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Research Article

Reactive aqueous two-phase systems for the production and purification of PEGylated proteins

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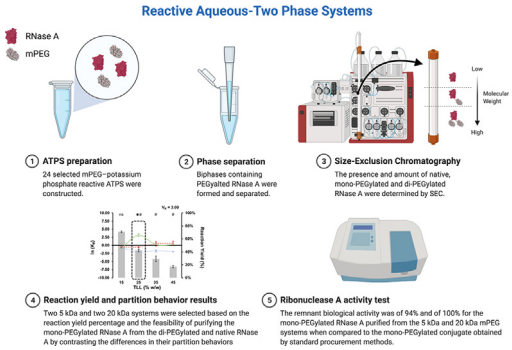
GRAPHICAL ABSTRACT



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ABSTRACT

Background: PEGylation, defined as the covalent attachment of polyethylene glycol, allows the synthesis of PEGylated therapeutic proteins with enhanced physicochemical properties. Traditional alkylating N-terminal PEGylation reactions on amine groups involve the use of modified linear mono-methoxy polyethylene glycol (mPEG) molecules looking for the synthesis of mono-PEGylated products. However, this approach requires different purification steps since inevitably undesired cross-linked products are synthesized. Herein, we propose the use of reactive aqueous two-phase systems (ATPS) to produce and purify PEGylated therapeutic conjugates using Ribonuclease A (RNase A) as a model protein.

Results: Selected linear 5 kDa and 20 kDa mPEG – potassium phosphate systems were produced according to equilibrium data obtained from constructed binodal curves. All reactive systems were able to generate biphasic systems and to PEGylate RNase A. Two 5 kDa and two 20 kDa systems were selected based on the reaction yield percentage and the feasibility of purifying the mono-PEGylated RNase A from the di-PEGylated and native RNase A by contrasting the differences in their partition behaviors. The remnant biological activity was of 94% and of 100% for the mono-PEGylated RNase A purified from the 5 kDa and 20 kDa mPEG systems when compared to the mono-PEGylated conjugate obtained by standard procurement methods.

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Conclusions: This novel approach using reactive ATPS shows that it is feasible to simultaneously produce and purify PEGylated therapeutic proteins with conserved biological activity and presents another example where reactive ATPS can be successfully implemented.

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1. Introduction

PEGylation is a reaction defined as the covalent attachment of at least one polyethylene glycol (PEG) chain to a molecule, usually a protein, resulting in a PEG-protein conjugate with enhanced physicochemical properties. The properties of PEGylated proteins with respect to their native forms include an increased size, solubility, and stability, which grant protection from proteolytic agents, decrease immunogenicity, reduce renal excretion and increase retention times in blood [1]. The properties of PEGylated products, and the fact that the use of PEG in foods, cosmetics and pharmaceuticals is approved by regulatory agencies for its negligible metabolic toxicity [2], makes PEGylation one of the most important protein enhancement technologies of our time.

In most cases, PEGylated products are synthesized using linear mono-methoxy PEG (mPEG) molecules modified with a variety of functional groups that react at a specific site of the targeted molecule [3]. However, even when the reactions of protein PEGylation are designed to be site-specific, PEG inevitably reacts at a certain degree with the functional groups of amino acid residues of the protein. This tendency of PEGylation reactions, besides the inner molecular weight dispersity of mPEG molecules, leads to the formation of a heterogeneous product comprised of the mono-PEGylated product (which is usually the most active conjugate) along with undesired PEGylated isomers, multi-PEGylated products, residual unreacted protein, and unreacted mPEG. This is an issue that affects primarily therapeutic products, where heterogeneity can have a significant impact on the pharmacokinetic behavior of the drug.

Different purification approaches have been implemented to address this issue depending on the nature of the PEGylated protein and the further processing steps needed to obtain the therapeutic formulation. The most widely used purification techniques for PEGylated proteins are based on differences in molecular size, shape, charge, solubility and hydrophobicity; where ion exchange and size exclusion chromatography are the ones that currently dominate the scene [4,5]. While chromatographic methods are preferred for their high resolution and easy implementation, there are multiple issues to address before a proper purification method is developed, including: sample dilution, long processing times, and low dynamic binding capacities [6]. Although promising results have been obtained using affinity-based strategies [7], the development of cost-effective chromatographic or non-chromatographic methods for the purification of mono-PEGylated therapeutic proteins remains a significant challenge.

In this context, purification approaches using aqueous two-phase systems (ATPS) are an attractive alternative to purify PEGylated therapeutics for being effective, biocompatible, integrative, easy to scale-up, and widely studied methods. Furthermore, purification protocols using ATPS are simple, efficient, rapid, flexible, economical, and can achieve equal or even higher purity and yield levels than traditional or more commonly used downstream operations [8]. ATPS are a liquid–liquid fractionation technique based on the incompatibility that certain hydrophilic solutes display above critical concentrations, leading to the formation of two phases where raw mixtures can fractionate: one containing particles and contaminants, and the other containing the product of interest [9]. ATPS are commonly formed by combining aqueous solutions of two polymers or a polymer and a salt, where the polymers are usually PEG, dextran or polypropylene glycol. The partitioning performance of ATPS is given by different factors, including salt ionic strength, pH, and concentration, and molecular weight of the polymers; these factors affect system parameters such as the partition coefficient (K_P) (i.e., the ratio of the concentration of the molecule of interest in the top and bottom phases) and the tie-line length (TLL) (i.e., the line length that connects the composition of the top and bottom phases in a phase diagram which indicates the product ratio of volume and density between them) [10].

There are some examples of the use of ATPS to purify PEGylated proteins, where most of them evaluate the effect that system parameters have in the recovery of the PEGylated proteins. Delgado et al. [11,12] studied the correlation among the molecular weight of the polymers and the value of K_P in a NaCl-enriched PEG – Dextran ATPS using the PEGylated forms of granulocyte–macrophage colony stimulation factor, bovine serum albumin and immunoglobulin G. More recently, Hernandez-Vargas and collaborators [13], evaluated the use of UCON as a novel polymer in ATPS, obtaining a recovery of 87% of mono-PEG lysozyme in the bottom phase of a PEG-UCON system.

Among the PEGylated protein therapeutics that can be used as models to evaluate ATPS, RNase A is convenient for being a small enzyme (13.7 kDa), whose biological activity has potential antitumoral effects, particularly on its PEGylated forms [14]. Our research group has previously reported the partition behavior of RNase A in PEG–potassium phosphate ATPS, obtaining a bottom phase recovery of up to 99% of native RNase A, and 98% and 88% of mono and di-PEGylated conjugates at the top phase, respectively, in optimized systems [15].

A novel approach denominated *in situ* ATPS takes advantage of the excess of mPEG used in a PEGylation reaction to form a biphasic system directly from the PEGylation reaction mixture. Mejía-Manzano and collaborators [16] used this approach to purify the PEGylated conjugates of lysozyme from residual unreacted protein in an ATPS comprised of a mixture of mPEG with a different polymer and ammonium sulfate. This report proved that it is possible to use residual mPEG to form a biphasic system, leading to the possibility of using mPEG instead of PEG to form ATPS where PEGylation reactions and product separations occur in the same operation.

The present study explores the possibility of using mPEG – potassium phosphate ATPS to produce and purify PEGylated proteins using RNase A as a model protein. This system will serve as both, a chemical reactor to produce RNase A mono and di-PEGylated conjugates, and as a purification system to separate the PEGylated proteins from undesired cross-linked products, residual unreacted RNase A and unreacted mPEG. This novel and integrative approach differentiates from previously reported efforts focused on using ATPS as a purification method for RNase A conjugates after PEGylation reactions [15]. The use of mPEG as a phase-forming chemical in PEG – potassium phosphate ATPS, allows the obtention of PEGylated RNase A while capitalizing the

purification power that has been achieved for RNase A in these systems [15]. The potential of this novel methodology has its greater impact on the industry, for the development and processing of PEGylated protein therapeutics.

2. Materials and methods

2.1. Materials

RNase A from bovine pancreas (cat. no. R6513) was purchased from MilliporeSigma (MA, USA). Methoxy-PEG-propionaldehyde with nominal molecular weight of 5 kDa (cat no. A3039-10) and methoxy-PEG-propionaldehyde with nominal molecular weight of 20 kDa (cat no. A3001-10) were obtained from JenKem Technologies (TX, USA). Sodium cyanoborohydride (cat. no. 1001911397) was purchased from Honeywell Fluka (MO, USA). Sodium phosphate, monobasic, monohydrate (cat no. 3820-01) and sodium phosphate, dibasic, 7-hydrate (cat no. 3817-01) of ultra-pure grade were purchased from J.T. Baker (NJ, USA). RNase activity kit, RNaseAlert™ Lab Test Kit, (cat. no. AM1964) was obtained from Thermo Fisher Scientific (MA, USA). All other used chemicals were at least of analytical grade.

2.2. RNase A PEGylation reaction for standard procurement

To obtain analytical standards, RNase A PEGylation reactions were conducted, separated and purified according to the procedure reported by Cisneros-Ruiz et al. [17]. Briefly, 2.0 mL of an RNase A solution (3.0 mg mL^{-1}) in a 100 mM phosphate pH 5.1 buffer with 20 mM sodium cyanoborohydride were added to a flask containing 30 mg of 5 kDa mPEG propionaldehyde or 20 kDa mPEG propionaldehyde. The PEGylation reaction mixture was stirred and incubated at 4°C for 17 h and the reaction was stopped by freezing the flask at −20°C. The PEGylation reaction mixture was purified using a size exclusion chromatography (SEC) approach, by injecting the samples into an Äkta Explorer 100 (Cytiva; MA, USA) chromatographer equipped with a HiPrepTM 16/60 chromatographic column prepacked with SephacrylTM S-300 high resolution resin (Cytiva). The fractions corresponding to the di-PEGylated, mono-PEGylated and native RNase A from each SEC peak were identified and collected. Then, fractions were pooled and concentrated by ultrafiltration under nitrogen atmosphere using 10 kDa Diaflo® ultrafiltration membranes (MilliporeSigma) coupled to an Amicon® ultrafiltration cell 8050 (MilliporeSigma) until 25 mL of each concentrated fraction were obtained. Finally, these concentrated fractions were lyophilized and stored at −4°C as previously reported by Cisneros-Ruiz et al. [17].

2.3. Reactive ATPS formation and partition behavior

The concentration of the phase forming chemicals that allow the formation of reactive mPEG – potassium phosphate ATPS was selected according to equilibrium data previously obtained by the generation of binodal curves using the turbidimetric titration approach [18]. In doing so, two different binodal curves were generated at pH 5.1 (i.e., the pH value needed for alkylating N-terminal specific PEGylation reactions), one using mPEG with nominal molecular weight of 5 kDa and the other using mPEG with nominal molecular weight of 20 kDa (Fig. S1). Based on the data gathered from the binodal curves, 24 selected mPEG–potassium phosphate ATPS were constructed (Table 1). It should be noted that in all systems, the polymer-rich phase represents a viable PEGylation environment since mPEG is present at above a 5-fold molar excess with respect to the protein. This concentration relationship considers that amine reactive PEG reagents tend to be used in stoichiometric excess to achieve optimal PEGylation conditions and yields [19]. Furthermore, these systems were selected as models to determine the mono-PEGylated RNase A reaction yield (defined as the % w/w of mono-PEGylated/native RNase A) and the partition behavior of native, mono- and di-PEGylated RNase A with respect to mPEG nominal molecular weight, volume ratio (V_R; defined as the relation between the volume of the top and bottom phases) and TLL parameters. The reactive ATPS were prepared by mixing the following components: a 50% w/w solution of mPEG with either nominal molecular weight of 5 kDa or 20 kDa, a 40% w/w (18:7) solution of disodium hydrogen orthophosphate/sodium di-hydrogen orthophosphate adjusted to pH 5.1, 0.2 g of a sample solution containing 3 mg mL^{-1} RNase A and 200 mM sodium cyanoborohydride and enough bi-distilled water to give a final total weight of 2.0 g per system. The solutions were mixed for 10 min, and the PEGylation reactions took place by placing the tubes in gentle mixing conditions and incubating at 4°C for 17 h. Complete phase separation was achieved by centrifugation at 5000× *g* for 10 min at 4°C using an Eppendorf 5804R centrifuge (Eppendorf; HH, Germany). The partition behavior in each system was studied according to both 5 kDa and 20 kDa mPEG molecular weights; TLL values of 15, 25, 35 or 45% w/w; and V_R values of 3.00, 1.00 or 0.33. Samples of the top and bottom phases were carefully taken from each system, where the interphase was considered as part of the bottom phase. SEC was used to determine the presence and amount of native, mono-PEGylated and di-PEGylated RNase A in each sample with calibration curves prepared with single injections of 0.25, 0.50, 0.75, 1.00 and 1.5 mg/mL of the native, mono and di-PEGylated standards previously prepared as described in Section 2.2. This allowed a precise estimation of the amount of each of the species in every phase and enabled the calculation of recovery yields and partition coefficients. In this sense, the recovery yield was estimated as the amount of each native or RNase A conjugate relative to the original amount of RNase A loaded into the system, while the partition coefficient (K_P) was calculated as the ratio of the protein concentration of each species between the top and the bottom phases. In summary, the parameters were calculated according to the following equations.

$$TLL = \sqrt{[BP_{salt} - TP_{salt}]^2 + [TP_{PEG} - BP_{PEG}]^2}$$

$$Kp = \frac{\text{Concentration of protein intop phase}}{\text{Concentration of protein in bottom phase}}$$

$$\text{Reaction yield}\left(\%\frac{w}{w}\right) = \frac{\text{Mono} - \text{PEGylated RNase A}}{\text{Native RNase A}} \times 100$$

$$\text{Recovery yield}\left(\%\frac{w}{w}\right) = \frac{\text{Protein purified in the ATPS}}{\text{Protein loaded into the ATPS}} \times 100$$

2.4. SDS-PAGE

Selected reactive ATPS based on the obtained recovery yield and partition coefficient results were evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in 15% (w/v) acrylamide gels using a Mini-PROTEAN® Electrophoresis System (Bio-Rad; CA, USA). 20 µL of the samples at 1.5 mg/mL were applied per well and separation took place by applying current at 90 V, 50 mA, 10 W during 2 h and 40 min. Staining of the resolved band proteins was achieved with 0.1% (w/v) Coomassie brilliant blue R-250 dissolved in methanol, acetic acid, and distilled water (4:1:5; v/v/v). Precision plus protein all blue standard (Bio-Rad) was used as protein molecular weight marker.

Table 1
Composition of selected polyethylene glycol (PEG) – sodium phosphate (PO_4) reactive ATPS for the fractionation and PEGylation of native RNase A. TLL, V_R and the composition of each system were estimated using the binodal curves shown in Fig. S1.

System	mPEG MW (g mol^{-1})	V_R	TLL (% w/w)	mPEG (% w/w)	PO_4 (% w/w)
1	5,000	3.00	15	14.82	10.90
2			25	19.38	10.52
3			35	23.38	11.39
4			45	28.22	12.44
5		1.00	15	12.00	12.80
6			25	14.80	13.20
7			35	21.00	12.50
8			45	25.65	14.00
9		0.33	15	10.50	14.00
10			25	11.00	15.50
11			35	13.50	17.00
12			45	20.00	17.50
13	20,000	3.00	15	18.00	07.20
14			25	19.00	07.80
15			35	20.00	08.40
16			45	21.00	09.10
17		1.00	15	12.80	09.20
18			25	12.00	09.70
19			35	11.00	10.40
20			45	10.50	11.30
21		0.33	15	12.00	09.00
22			25	11.00	10.00
23			35	10.50	10.50
24			45	10.00	11.40

2.5. RNase A activity test

RNase A activity was tested on selected samples from selected reactive ATPS systems based on the obtained recovery yield and partition coefficient results. Samples of mono-PEGylated RNase A were collected using SEC, preventing other PEGylation reagents in excess from interfering with the analysis. RNase A activity was measured by detecting the liberated fluorophore from a mixture of fluorescent and quencher-labeled RNA using the RNaseAlert™ Lab Test Kit. The reaction mixture comprised 200 ng of native or mono-PEGylated RNase A, RNaseAlert® substrate v2, RNaseAlert® lab test buffer and nuclease-free water in a total reaction volume of 50 μL. The liberated fluorescence of the samples was recorded using excitation/emission at 490/520 nm in a Synergy HT fluorescent spectrophotomer (BioTek Instruments; VT, USA). The samples were equilibrated at 37°C, and the fluorescence was read every three minutes for up to 1 h.

2.6. Statistical analyses.

Results of all analyses are reported as means of samples analyzed by triplicate, including standard errors stated as intervals on raw data or as bar errors on graphs. JMP version 15.2.1 software (SAS Institute Inc.; NC, USA) was used to assess the variability of partition coefficient results, using one-way analysis of variance (ANOVA) with a 95% confidence level [20].

3. Results and discussion

3.1. Partition behavior of reactive ATPS

To evaluate the possibility of using reactive ATPS to produce and purify PEGylated proteins in a single operation, selected 5 kDa and 20 kDa mPEG – potassium phosphate systems were constructed based on data gathered from binodal curves generated at pH 5.1. Partition behavior and reaction yields were calculated by chromatographic analyses of both phases in each system as previously described. In total, 24 reactive ATPS were constructed, 12 corresponding to 5 kDa mPEG systems and 12 corresponding to 20 kDa mPEG systems. It should be noted that tie lines in the binodal curve for the systems generated with 5 kDa mPEG follow a parallel pattern, which was not the case for the binodal curve generated with 20 kDa mPEG where the lines presented a fan-like pattern (Fig. S1). However, this behavior has been observed in many previously described PEG – salt ATPS where the tie line slope needs to be calculated accordingly as in our case [21]. Baskaran and colleagues predicted a similar behavior in binodal curves of PEG – potassium phosphate ATPS using empirical equations and correlated this pattern experimentally [22]. The results for the 5 kDa mPEG (Fig. 1) and 20 kDa mPEG (Fig. 2) ATPS are reported as two axe graphs showing the reaction yield percentage of mono-PEGylated RNase and the natural logarithm of the partition coefficient ($\ln(K_P)$) of di-PEGylated, mono-PEGylated and native RNase A. Positive logarithmic values of $\ln(K_P)$ demonstrate a partition preference towards the top phase while negative values suggest a partition preference towards the bottom one. It should be noted that all reactive ATPS were able to generate biphasic systems and were able to PEGylate RNase A, as well as to separate native, di-PEGylated and mono-PEGylated RNase A with different partition coefficients.

The observed differences in partition behavior of native RNase A and its PEGylated conjugates are explained by their solubility (hydrophobicity) differences, which has shown to correlate directly with the partition coefficient of proteins in ATPS [23]. RNase A is an extremely hydrophilic protein, with an isoelectric point of 9.6 [24], but once a protein is PEGylated it is known that it loses positive charges because of the conversion of its lysine residues into amides [25]. The increment in hydrophobicity of PEGylated RNase A conjugates promotes their partition towards the least hydrophilic phase, which in this case is the top (polymer-rich) phase, in comparison to the bottom (salt-rich) phase [26]. Furthermore, it has been shown that this preference for the polymer-rich phase increases proportionally to the number of grafted PEG chains to the protein structure [27]. In general, mono-PEGylated RNase was purified preferably in the top phase of the 5 kDa mPEG ATPS, while it was purified preferably in the bottom phase of the 20 kDa mPEG ATPS. This partition behavior is in accordance with previous

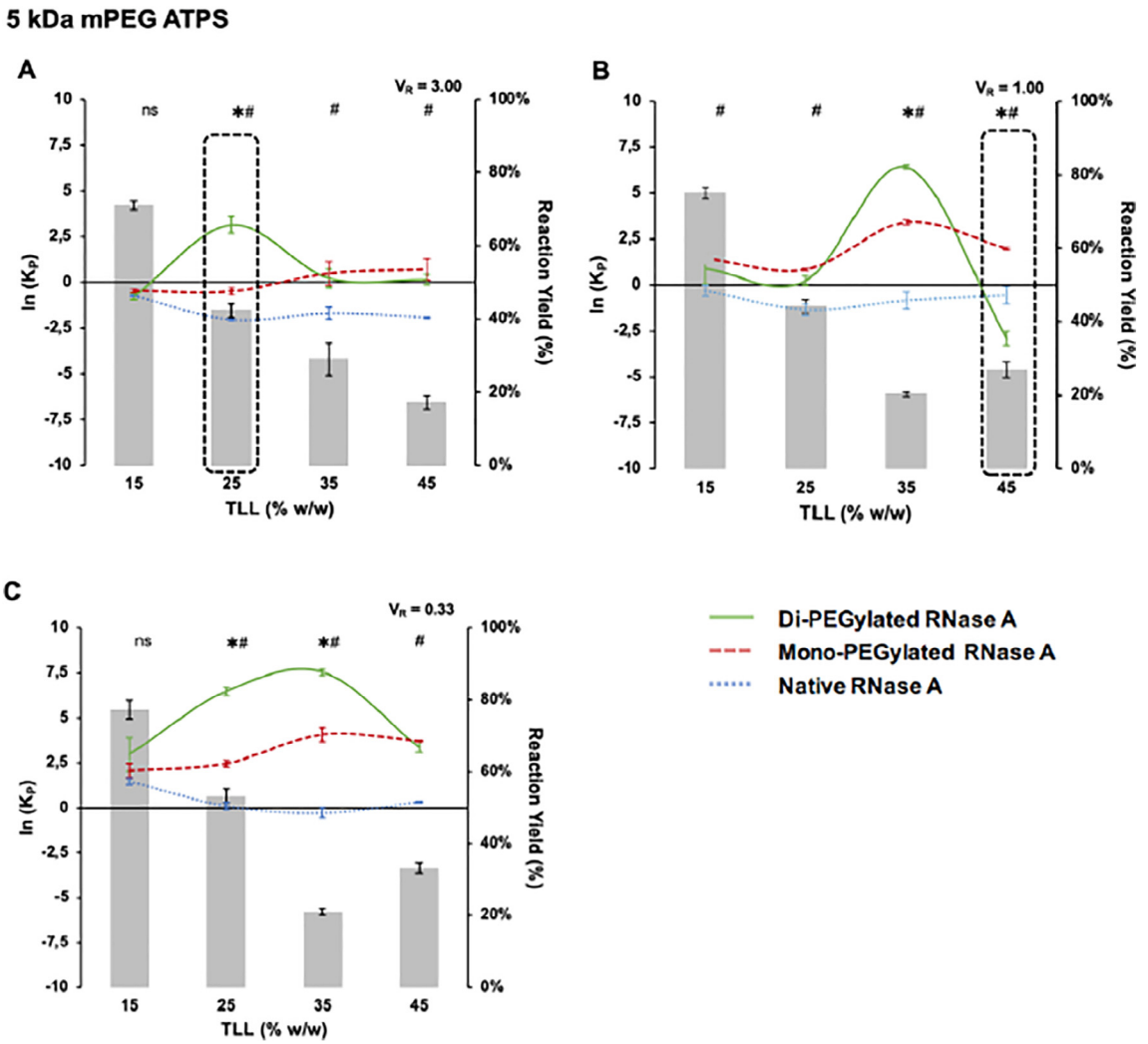

Fig. 1. Reaction yield percentage and conjugate partition coefficients in 5 kDA mPEG ATPS. System groups with volume ratios of 3.00 (A), 1.00 (B) and 0.33 (C) each at TLL of 15, 25, 35 and 45% w/w are presented. Based on the partition coefficient and reaction yield for di-PEGylated, mono-PEGylated and native RNase A selected ATPS are shown in a box. ns: ln(K_P) differences are not statistically significant (p-value > 0.05); (*): mono-PEGylated RNase A ln(K_P) is statistically different from di-PEGylated RNase A ln(K_P) (p-value $\leq$ 0.05); (#): mono-PEGylated RNase A ln(K_P) is statistically different from native RNase A ln(K_P) (p-value $\leq$ 0.05).

reports from our research team that indicate a preference of mono-PEGylated RNase towards the top phase when low molecular weight PEGs are used as components of the ATPS [15,28], as well as in a recent report by da Silva and colleagues where different organic compounds were tested as ATPS additives using different model proteins, including RNase A [29].

The results of 5 kDa mPEG ATPS show an overall negative relationship between TLL and the reaction yield percentage of mono-PEGylated RNase, except for the V_R 0.33, TLL 45%, and V_R 1.00, TLL 45% systems where the reaction yield percentage slightly increased with respect to the V_R 0.33, TLL 35%, and V_R 1.00, TLL 35% systems by approximately 12 and 7%, respectively (Fig. 1). TLL has been widely used as a parameter to account for concentration and compatibility differences between the phase-forming chemicals at the top and the bottom phases [30], since it indicates how incompatible these components are [31]. The results suggest that the more incompatible the components, the lower the reaction yield of mono-PEGylated RNase. This also suggests that higher salt concentrations limit the extent of the PEGylation reaction, which can be explained by the salting-out effect that is created when high ion concentrations increase the hydrophobic interactions of RNase A, decreasing its solubility and ultimately affecting its availability for PEGylation.

In addition to reaction yield percentage, the separation of di-PEGylated, mono-PEGylated and native RNase A in the 5 kDa mPEG ATPS showed, as expected, a direct relationship between ln(K_P) and the PEGylation degree of RNase A (Fig. 1). Similar partition behaviors have been reported in PEGylated granulocyte–macrophage colony stimulating factor in a research by Delgado and collaborators [32], where the partition coefficient increased linearly with the number of PEGs attached to the molecule in PEG/dextran ATPS. The same has been observed in different works performed in our research group where di-PEGylated conjugates showed higher partition coefficients in comparison to the mono-PEGylated forms, including RNase A [17], as well as lysozyme [16].

Regarding the 20 kDa mPEG ATPS, there was not a clear pattern among the partition behavior and the different ATPS parameters, except for the V_R 1.00 system, in which mono-PEGylated RNase A presented a negative ln(K_P) value of at least two units when compared to the di-PEGylated and native RNase A conjugates among the tested TLL values (Fig. 2). These results contrast with a research published by Santos et al. [33], at which above 90% of a 20 kDa PEGylated cytochrome c was recovered in the top phase of PEG/Citrate ATPS, while less than 10% was recovered in the bottom phase. This difference in partition behavior is explained by the different pH, salts and polymers used for the generation of the ATPS.

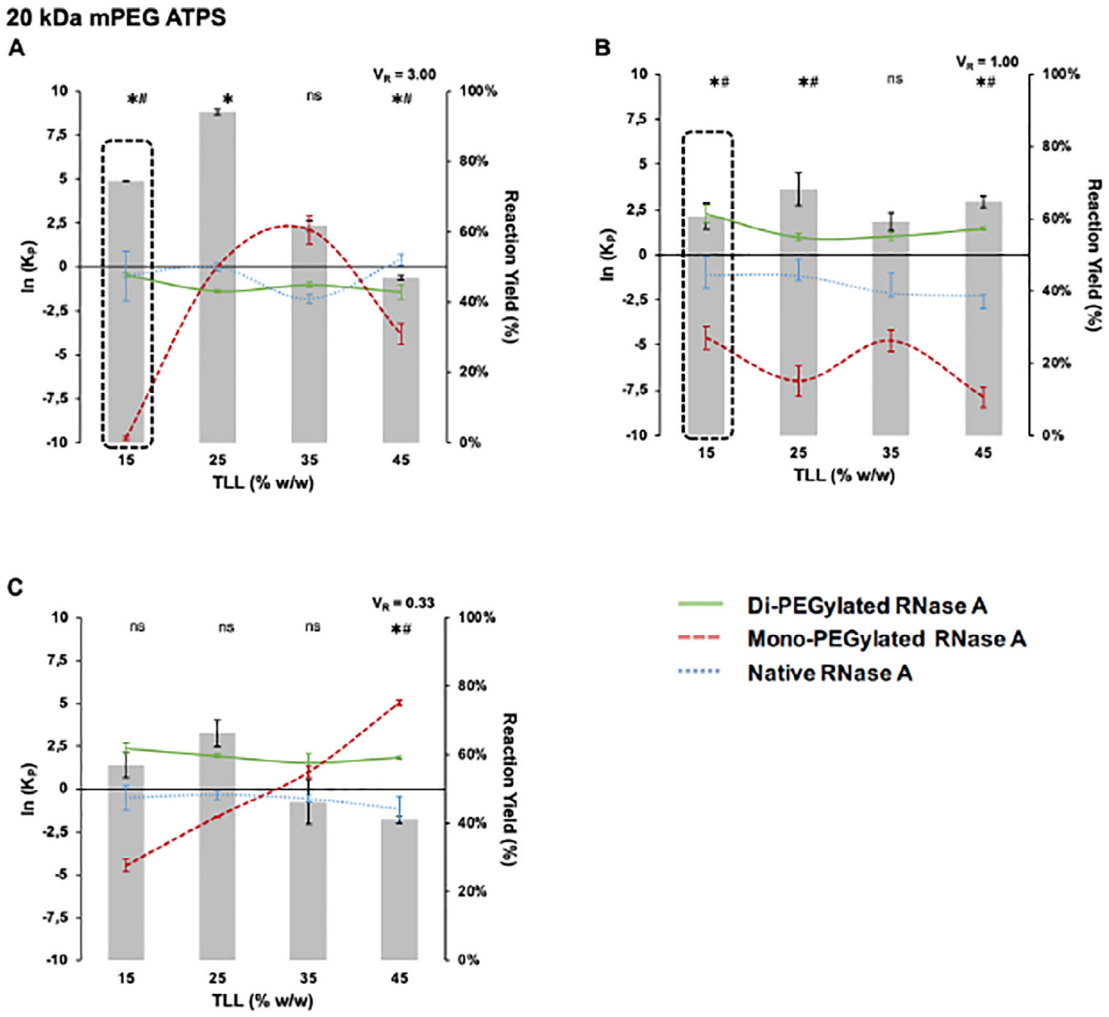

Fig. 2. Reaction yield percentage and conjugate partition coefficients in 20 kDa mPEG ATPS. System groups with volume ratios of 3.00 (A), 1.00 (B) and 0.33 (C) each at TLL of 15, 25, 35 and 45% w/w are presented. Based on the partition coefficient and reaction yield for di-PEGylated, mono-PEGylated and native RNase A selected ATPS are shown in a box. ns: ln(K_P) differences are not statistically significant (p-value > 0.05); (*): mono-PEGylated RNase A ln(K_P) is statistically different from di-PEGylated RNase A ln(K_P) (p-value ≤ 0.05); (#): mono-PEGylated RNase A ln(K_P) is statistically different from native RNase A ln(K_P) (p-value ≤ 0.05).

For instance, in this work the ATPS was formed at pH 5.1 as a strategy to promote N-terminus PEGylation [34], while citrate buffer at pH 7.0 was used as a phase forming chemical for the PEG/Citrate ATPS in said study.

Two 5 kDa and two 20 kDa mPEG ATPS were selected based on the obtained reaction yields and the feasibility of purifying mono-PEGylated RNase A from the di-PEGylated conjugates and remnant species as determined by differences in the resulting ln(K_P) values. For the 5 kDa mPEG reactive systems the V_R 1.00, TLL 45% w/w, and the V_R 3.00, TLL 25% w/w ATPS were selected; while for 20 kDa mPEG systems the V_R 1.00, TLL 15% w/w, and the V_R 3.00, TLL 15% w/w ATPS were selected. The four selected systems showed statistically significant differences (p-value ≤0.05) between the ln(K_P) of the mono-PEGylated RNase A in regard to the obtained values for the di-PEGylated and native RNase A (Table 2).

Table 2
Recovery yield percentage, reaction yield percentage and partition behavior of selected reactive 5 kDa and 20 kDa ATPS.

System	mPEG MW (g mol^{-1})	Mono-PEG RNAse A Recovery Yield (%)	Reaction Yield (%)	ln(K_P)			
				Di-PEG RNase A	Mono-PEG Rnase A	Native RNase A	p-value
2	5,000	27.08 ± 0.83 (TP) 15.19 ± 2.68 (BP)	42.27 ± 1.85	3.14 ± 0.45	−0.49 ± 0.20	−2.04 ± 0.50	* = 0.009 # = 0.006
8		22.96 ± 0.27 (TP) 4.03 ± 2.06 (BP)	26.99 ± 2.11	−2.92 ± 0.40	1.98 ± 0.47	−0.55 ± 0.07	* = 0.016 # = 0.002
13	20,000	48.35 ± 2.56 (TP) 26.01 ± 1.56 (BP)	74.36 ± 0.26	−0.48 ± 0.11	−9.73 ± 0.10	−0.50 ± 1.41	* = 0.034 # = 0.050
17		43.49 ± 10.13 (TP) 17.15 ± 0.25 (BP)	60.65 ± 3.59	2.28 ± 0.51	−4.60 ± 1.07	−1.14 ± 0.63	* = 0.001 # = 0.003

TP: top phase; BP bottom phase; (*): mono-PEGylated RNase A ln(K_P) is statistically significant different from di-PEGylated RNase A ln(K_P) (p-value ≤ 0.05); (#): mono-PEGylated RNase A ln(K_P) is statistically significant different from native RNase A ln(K_P) (p-value ≤ 0.05).

The partition behavior of the four selected ATPS was corroborated by SDS-PAGE, showing qualitative results consistent with the calculated ln(K_P) values for the di-PEGylated, the mono-PEGylated and native RNase A as well as with the mono-PEGylated RNase A recovery yield. Sample electrophoretic migration compared to the protein molecular weight markers allowed to determine the presence of native RNase A and its PEGylated conjugates. Fig. S2 shows SDS-PAGE analysis of a selected ATPS with mPEG 20.0 kDa, TLL 15%, V_R 1.0 (System 17). Mono-PEGylated RNase A is majorly found on the top phase, this correlates with the mono-PEGylated RNase A recovery yield obtained in system 17, which was 43.49% for the top phase and 17.15% for the bottom phase (Table 2).

3.2. RNase A activity test

One of the major concerns on developing a PEGylated therapeutic product is the loss of activity that is usually accompanied with this chemical modification [35]. This loss of activity is often attributed to the covalent attachment of PEG that causes a modification in its active site, steric hindrance near its surface; or as reported more recently, alteration in its structure, its dynamics or alteration in the microenvironment of its surface [36]. Considering that PEGylated RNase A conjugates could have their activity hindered as result of the reactive ATPS conditions (i.e., pH and salt concentration), purified fractions of the mono-PEGylated RNase A obtained from the selected reactive 5 kDa and 20 kDa ATPS were tested for enzymatic activity. For this, a modification of the standard protocol using the RNaseAlert™ Lab Test Kit to allow for a quantitative analysis was performed as previously described. Similar modifications of the protocol have been used before for this purpose, reporting that RNase A activity can be measured by using an excess of labeled RNA in in-vitro studies [37,38].

RNase A activity results are reported by comparing the remnant enzymatic activities of mono-PEGylated RNase A synthesized and purified following the standard procurement protocols as described in Section 2.2 with those observed in the species obtained in the reactive ATPS. This approach allowed to assess if reactive ATPS have a direct effect on PEGylated RNase A activity when compared to traditional reaction protocols, showing that RNase A activity is retained with values of above 87% for the 5 kDa mPEG ATPS and above 78% for the 20 kDa mPEG ATPS (Fig. 3). A similar behavior for RNase A kinetic parameters (i.e. K_M and K_c) is expected, since PEGylation not only usually affects RNase A biological activity, but also its catalytic efficiency [39].

It should be mentioned that in accordance with the obtained K_P values, the mono-PEGylated RNase A was found almost exclusively in the bottom phase at the 20 kDa mPEG ATPS, while at the 5 kDa mPEG ATPS, it was recovered from both the top and bottom phases. The most active mono-PEGylated RNase A was found in the bottom phase of the 20 kDa mPEG, V_R 1.00, TLL 15% w/w ATPS (system 17), while the least active mono-PEGylated RNase A was found in the bottom phase of 20 kDa mPEG, V_R 3.00, TLL 15% w/w ATPS (system 13). These results suggest that both structure and biological activity of mono-PEGylated RNase A is affected by the different V_R values used in the reactive systems. It is known, that ATPS that present a higher V_R value tend to take more time to settle because of differences in density and viscosity between both phases [40,41]. This viscosity differences might result in protein structural changes that ultimately influence biological activity. On the other hand, the biological activity of the mono-PEGylated RNase A produced from 5 kDa mPEG ATPS showed slight activity differences, with values ranging from 87 to 94%. This further supports the role of viscosity in RNase A structure since viscosity of ATPS increases as the polymer molecular weight increases at the same PEG concentration [42].

In addition to having equal biological activity than the mono-PEGylated RNase A produced from standard procurement, the mono-PEGylated RNase A from system 17 showed favorable partition behavior with statistically significant differences against di-PEGylated and native RNase A with *p*-values of 0.001 and 0.003, respectively (Table 2). Furthermore, the composition of system 17 is convenient for developing large scale ATPS to produce PEGylated therapeutic proteins, as low volume ratios and tie-line lengths provide better conditions for faster and continuous processes [43].

In this regard, reduced concentrations of mPEG used to produce the selected reactive ATPS were tested looking to lower the cost for its potential use in larger scales. Combinations of 5 kDa and 20 kDa mPEG and non-reactive PEG with similar nominal molecular weights were tested at ranges between 50% and 1% mPEG to conserve conditions and partition behavior of the reactive ATPS. However, these combinations reduced the reaction yield percentage to less than 10% of the observed yields in systems constructed with 100% mPEG (data not shown). Further analyses are needed to discard the possibility of using combinations of mPEG and PEG to develop reactive ATPS or to implement optimization strategies to increase the mass recovery of PEGylated conjugates in these reactive systems.

The capabilities of reactive ATPS can be further optimized by exploring the possibility of reusing and recycling the phase form-

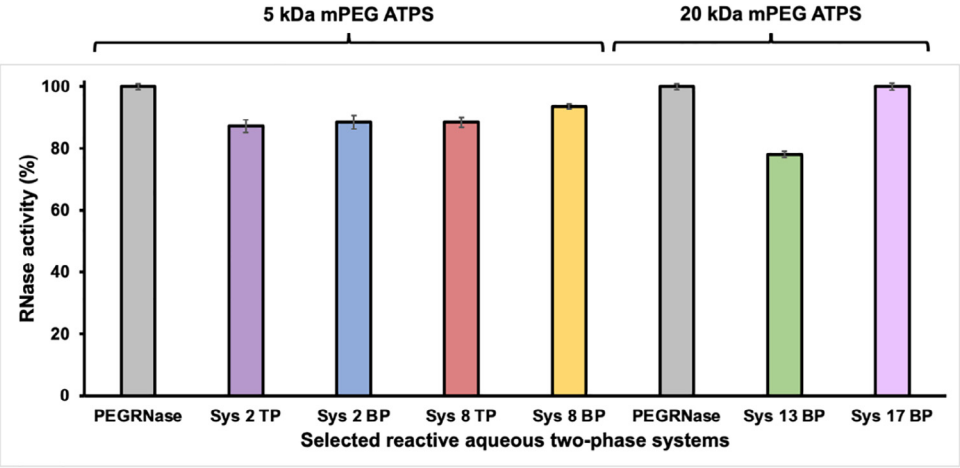

Fig. 3. RNase activity percentage of selected reactive ATPS. Sys: system; TP: top phase; BP bottom phase.

ing elements in additional reaction cycles. Our research group has proposed an ATPS strategy for the recovery of mono-PEGylated lysozyme from the rest of the PEGylation reaction products by recycling UCON as a phase forming element [13]. A similar strategy can be developed in selected reactive ATPS by evaluating the mono-PEGylated RNase A recovery yield obtained in each cycle. This approach could serve as a lower cost and environmentally-friendly alternative, taking advantage of unreacted mPEG as well as the surplus of the phase forming elements.

Reactive ATPS represent an attractive alternative for the production of PEGylated therapeutic proteins. As mentioned, ATPS present many advantages over conventional extraction techniques, including, using low-cost components, compatibility with continuous operation strategies and scale up feasibility [44]. Reactive ATPS benefits from these advantages while furtherly integrating, in this case, a PEGylation reaction during the phase forming process, allowing to obtain and purify PEGylated proteins in a single step.

4. Conclusions

One of the challenges of the development of a manufacturing process that generates PEGylated therapeutic proteins is to efficiently produce and purify the mono-PEGylated form without sacrificing the biological activity of the drug. Here, we proposed the use of reactive aqueous two-phase systems, an integrative method that allowed simultaneous production and purification of mono-PEGylated RNase A from di-PEGylated and remnant native species. This method represents an advancement from previous efforts where PEGylated RNase A has been purified by ATPS, but PEGylation was performed separately in a preceding step.

Different reactive aqueous two-phase systems were developed, obtaining particularly relevant results on the 20 kDa linear monomethoxy polyethylene glycol systems, with reaction yields of above 70% and an equivalent biological activity than the mono-PEGylated RNase A obtained following a standard procurement methodology.

This study represents an alternative to traditional PEGylation reaction processes, with potential application on the pharmaceutical industry for being an integrative methodology that proved to be efficient on a laboratory scale. Further studies on large scale reactive aqueous two-phase systems will explore the feasibility of using this approach on industrial settings, particularly on the selected systems that show high reaction yield percentages and favorable partition behavior towards the mono-PEGylated form.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary material

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