 600–14.3% (w/w) DEX 40 in 15 mM phosphate buffer pH 7.4. (A) Partition coefficient of pUC19 (circles) and LacI–His_6–GFP (squares) in each system contained constant amount of pUC19 (2×10^{-12} mol, 3.6 μg) and LacI–His_6–GFP (1.9×10^{-10}, 54.2 μg). (B) DNA gel electrophoresis of top and bottom phase of systems described in A; (1) pUC19 plasmid (control); (2) DNA marker (see Fig. 4); (3) top phase (TP) and bottom phase (BP) of system composed with pUC19 and PEG–IDA–Cu(II) (12% total PEG); (4–7) 0.15, 0.71, 1.5, 12% of modified PEG added, respectively.

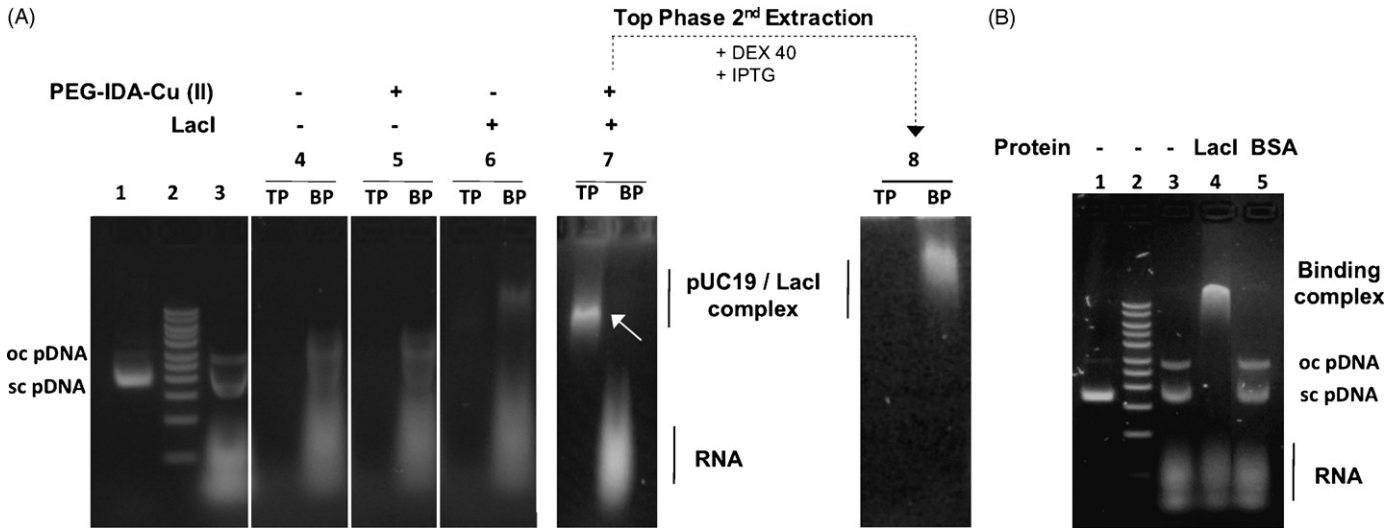

Fig. 6. Agarose gel analysis of the interaction between LacI–His_6–GFP protein and bacterial lysate in ATPS (A) and in solution (B). (A) Top phase (TP) and bottom phase (BP) of systems composed with bacterial lysate and LacI fusion protein. Systems composed by 16% (w/w) PEG 600–13.3% (w/w) DEX 40 (0.148% of the PEG was replaced with PEG–IDA–Cu(II)); (1) pUC19 plasmid; (2) marker; (3) bacterial lysate containing pUC19; (4) top and bottom phase system with only bacterial lysate; (5) system with bacterial lysate and PEG–IDA–Cu(II); (6) systems with pUC19 and LacI–His_6–GFP protein (without modified PEG); (7) system with bacterial lysate, 2.73×10^{-10} mol LacI fusion protein and 0.14% of PEG–IDA–Cu(II); (8) back extraction of system shown in lane 7 to a new DEX 40, 2.5 mM IPTG. (B) Analysis of pUC19 lysate with LacI protein and BSA in solution. (1) pUC19; (2) marker; (3) bacterial lysate; (4) bacterial lysate and LacI–His_6–GFP; (5) bacterial lysate and BSA.

plasmid in solution. As shown for ATPS, the LacI fusion protein binds preferentially to the pDNA instead of RNA and no interaction is observed with BSA. Although no genomic DNA is present in this pUC19 lysates, as no high MW bands in the gels are present, it is also known that the LacI fusion protein binds almost 4×10^6-fold more strongly to its operator compared to other non-specific sites within the DNA sequence [20]. Although the his-tagged LacI might bind to one operator it requires two operators, *Lac*O_3/*Lac*O_1 in the pUC19 plasmid, in order for the protein/DNA complex for optimal binding.

Here it was accomplished one step elimination of RNA and genomic DNA, two major pDNA contaminants. For the therapeutically use of plasmid DNA a lack of contamination by RNA, genomic DNA, host proteins, and endotoxin is necessary to meet the stringent specifications of regulatory agencies [26].

3.4. Second extraction/pUC19 elution

To elute the pUC19 plasmid from its LacI–His_6–GFP affinity protein and remove excess of the co-purified host proteins, a second extraction stage was performed with either DEX 40 polymer or a potassium phosphate salt buffer as the second phase. The top phase of ATPS with composition shown on system 6 (Table 3) was collected and, additionally to the phase forming polymer/salt, IPTG or NaCl were also added. IPTG is known to induce an allosteric change in the LacI protein, allowing the release of the plasmid, whereas NaCl addition increases the ionic strength of the solution and weakens the electrostatic interactions that mediate the binding of the protein to the DNA [20].

As seen in Table 4, the use of dextran as the second phase resulted in the accumulation of the pDNA in the bottom phase, $K_{DNA} = 0.09$, for systems with 2.5 mM IPTG/0.3 M NaCl, and $K_{DNA} = 0.04$ when only IPTG was added. Total protein partitioned to the opposite PEG phase with $K_{protein} = 2.91$ and $K_{protein} = 2.63$, respectively for each system, whereas in both systems LacI partitioned to the bottom with the DNA. Agarose gel analysis of the bottom phase of the second extraction system showed that the pDNA remained in the bound form (Fig. 6A, lane 8-BP) and thus elution had not been properly achieved. Further attempts to elute the DNA were made by adding PEG–IDA–Cu(II) to the systems or by increasing the IPTG concentration but no improvement to the pDNA elution was observed. In both cases more than 75% of total protein was removed (85% for back-extraction systems without NaCl). Much greater protein removal would have been achieved if the pUC19 plasmid had been eluted from the LacI protein since under these conditions the LacI protein would partition to the PEG–IDA–Cu(II) rich phase, as shown in Fig. 2A.

We speculated that polymer–salt systems might improve upon the pDNA elution since a high salt concentration may impair the pDNA/protein interaction. As shown in Table 4, however, sim-

Table 3
Effect of increased concentration of LacI–His_6–GFP on the pUC19 plasmid partition from a desalted bacterial cell lysate. System composed by 18% (w/w) PEG 600–13.3% (w/w) DEX 40 in 15 mM phosphate buffer pH 7.8.

System	PEG–IDA–Cu(II) (%, w/w)	LacI ($\times 10^{-10}$ mol)	Partition coefficient (K)			DNA PP top[a] (%)	DNA yield (%)
			Total protein	LacI	pUC19		
1	–	–	0.78	–	0.022	3.6 ± 0.2	1.7 ± 0.1
2	0.14	–	0.96	–	BP[b]	BP[b]	BP[b]
3	–	1.36	0.99	0.77	0.14	17.4 ± 1.2	8.0 ± 0.4
4	0.14	0.82	1.32	0.72	0.13	16.5 ± 1.8	12.2 ± 0.6
5	0.14	1.36	3.45	1.24	1.00	59.8 ± 1.6	36.7 ± 1.2
6	0.14	2.73	4.17	2.35	1.54	72.8 ± 4.3	49.2 ± 3.1

pUC19 lysate = 109.6 μg/mL protein and 11.9 μg/mL DNA.
[a] PP top—partition percentage on top phase.
[b] BP—bottom phase only.

Table 4
Second extractions of pUC19 plasmid using the top phase of the system 16% (w/w) PEG 600–13.3% (w/w) DEX 40 with bacterial cell lysate, 2.73×10^{-10} mol LacI–His_6–GFP protein and 0.14% of PEG–IDA–Cu(II) in 15 mM phosphate buffer pH 7.8.

Second phase	IPTG (mM)	NaCl (M)	Partition coefficient			Protein removal (%)	DNA PP[a] (%)	DNA yield (%)
			Total protein	LacI	DNA			
DEX40 (pH 7.4)	2.5	0.3	2.91	0.71	0.09	76.3 ± 0.79 (bottom)	81.8 ± 3.9 (bottom)	27.3 ± 1.3 (bottom)
	2.5	–	2.63	0.51	0.04	85.9 ± 1.6 (bottom)	89.2 ± 8.4 (bottom)	25.5 ± 2.7 (bottom)
K_2HPO_4–KH_2PO_4 (pH 7.4)	1.2	–	0.31	TB[b]	12.86	74.4 ± 2.6 (top)	94.7 ± 5.9 (top)	23.8 ± 1.5 (top)
	–	–	0.33	TB[b]	2.90	76.7 ± 0.8 (top)	81.3 ± 2.2 (top)	9.0 ± 0.3 (top)

[a] DNA PP—DNA partition percentage on top phase.
[b] TB—top phase only.

ilar results were obtained and no elution was achieved with K_2HPO_4–KH_2PO_4 solution. With salt as the second phase, the DNA and LacI partitioned to the PEG-rich phase rather than to the salt phase where total protein accumulated ($K_{protein} \approx 0.3$). A slight improvement in the final pDNA yield from 9.0% to 23.8% was attained when 1.2 mM IPTG was added but in both cases the pDNA was still in the bound form.

The lack of DNA elution by IPTG is confusing and requires further study. The allosteric nature of LacI repressor protein imposes two distinct conformations that correspond to the induced and repressed states, which are regulated by the concentration of IPTG. When IPTG binds a structural conformation is induced in the protein that reduces in several orders of magnitude its affinity to the DNA operator increasing the amount of unbound DNA. As in the present case elution is not observed it is probable that the binding of IPTG inducer to its protein target is prevented by the specific characteristics of the ATPS. For instance it is conceivable that steric hindrance by PEG may impede the access of IPTG to the binding site on LacI and thus not induce the conformational change in the 3D structure of LacI. A similar effect would result if IPTG partitioned to the opposite phase to the LacI/pDNA complex or if the protein/pDNA complex aggregated during the extraction process, hindering access to the site for IPTG binding. These hypotheses are being investigated and the protocol is being optimised for pUC19 elution. An alternative strategy would be to design plasmids with lower LacI binding affinity (Lac repressor has rather low dissociation constant, $K_D = 10^{-13}$ M [27]), by modifying some bases on LacI recognition sequences. It is predicable that the elution would then be easier to perform by either using the IPTG inducer or with simple increase of salt strength in the system.

Nonetheless with this current protocol, 75% of total protein was removed without either genomic DNA nor RNA contamination, two of the major pDNA impurities present in bacterial cell lysates. Although an overall yield of ~25% was obtained, this represents about 1.4 mg of pDNA per 200 mL desalted lysate which is about a 9-fold improvement compared to that reported for LacI–His_6–GFP immobilized into an chromatography column [21]. In addition to the higher capacity, this method also avoids the long binding kinetics of other chromatographic approaches using high selective sequence-specific ligands such as triple-helix affinity methods ([28,29].

Although DNA yields obtained here were below the ones obtained using PEI as ligand (100%) [8] this protein-based affinity methodology developed is more selective to the target plasmid DNA as compared to the non-specific ligand used before such polyethylamine (PEI) used in ATPS. When PEI is used in ATPS the co-purification of RNA is observed unless 0.2–0.5 M ammonium sulfate is present in the systems, since the non-specific ligand binds electrostatically to negatively charged molecules. The DNA sequence specificity of our method avoids this problem since it isolates pDNA from the bottom phase without co-purification of single-stranded RNA. In both methods pDNA is recovered in the complex form with the ligand and with small amounts of protein impurities, which in this work should be significantly reduced with the optimisation of pDNA elution from the LacI protein.

As we also demonstrated in our previous work using a GST–ZnF protein [9], we have shown here that the utilisation of protein-based affinity ligands can enhance significantly the pDNA partitioning to the top phase of PEG–dextran systems. Since these systems can be easily selected for the partitioning of the main pDNA impurities to the bottom phase, the protein-based ligands can be used to efficiently steer the pDNA to a phase with less impurities so increasing its purification efficiency.

Although the proof-of concept for a pDNA affinity extraction process was also demonstrated using the GST–ZnF protein, no further assays were carried out using bacterial cell lysates with this ligand due to the need for the previously covalent PEGylation of the ligand to efficiently steer the pDNA to a phase where less impurities accumulate, contrasting with the dual affinity method developed here where the PEGylation is performed *in situ*.

4. Conclusions

A dual affinity partitioning strategy is presented for the purification of pUC19 in PEG–dextran ATPS. A system composed of PEG–IDA–Cu(II)/LacI–His_6–GFP has been shown to be effective for the affinity purification of pDNA from bacterial alkaline lysates without the need for covalent PEGylation of the ligand. This represents an advantage over this process since there is no need to perform the expensive and time consuming PEGylation reactions or the subsequent PEG–ligand purification steps. Although pDNA elution from the complex proves to be difficult we believe that with further improvements this method may be appropriate for the development of large-scale manufacturing of pDNA for gene therapy applications or DNA vaccines.

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