INSTITUTO TECNOLÓGICO Y DE ESTUDIOS SUPERIORES DE MONTERREY

CAMPUS MONTERREY

DIVISIÓN DE BIOTECNOLOGÍA Y ALIMENTOS

PROGRAMA DE GRADUADOS EN BIOTECNOLOGÍA

"Cuantificación y Caracterización del Comportamiento de Partición de la Ribonucleasa A y sus Conjugados Polímero – Proteína en Sistemas de Dos Fases Acuosas Polímero – Sal"

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POR:

JOSÉ GUILLERMO GONZÁLEZ VALDEZ

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To those who have found that while
little science estranges people from God,
a lot of science takes them back to Him.

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"Quantification and Characterization of the Partition Behavior of Ribonuclease A and its Polymer – Protein Conjugates in Polymer – Salt Aqueous Two Phase Systems"

INDEX

SUMMARY

One of the alternatives to extend the half-life and antitumoral activity of RNase A inside the human body is through a process called PEGylation. This process consists in the modification of a peptidic or non-peptidic molecule attaching at least one chain of polyethylene glycol (PEG). PEGylation reactions produce mixtures of the remaining native specie and polymer conjugates that vary in the number of PEG chains grafted to the protein. These mixtures are difficult to separate and purify. However, Aqueous Two-Phase Systems (ATPS) have shown to have potential for the fractionation of native proteins, such as bovine serum albumin, immunoglobulin G and granulocyte-macrophage colony stimulation factor, from their PEGylated conjugates. In this context, the characterization of the partition behavior of RNase A and its PEGylated conjugates results of scientific interest.

As a first step to study the partition behavior of native RNase A and its polymer conjugates on polyethylene glycol-potassium phosphate ATPS, an easy to implement quantification methodology based in UV light absorbance at 280 nm on polymer-salt rich environments was developed. This quantification technique takes into consideration the effects of phase composition in ATPS environments and grafted polymer chains in the protein upon its UV absorbance. This method was utilized in order to characterize the partition behavior of native, mono and di-PEGylated RNase A, individually, on ATPS. The effect of system parameters such as tie – line length (TLL, 15-45% w/w) and PEG molecular weight (400-8000 g/mol) on partition behavior were studied. ATPS constructed with low molecular weight PEG (400 g/mol) and a high TLL (45% w/w) allowed the partition of the RNase A from its PEGylated conjugates on opposite phases, achieving high recovery yields (> 84%) and showing the potential that ATPS have on the selective recovery of these compounds.

Quantification of RNase A and its PEGylated conjugates on polymer – salt rich environments using absorbance at 280 nm.

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Running Title: Quantification of RNase A and its PEGylated conjugates under aqueous two-phase system conditions

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ABSTRACT

Ribonuclease A from bovine pancreas and its PEGylated conjugates has proven to have several potential therapeutic applications. Aqueous Two-Phase Systems (ATPS) is a promising primary recovery strategy for the fractionation of proteins and their PEGylated conjugates. However, in order to characterize the partition behavior of these molecules in ATPS, an easy to implement method is needed to estimate protein concentration in each phase. Because RNase A quantification based on colorimetric methods usually renders poor sensitivity, this paper presents a methodology based on UV absorbance to quantify RNase A and its PEGylated conjugates on polymer (polyethylene glycol; PEG) and salt (potassium phosphate; PO_4) rich environments, simulating conditions found on polymer – salt ATPS. The effect of PEG and PO_4 concentrations and the effect of grafted PEG chains on RNase A upon UV absorbance were evaluated. Polymer and salt concentrations have a significant effect on RNase A absorbance, reflecting the need of specific standard curves in order to consider the impact of the chemical forming phase upon the extinction coefficient. The absorbance ratios at 280 nm between mono-PEGylated RNase A/native RNase A and di-PEGylated RNase A/native RNase A in PEG and potassium phosphate free environments were found to be 0.32 and 0.46, respectively, demonstrating that the grafted PEG chains have also an important effect upon protein light absorbance. The method presented results in an easy-to-implement alternative, to chromatographic approaches, since just an UV-visible spectrophotometer or plate reader is required.

KEYWORDS: RNase A, PEGylation, quantification, UV spectrophotometry, Aqueous Two-Phase Systems

1. INTRODUCTION

Ribonuclease A (RNase A) from bovine pancreas is a small enzyme formed by 124 amino acids and a molecular weight of 13,686 Da [1]. This enzyme has therapeutic applications as an aspermatogenic and antitumoral agent. Inside the cell, RNase A complexes with its inhibitory protein, Ribonuclease Inhibitor (RI), and its surface mutates avoiding recognition by its cytosolic inhibitor, thus becoming toxic to cancer cells by degradation of intracellular molecular RNA [2]. Furthermore, it has been shown that the PEGylation of this protein greatly improves its therapeutic effects [3].

PEGylation consists in the modification of a peptidic or non-peptidic molecule by attaching at least one chain of polyethylene glycol (PEG) of certain molecular weight, hence the name of the process [4]. Described initially in 1977 by Abuchowski and collaborators, the discovery of this technique became a milestone because for the first time in history an enzyme was being synthetically modified without losing its properties [5], characteristic that can be exploited in proteins with pharmaceutical applications. Protein PEGylation allows a milder attack by proteolytic enzymes to the modified molecule once it has been administrated. Grafted PEG chains lower the absorption rate of the protein in the kidneys, expanding its circulating lifetime and biodistribution due to the increase in molecular weight. PEGylation also reduces the generation of specific antibodies to this molecule, decreasing its reticuloendothelial elimination and increasing its solubility. Additionally, the shelf life of the product is also noticeably improved [6,7].

The PEGylation reaction of RNase A results in 3 main reported species: the residual native RNase A, mono-PEG RNase A (which exhibits the best therapeutic effects and where a single PEG chain is attached to the N-terminal lysine of the protein), and di-PEG RNase A (that appears once the mono-PEG RNase A is synthesized and continues reacting by adding

another PEG molecule to one of its 9 available lysines) [8]. The proper characterization of PEGylated conjugates requires a precise quantification method. Typically, the quantification of PEGylated proteins using techniques based in colorimetric essays is preferred [9]. Nevertheless, the selection of an essay depends on the needs of the user and the particular protein that is being analyzed. In the particular case of RNase A and its polymer conjugates, previous experience in our research group has demonstrated that the quantification of these compounds is difficult when colorimetric methods (i.e. Bradford and Biuret assays) are used. Such findings are explained due to the lack of tryptophan and the considerable low molecular weight of RNase A and its polymer conjugates. The application of such colorimetric assays resulted in extremely low absorbance readings even at high protein concentration (> 10 mg/ml). Additionally, high noise/signal ratios were observed, increasing experimental variability beyond acceptance. On the other hand, it is well known that UV spectrophotometry (λ = 280 nm) can be used for the quantification of proteins based on their content of aromatic residues and disulfide bonds. Thus, it would be practical to develop an assay based on UV spectrophotometry to quantify RNase A and its polymer conjugates. Protein quantification using UV absorbance is one of the most used methods due mainly to its wide availability, usage facility and rapidness. Additionally, since the sample is not destroyed during the assay it may be subsequently reutilized.

In the field of PEGylated proteins separation, there are several chromatographic approaches that have been used to separate such protein-polymer conjugates. Size exclusion chromatography (SEC) separates these molecules based in molecular size. SEC separation is favored by the increase in the hydrodynamic radius of the conjugates, which reduces their accessibility trough the porous media in the column and alters its elution times [10]. Besides size and molecular weight, other properties change within polymer conjugates.

PEGylated proteins lose one positive charge for each PEG molecule grafted to the protein [11]. Therefore, ion exchange chromatography (IEX) is also used in separating these molecules. Hydrophobicity is also altered in PEGylated molecules making it possible to separate them through hydrophobic interaction chromatography (HIC) or reversed-phase high performance liquid chromatography (RP-HPLC) [11]. After SEC, other non-chromatographic methods based in molecular size are ultrafiltration and diafiltration used mainly to concentrate molecules [12]. In this context, aqueous two-phase systems (ATPS) represent an interesting alternative for the separation of PEGylated proteins.

ATPS constitute a liquid-liquid fractionation technique based in the differences between the partition coefficients (K_P) of the different species that are being separated [13]. This technique has been used for the primary recovery and partial purification of biological compounds like proteins, genetic material, low molecular weight products, cells and organelles [14]. There are some works that document the characterization of the partition behavior of PEGylated proteins in ATPS, demonstrating that their fractionation and recovery is feasible [15,16]. Even more, compared to chromatographic techniques ATPS are cheaper to implement and are able to generate more biocompatible, robust and scalable processes [14]. However, during preliminary fractionation studies of RNase A and its PEGylated conjugates on ATPS in our research group, enzyme concentrations on the top and bottom phases of selected polymer (polyethylene glycol; PEG) – salt (potassium phosphate) systems could not be adequately quantified by UV spectrophotometry at 280 nm. As known, the extinction coefficient of a solute may be highly dependent on the nature of the solvent (hydrophobicity, ionic force, pH, etc.) in which it is dissolved [17]. Therefore, it becomes evident that in order to properly quantify this enzyme and its conjugates, the effect of PEG and potassium phosphate concentrations on UV absorbance

must be studied to outcome these quantification inconsistencies. Furthermore, it could be expected that the linked PEG chains in the conjugates may interact with the protein generating an absorbance deviation that must be characterized to be taken into consideration during quantification.

The main purpose of this study was to evaluate the effect of PEG (at four different molecular weights) and potassium phosphate solvent concentrations upon the absorbance at 280 nm of RNase A. Additionally, the absorbance effect of the polymer chains grafted to the protein through PEGylation at 280 nm of mono-PEG RNase A and di-PEG RNase A was also evaluated. The characterization of the effects of the chemical forming phases and the presence of polymer chains upon this parameter were then used to establish an easy-to-implement quantification methodology for RNase A and its PEGylated conjugates at polymer-salt ATPS environments.

2. MATERIALS AND METHODS

2.1 PEGylation of RNase A

The reaction was made under conditions that maximize the addition of PEG chains to the N-terminal lysine of the molecule in order to minimize PEG interference with other aminoacid residues in the protein [18] according to the procedure reported by Cisneros-Ruiz *et al* [19]. Briefly, 2.0 ml of an RNase A (Sigma, MO, USA) solution at 3.0 mg/ml in a pH 5.1, 100 mM phosphate buffer with 20 mM sodium cyanoborohydride (Fluka, Switzerland) was added to a flask containing 30 mg of 20 kDa mPEG propionaldehyde (Jen Kem Technologies, China). The mixture was vigorously stirred for 17 - 19 hr at 4 °C

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with a magnetic stirrer. The reaction mixture was then stored for less than 3 days at 4 °C for further separation of the conjugates by size exclusion chromatography.

2.2 Recovery and purification of the PEGylated conjugates by size exclusion chromatography

The PEGylation reaction mixture was injected into an Äkta Explorer 100 (GE Healthcare, United Kingdom) size exclusion chromatographer equipped with a HiPrep™ 16/60 chromatographic column prepacked with Sephacryl™ S-300 High Resolution resin (GE Healthcare, United Kingdom) in order to separate each conjugate and the unreacted RNase A. Each SEC peak had been previously analyzed by Cisneros-Ruiz *et al*. [19] through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) to identify and assure the purity of di-PEGylated, mono-PEGylated and native RNase A species [19]. Fractions collected for each PEGylated conjugate were pooled and concentrated by ultrafiltration under nitrogen atmosphere using an Amicon® ultrafiltration cell 8050 (Amicon INC., MA, USA) with a 10 kDa Diaflo® ultrafiltration membrane (Amicon INC., MA, USA) until 25 ml of concentrated fractions were obtained. Concentrated fractions were frozen at -80 °C in a REVCO Ultima II ultra low freezer (Thermo Fisher Scientific, MA, USA). Finally, frozen fractions were transferred to a Sentry 2.0 lyophilizer (Virtis, NY, USA) at -80 °C and less than 100 mTorr until all water was removed. Purified fractions of mono and di-PEGylated RNase A were stored at -20 °C until used.

2.3 Effect of polyethylene glycol and potassium phosphate concentration and polyethylene glycol molecular weight upon absorbance of native RNase A at 280 nm

In order to study the effect of PEG and potassium phosphate concentrations and the effects of different PEG molecular weights upon RNase A absorbance, a set of 16 PEG - phosphate biphasic systems (1-16, **Table 1**) were constructed on a mass basis (50 g total weight) using the corresponding binodal curves, from which the top and bottom phase compositions were also estimated [20]. PEG (Sigma, MO, USA) of nominal molecular weights of 400, 1 000, 3 350 and 8 000 g/mol (50% w/w stock solution, except PEG 400 which is liquid at room temperature and was used at 100% w/w) and a dipotassium hydrogen orthophosphate / potassium di-hydrogen orthophosphate (18:7) solution (DEQ, Mexico) (40% w/w stock solution, pH 7) were used to construct the ATPS. Volume ratio (V_R, defined as the volume ratio between the top and bottom phases) and pH were kept constant at 1 and 7, respectively. Systems were vigorously mixed during 10 min using a Glas-Col 099A-RD4512 Tissue Culture Rotator (Glas-Col LLC, IN, USA) in order to assure contact of all components. ATPS were then centrifuged at 5 000 *g* for 10 min at 25 ºC using an Eppendorf 5804R centrifuge (Eppendorf, Germany) to address phase equilibrium. Top and bottom phases from each system were then carefully separated, stored and used to prepare 0.25, 0.50, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml solutions of RNase A (Sigma, MO, USA) to obtain a standard curve for each phase at 280 nm. Phases without protein were used as blanks.

2.4 Effect of PEGylation upon absorbance of RNase A at 280 nm

Considering molecular weights of 13 686 Da for native RNase A, 33 686 Da for mono-PEG RNase A and 53 686 Da for di-PEG RNase A, the protein fraction of mono-

PEGylated and di-PEGylated RNase A was estimated to be 0.4062 and 0.2549, respectively, based on the RNase A molecular weight / PEGylated conjugate molecular weight ratio. Molecular weight of each PEGylated species was calculated as the sum of the molecular mass of the native RNase A and each grafted mPEG chain. In order to determine the effect of grafted PEG chains on RNase A UV absorbance at 280 nm, separate readings of different solutions of native RNase A and its PEGylated conjugates at equal total protein concentrations were compared. Using the protein fraction values of each conjugate, absorbance at 280 nm was measured to RNase A, mono-PEG RNase A and di-PEG RNase A solutions with a total protein concentration of 0.50, 0.75, 1.00, 1.50 and 2.00 mg/ml (regardless the molecular weight of the conjugate and not considering the PEG mass fraction). This corresponds to concentrations of 1.23, 1.84, 2.46, 3.69, 4.92 mg/ml of mono-PEG RNase A and to concentrations of 1.96, 2.94, 3.92, 5.88, 7.84 mg/ml of di-PEG RNase A, respectively. Enzymes were diluted in a 20 mM pH 7 dipotassium hydrogen orthophosphate / potassium di-hydrogen orthophosphate (18:7) buffer (DEQ, Mexico). Buffer without protein was used as blank. In order to determine possible absorbance deviations as result of the grafted PEG chains a correction factor ζ, defined as the ratio between absorbance readings of the PEGylated conjugate and the absorbance readings of native RNase A at one same concentration (ζ = Abs @ 280 nm of PEGylated protein / Abs @ 280 nm of native protein), was calculated.

2.5 Analytical and statistical procedures

All absorbance measurements were made in a Beckman Coulter DU800 Spectrophotometer (Beckman Instruments, CA, USA) using 1.5 ml PMMA cuvettes (VWR International, PA,

USA). Protein was weighted in a Mettler-Toledo X564 analytical balance (Mettler-Toledo International, OH, USA).

All experiments were carried out at least by triplicate. Results presented are the mean value of the independent replicas, and the standard error was calculated as the standard deviation divided by the square root of the number of replicas. Statistical calculations and data analysis were made using JMP™ Statistical Discovery™ Software Version 7 (SAS Institute Inc., NC, USA).

3. RESULTS AND DISCUSSION

3.1 Effect of polyethylene glycol and potassium phosphate concentration and polyethylene glycol molecular weight upon absorbance of native RNase A at 280 nm

Selected ATPS designed with four different PEG molecular weights and four different tie-line lengths (TLL, 15, 25, 35 and 45% w/w; see **Table 1**) were used to evaluate the effect of PEG and potassium phosphate concentration and the effect of polymer molecular weight (PEG MW) on the absorbance of RNase A at 280 nm. Standard curves for RNase A at 280 nm on the different top and bottom phases studied are presented in **Figure 1**. PEG and potassium phosphate concentrations (% w/w) in each phase are described in **Table 1**. **Table 2** presents the extinction coefficients (a; ml mg^{-1} cm^{-1}) calculated from the slopes in the standard curves on **Figure 1**. It must be mentioned that the extinction coefficient for RNase A on 20 mM potassium phosphate pH 7 buffer was calculated to be 0.5271 ml mg^{-1} cm^{-1}.

The standard curves for the top and bottom phases of selected PEG 400 systems are depicted in **Figure 1a**. It is clear that for the top phase, as TLL increases the slope in these curves decreases (from 0.7899 to 0.5934 ml mg^{-1} cm^{-1}) and that for the bottom phase, in

3. RESULTS AND DISCUSSION

The partition behavior of RNase A on the selected ATPS is depicted in **Table 2**. It is clear that RNase A exhibited a top phase preference when the lowest molecular weight of PEG (i.e. PEG MW 400) was used. An increase in TLL in PEG 400 – potassium phosphate systems increased the top phase preference of the native RNase A. As a result the top phase protein recovery increases from 69% to 94%. As TLL increases, PEG concentration in the top phase and potassium phosphate concentration in the bottom phase increase. These changes alter the free volume available in each phase for protein partition, alter the surface tension at the interphase and the density of the phases [15]. These effects change the partition behavior of native RNase A specifically promoting it to the top phase.

An increase in PEG MW causes a shift of native RNase A partition towards the bottom phase (see **Table 2** and **Figure 1**). As PEG MW increments, the hydrophobicity of the phase increases. Since RNase A is a highly hydrophilic protein [9], it is then expected to find greater protein concentrations at the bottom phase as PEG MW increases. This behavior may also be explained in terms of the free volume available in each phase. As PEG chain lengths increase, free volume decreases generating steric effects that change protein partition towards the bottom phase. RNase A exhibited bottom phase preference when ATPS of PEG molecular weight of 1 000 g/mol or higher were used (systems 5 to 16 in **Table 2**). In these cases an increase in TLL did not change the phase preference of the protein. In fact, only for the specific case of PEG 400 systems an increment in RNase A recovery yield at the top phase was observed as TLL increased. For the rest of the systems (PEG 1 000, 3 350, and 8 000 g/mol) as TLL increased, a mixed effect was observed probably due to the mixture of hydrophobic, ionic charge and free volume interactions with RNase A. In general, for systems of PEG 1 000, 3 350 and 8 000 g/mol bottom phase

recoveries higher than 59% were obtained. In the particular case of system 8 (PEG 1 000 g/mol, TLL 45%), interestingly, a high top phase recovery yield (75.3%) was obtained. This behavior may indicate that salt concentration at the bottom phase is high enough to counteract the effect of excluded volume in the top phase, shifting protein partition towards such phase.

The partition behavior of mono-PEGylated RNase A is presented in **Table 3**. For all the systems selected, mono-PEGylated RNase A exhibited a bottom phase preference. Regardless of PEG MW and TLL, high bottom phase protein recovery (> 70%) was obtained. Since ATPS are highly hydrophilic, the increment in hydrophobicity granted by the grafted PEG chains promotes the partition of the conjugates towards the bottom phase (particularly towards the interphase, considered part of the bottom phase) [26]. Additionally, the grafted PEG chain of the mono-PEGylated RNase A increases significantly its molecular size when compared with native RNase A. Therefore, the behavior observed may be as well explained on the basis of the available free volume in each phase. PEG MW seems to have no extensive effect on mono-PEG RNase A recovery in the bottom phase. TLL increments in each of the different PEG MW studied did not present a clear effect except for PEG 400 systems where as TLL increases a minor protein partition increment towards the top phase is observed.

Table 4 presents the recovery percentages and the logarithm of the partition coefficients ($\ln K_P$) of di-PEGylated RNase A. As for the mono-PEGylated RNase A most conjugate is recovered in the bottom phase of the system, regardless PEG MW and TLL. However, recovery yields in the top PEG rich phase are slightly higher than those of the mono-PEGylated conjugate. Once again, the observed partition behavior may be explained in terms of phase hydrophobicity and available free volume.

However, as the number of chains linked to the protein increase, it is expected that partition behavior on ATPS may be influenced by free energy considerations that involve net interactions with the polymer rich regions in the system and within the conjugate. These interactions vary depending on the characteristics of the polymer and its concentration [21]. Previous PEGylated protein partition studies in PEG – dextran ATPS show that partition coefficients increase exponentially with the amount of PEG bound to the protein and that a linear relationship between log K_P values and the number of grafted PEG chains is preserved [27]. For these previously reported systems, an increase in partition towards the top phase is observed as the number of PEG chains increases accounting the modified amino groups in each protein. The proteins tested in these previous reports are granulocyte-macrophage colony stimulation factor, bovine serum albumin and immunoglobulin G (all with a large amount of PEGylation sites) and it has been found that the proportionality between log K_P and the number of PEG molecules attached is only compromised for conjugates having relatively large number of grafted PEG molecules in biphasic systems of high concentration of polymers, where a partial precipitation of the conjugates towards the dextran rich phase is observed [27]. This effect cannot be whatsoever compared in PEG – potassium phosphate systems by the study of the increment in TLL values and thus in the increments of the concentrations of the system components. It must also be noted that grafted PEG chains in proteins may interact with one another, with water molecules, ions and might as well form hydrogen bonds [21]. All these possible interactions as the kind of ATPS involved (i.e. polymer – polymer or polymer – salt) may also have an impact on the fractionation of each of the conjugates.

The particular effect of PEG MW on ln K_P of each studied specie at TLL 45% w/w is depicted in **Figure 1**. As it is seen, at PEG MW 400 g/mol, the ln K_P value for native

RNase A has the highest positive value (i.e. 2.5779) indicating its preference for the top phase, while the conjugates present both a negative value and thus showing a preference towards the bottom phase. Therefore, in order to achieve a selective partition of native RNase A from its PEGylated conjugates, it becomes evident that low PEG MW (400 g/mol) systems must be selected. System 4 (PEG 400, TLL 45%) allows the recovery of most of the native RNase A at the top phase (i.e. 95%), while in the bottom phase mono and di-PEGylated conjugates are recovered with yields superior to 84%.

A practical approach to fractionate native RNase A from its mono and di-PEGylated conjugates may involve the use of ATPS of low PEG MW and high TLL (system 4 in **Table 1**). **Figure 2** presents a simplified scheme of the proposed strategy where the expected final top and bottom phase recovery yields of each of the three species are reported. The results reported here, highlight the advantages of the potential application of an ATPS approach to establish the conditions for a single stage separation of RNase A and its conjugates. To date, no previous studies known by the authors have been reported that exploit the potential use of ATPS for this purpose.

4. CONCLUSIONS

PEG MW and TLL have significant effect on the partition behavior of native RNase A and its PEGylated conjugates on polymer – salt ATPS. Native RNase A can be predominantly recovered from the top phase of low PEG MW ATPS while mono and di-PEGylated RNase A have a preference to partition towards the bottom phase regardless of PEG MW and TLL. The characterization of the partition behavior of these species on ATPS resulted in the establishment of a potential strategy to fractionate unreacted native RNase A from its polymer conjugates in a PEGylation reaction mixture. A PEG 400 g/mol, TLL 45%, V_R 1

curves considering phase composition. The concentration of the PEGylated conjugates of RNase A in a phase of a particular ATPS can be obtained by:

1. Multiplying the absorbance value read experimentally of a sample with unknown concentration of the conjugate by its corresponding ζ factor.
2. Using this corrected absorbance value in the phase-specific standard curves designed for native RNase A to take into consideration the interactions between the protein and the phase in which it is diluted.
3. Dividing the estimated concentration obtained from the used curve by the protein mass fraction specific for each conjugate (0.4062 for mono-PEG RNase A and 0.2549 for di-PEG RNase A) in order to obtain the real concentration of the conjugate based on the protein fraction concentration.

Figure 3 graphically resumes the described methodology in which the ζ correction factor and the phase-specific standard curves for RNase A jointly allow a correct quantification of mono-PEG RNase A and di-PEG RNase A in ATPS environments. However, it must be remarked that this procedure, although easy to implement with widely available laboratory equipment (UV-visible spectrophotometer) can only be used for quantifying pure species, rather than a combination of native RNase A and its PEGylated conjugates. Quantification of pure species allows the further study of the partition behavior of such enzymes in ATPS.

4. CONCLUSIONS

In order to establish the potential of Aqueous Two-Phase Systems for the fractionation of RNase A and its PEGylated conjugates, an easy-to-implement quantification method based on UV absorbance at 280 nm was developed. The effect of PEG and potassium phosphate

concentration together with PEG MW upon RNase A absorbance was determined and the need to obtain standard curves specific for each phase from the biphasic aqueous systems was demonstrated. Furthermore, the effect of grafted PEG chains on protein absorbance was established, and correction factors to account for such effects were calculated. The developed method allows the individual quantification of native RNase A, and the mono and di-PEGylated RNase A conjugates, which will allow, amongst other things, the characterization of the partition behavior of these species in polymer – salt ATPS.

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FIGURE CAPTIONS

Figure 1. Effect of Polyethylene glycol molecular weight (PEG MW) and Tie-Line Length (TLL) on the absorbance of RNase A at 280 nm on top and bottom phases of selected Polyethylene glycol – potassium phosphate ATPS. For every PEG MW and each phase curves for TLL 15% (◆), TLL 25% (■), TLL 35% (▲) and TLL 45% (●) are shown. **1a**. Standard curves for PEG 400 systems are presented. **1b**. Standard curves for PEG 1 000 systems are presented. **1c**. Standard curves for PEG 3 350 systems are presented **1d**. Standard curves for PEG 8000 systems are presented. Curves were calculated measuring absorbance at 280 nm to solutions of 0.25, 0.5, 0.75, 1.00, 1.50 and 2.00 mg/ml of RNase A using the separated phases of each system as solvent.

Figure 2. Box Plots and Tukey – Kramer Plots for the one-way analysis of correction factor ζ for mono-PEG RNase A and di-PEG RNase A. Solutions of 0.50, 0.75, 1.00, 1.50 and 2.00 mg/ml considering only the total protein mass fraction of each analyzed specie were prepared in a 20 mM pH 7 dipotassium hydrogen orthophosphate / potassium di-hydrogen orthophosphate (18:7) buffer and read at 280 nm. The ζ correction factor was calculated as the ratio between absorbance readings of each conjugate and the absorbance readings of native RNase A.

Figure 3. Flow diagram of the proposed methodology to quantify RNase A and its polymer conjugates considering the effects of solvent interactions and PEGylation upon UV absorbance.

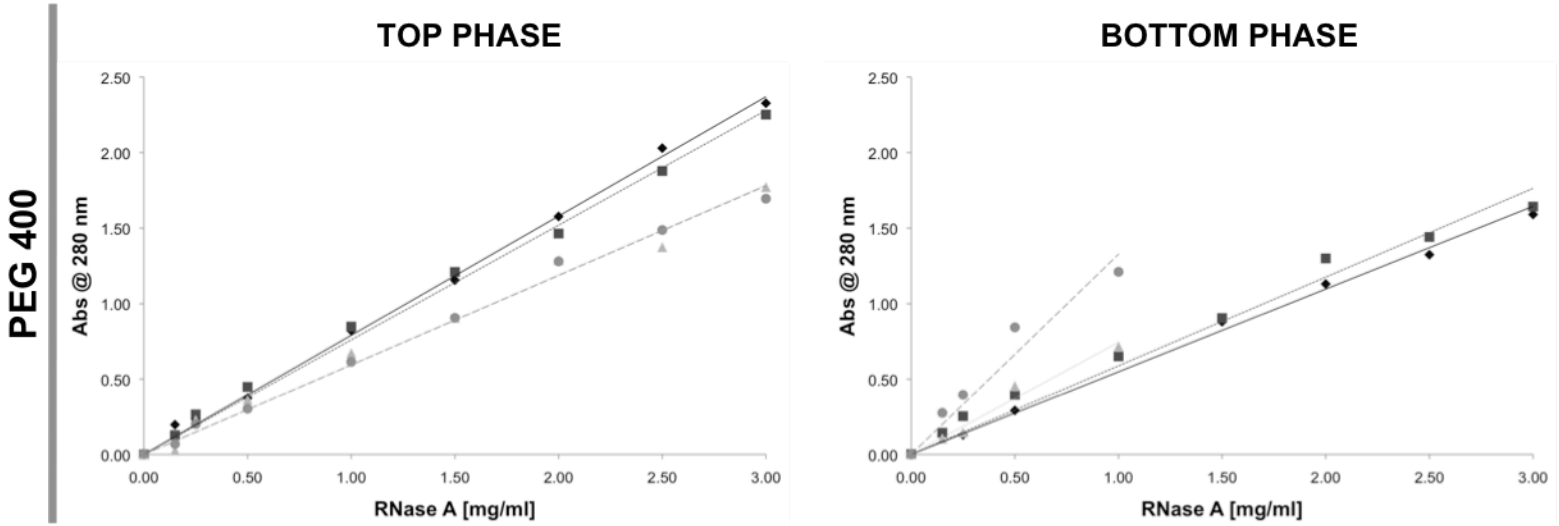
PEG 400
TOP PHASE
BOTTOM PHASE
Abs @ 280 nm
RNase A [mg/ml]
0.00
0.50
1.00
1.50
2.00
2.50
3.00

FIGURE 1a

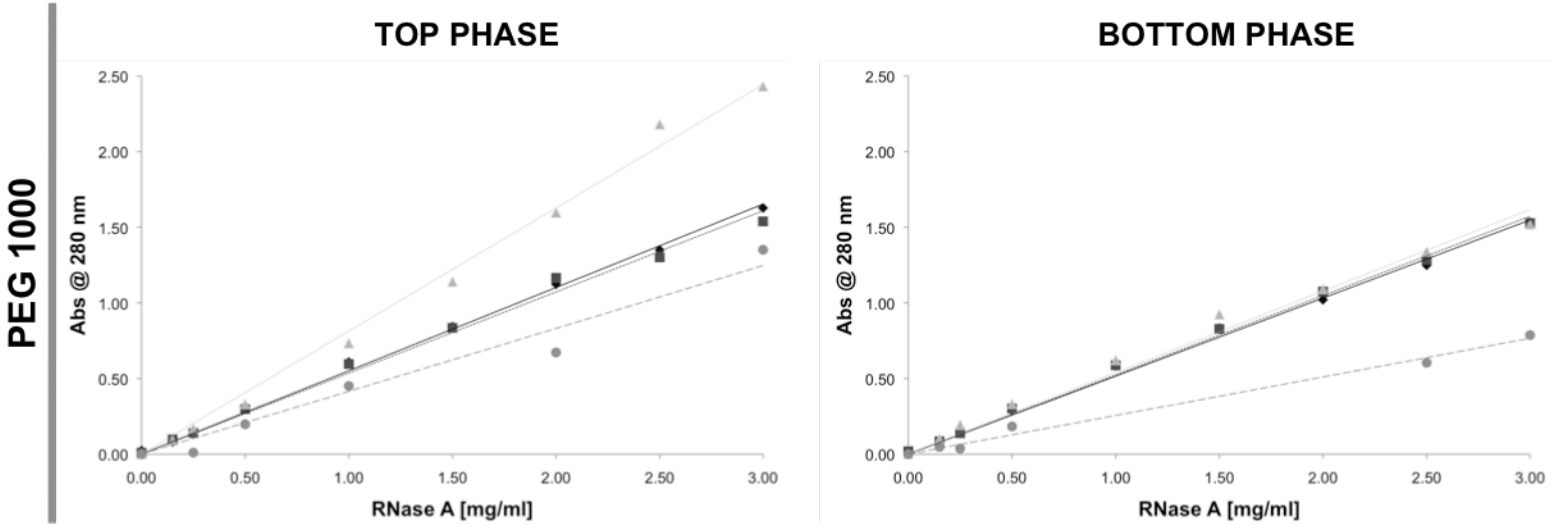
PEG 1000
TOP PHASE
BOTTOM PHASE
Abs @ 280 nm
RNase A [mg/ml]
0.00
0.50
1.00
1.50
2.00
2.50
3.00

FIGURE 1b

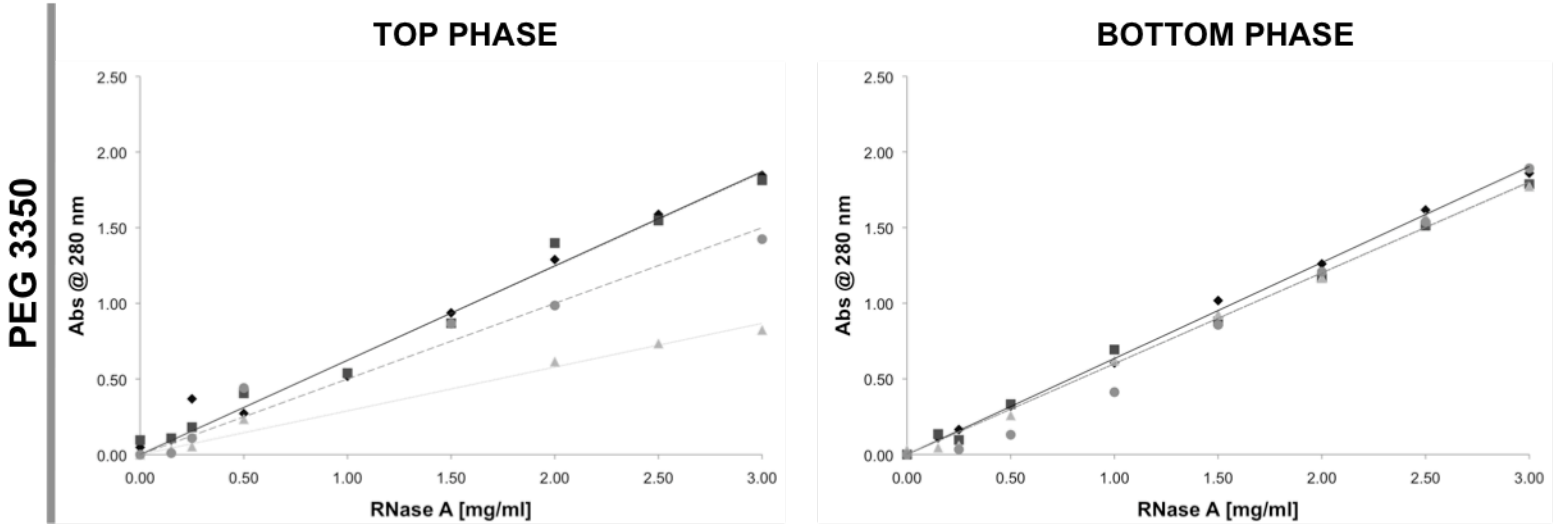
PEG 3350
TOP PHASE
BOTTOM PHASE
Abs @ 280 nm
RNase A [mg/ml]
0.00
0.50
1.00
1.50
2.00
2.50
3.00

FIGURE 1c

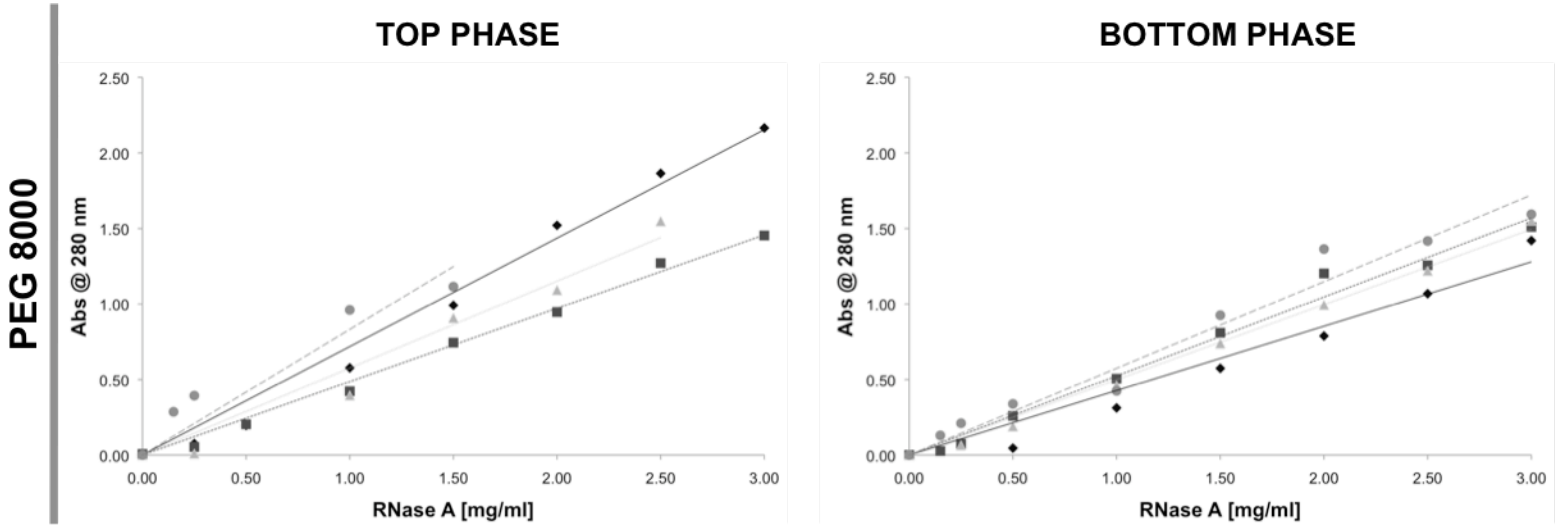
PEG 8000
TOP PHASE
BOTTOM PHASE
Abs @ 280 nm
RNase A [mg/ml]
0.00
0.50
1.00
1.50
2.00
2.50
3.00

FIGURE 1d

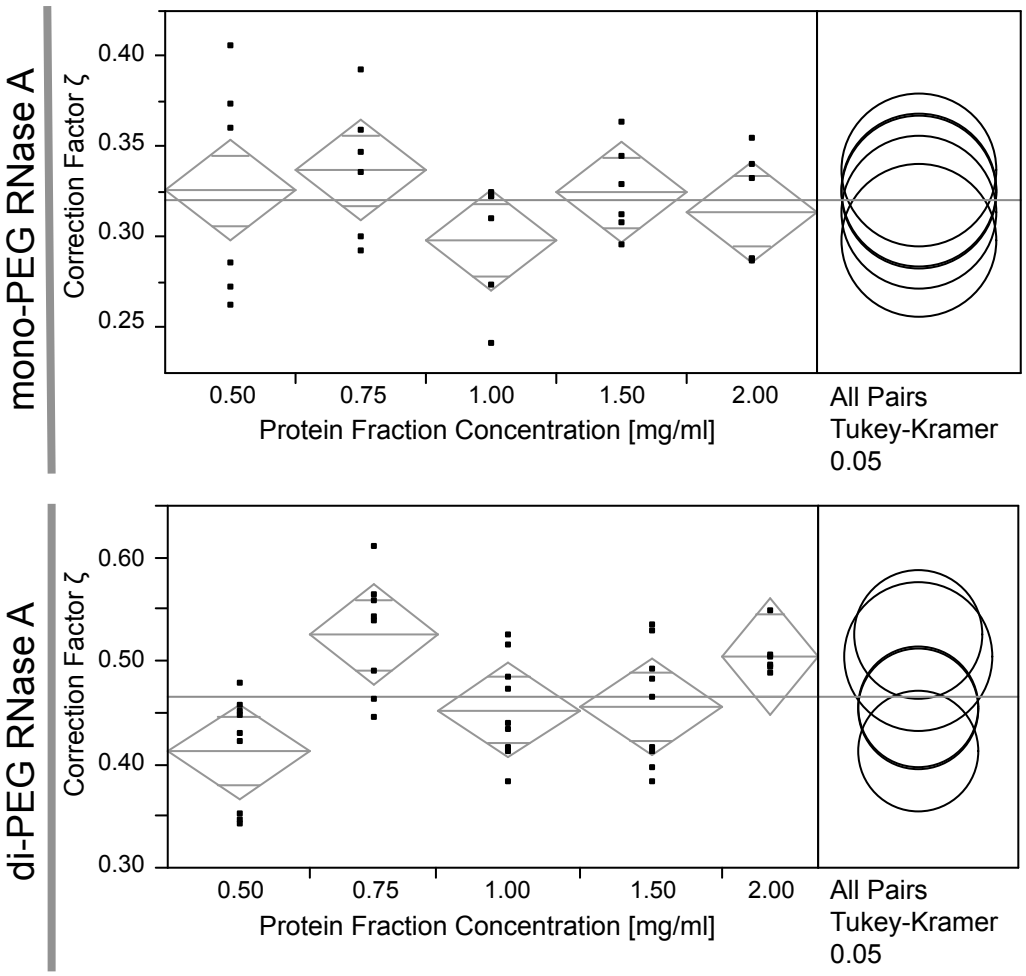
mono-PEG RNase A
Correction Factor ζ
0.40
0.35
0.30
0.25
0.50
0.75
1.00
1.50
2.00
Protein Fraction Concentration [mg/ml]
All Pairs
Tukey-Kramer
0.05
di-PEG RNase A
Correction Factor ζ
0.60
0.50
0.40
0.30
0.50
0.75
1.00
1.50
2.00
Protein Fraction Concentration [mg/ml]
All Pairs
Tukey-Kramer
0.05

FIGURE 2

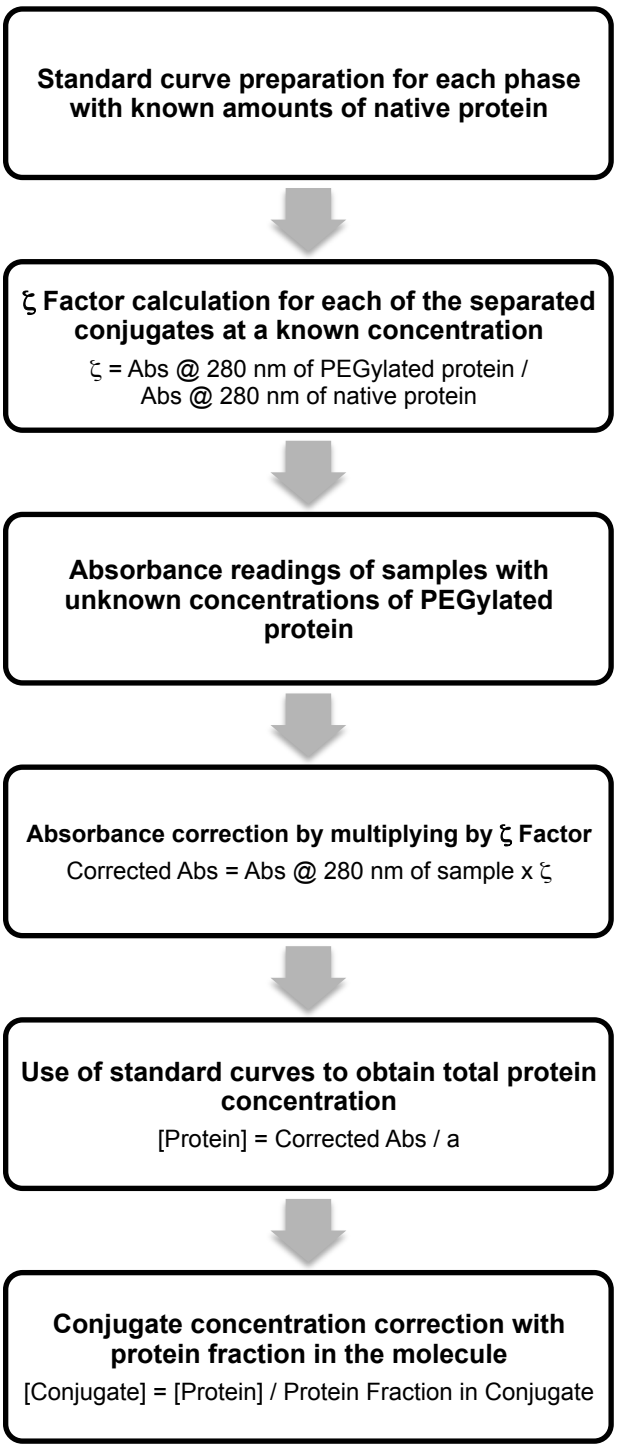
Standard curve preparation for each phase with known amounts of native protein
ζ Factor calculation for each of the separated conjugates at a known concentration
ζ = Abs @ 280 nm of PEGylated protein / Abs @ 280 nm of native protein
Absorbance readings of samples with unknown concentrations of PEGylated protein
Absorbance correction by multiplying by ζ Factor
Corrected Abs = Abs @ 280 nm of sample x ζ
Use of standard curves to obtain total protein concentration
[Protein] = Corrected Abs / a
Conjugate concentration correction with protein fraction in the molecule
[Conjugate] = [Protein] / Protein Fraction in Conjugate

FIGURE 3

Table 1. System characterization by polymer molecular weight and Tie Line Length (TLL) with global and phase PEG and phosphate compositions.

System	PEG MW (g/mol)	TLL (%,w/w)	Global		Top Phase		Bottom Phase	
			PEG (%, w/w)	PO_4 (%, w/w)	PEG (%, w/w)	PO_4 (%, w/w)	PEG (%, w/w)	PO_4 (%, w/w)
1	400	15	16.5	16.0	20.5	11.5	9.0	21.6
2		25	17.2	15.5	24.2	9.0	6.5	26.0
3		35	21.0	16.9	29.0	7.0	5.3	31.8
4		45	22.0	17.0	33.9	6.0	2.0	36.0
5	1 000	15	12.0	19.6	20.0	9.2	7.0	17.8
6		25	13.3	15.0	29.0	8.5	3.0	21.6
7		35	16.0	16.0	30.0	5.4	1.0	25.5
8		45	19.2	17.1	36.2	4.0	0.7	31.0
9	3 350	15	10.0	10.8	16.2	7.6	5.2	14.0
10		25	16.9	11.8	23.0	5.5	1.8	17.0
11		35	15.0	13.2	31.0	4.0	0.8	21.8
12		45	18.8	15.0	38.0	3.0	0.2	26.9
13	8 000	15	10.2	8.8	17.5	5.6	5.2	12.0
14		25	13.0	10.4	29.0	4.8	2.0	15.8
15		35	16.1	12.3	31.5	3.6	1.5	21.4
16		45	20.2	14.8	39.2	2.2	1.0	27.3

Systems were designed as described in section 2. TLL and compositions for each system were estimated using the binodal curves presented by Zaslavsky [20]

Table 2. Calculated extinction coefficients (a) of the standard curves for each phase in the 16 designed ATPS.

System	PEG MW (g/mol)	TLL % (w/w)	Top phase		Bottom phase	
			a ($mlmg^{-1}$ cm^{-1})	R^2	a ($mlmg^{-1}$ cm^{-1})	R^2
1	400	15	0.7899	0.9975	0.5483	0.9907
2		25	0.7598	0.9947	0.5877	0.9794
3		35	0.5950	0.9868	0.5512	0.9617
4		45	0.5934	0.9939	1.3273	0.9400
5	1 000	15	0.5512	0.9971	0.5163	0.9953
6		25	0.5363	0.9920	0.5243	0.9954
7		35	0.8141	0.9935	0.5392	0.9852
8		45	0.4157	0.9649	0.2553	0.9903
9	3 350	15	0.6241	0.9822	0.6345	0.9977
10		25	0.6224	0.9838	0.5998	0.9950
11		35	0.2893	0.9846	0.6011	0.9941
12		45	0.4998	0.9676	0.6006	0.9760
13	8 000	15	0.7182	0.9846	0.4266	0.9584
14		25	0.4860	0.9932	0.5234	0.9863
15		35	0.5758	0.9651	0.4983	0.9944
16		45	0.8321	0.8942	0.5741	0.9678

R^2 values were obtained by linear regression. The extinction coefficient of RNase A on 20 mM potassium phosphate pH 7 buffer was found to be 0.5271 with an R^2 value of 0.9996.

Potential application of Aqueous Two-Phase Systems for the fractionation of RNase A from its PEGylated conjugates

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Running Title: Fractionation of RNase A from its PEGylated conjugates using Aqueous Two-Phase Systems

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ABSTRACT

Native RNase A but mainly its two main PEGylated conjugates (mono-PEG RNase A and di-PEG RNase A) have demonstrated great therapeutic potential as antitumoral agents. Aqueous Two-Phase Systems (ATPS) have shown potential for the fractionation of native proteins from its PEGylated conjugates. In the present study the partition behavior of RNase A and its mono and di-PEGylated conjugates on polyethylene glycol (PEG) – potassium phosphate ATPS was characterized. Such partition behavior was then used to establish a potential strategy to separate unreacted native protein from the PEGylated species. The effect of PEG molecular weight (400 – 8 000 g/mol) and tie - line length (15 – 45% w/w) on native and PEGylated proteins partition behavior was studied. The use of ATPS constructed with low PEG molecular weight (400 g/mol) and high tie-line lengths (45% w/w) allowed the selective fractionation of native RNase A and its PEGylated conjugates on opposite phases. Such conditions resulted in an RNase A top phase recovery of 95%, while a 87% and 85% recovery yield was achieved for the mono and di-PEGylated conjugates, respectively, at the bottom phase. The results reported here demonstrated the potential application of ATPS for the fractionation of native RNase A from its PEGylated conjugates.

KEYWORDS: RNase A, PEGylation, partition, Aqueous Two-Phase Systems

1. INTRODUCTION

PEGylation was initially described in 1977 [1] and it is defined as the modification with at least one polyethylene glycol (PEG) chain of a peptidic or non-peptidic molecule without losing the properties of the target molecule [2]. PEGylation enhances the therapeutic properties of the modified molecules improving the pharmacokinetic parameters inside the human body [3,4]. In fact, over the last decades, new drugs developed using "PEGnology" with the aim to treat different kinds of cancer have gone into the market in an enormous international effort to better diagnose and treat this disease [5].

Drug PEGylation is usually carried out using mono-methoxy PEG (mPEG), which has only one reactive hydroxile group (which avoids the formation of crossed linked products) and has been approved for its use in pharmaceutical preparations [6]. The reaction must be made under conditions that maximize the addition of PEG chains to the N-terminal lysine of the molecule, since it is sought to minimize PEG interference with other aminoacid residues in the protein. This can be achieved using mPEG derivates in acidic pH conditions. This strategy is based on the difference in pKa values between the α-amino and ε-amino groups of the 10 lysine residues in the protein [7]. After reaction, purification of PEGylated proteins implies two main challenges: the separation of PEGylated conjugates from the rest of the reaction species and the subsequent fractionation of the PEGylated conjugates according to the number of PEG chains grafted to them and their positional isomerism [8].

Ribonuclease A (RNase A) is a small (13,686 Da) model enzyme [9] that has proven to have several potential therapeutical applications *in vivo* as an antitumoral agent mainly in its PEGylated form [10]. PEGylated RNase A becomes protected from Ribonuclease Inhibitor (RI) factors once it has been administrated thanks to the polymer chains grafted to it. These chains increase RNase A circulation time inside the body, greatly enhancing their

utilization due to their extended half-life time. Additionally, it has been found that the PEGylated RNase A binds fewer antibodies than its native form allowing it to increase its therapeutic effects [10]. For the particular case of RNase A, three main species result after PEGylation: mono-PEGylated RNase A, di-PEGylated RNase A and the residual native RNase A. From the two PEGylated conjugates mono-PEGylated RNase A exhibits the best therapeutic effects and has only one grafted chain to the N-terminal lysine of the protein. Di-PEGylated RNase A appears once the mono-PEGylated RNase A is synthesized and continues reacting by grafting another mPEG chain to one of its other 9 available lysines [11].

Most purification methods for PEGylated conjugates are based on molecular size. For example, size exclusion chromatography (SEC) fractionation is favored by the increment in the hydrodynamic radius of the PEGylated proteins that alters their elution times. However, this technique is incapable of separating positional isomers [12]. PEGylated proteins also present altered charge nature, hydrophobicity, metal chelation, hydrogen bonding, and affinity interactions [8]. Therefore other preparative and analytical separation methods like reversed-phase (RP), hydrophobic Interaction (HI), ion exchange (IEX) and immobilized metal affinity (IMA) chromatographies, and even gel electrophoresis may be exploited to separate these molecules [8,13]. However, these methods present their own disadvantages. For RP chromatography low resolution has been obtained while trying to separate the conjugates, producing at the same time structural changes due to the extreme hydrophobic environment [11]. HI chromatography presents as well a low processing capacity and little discrimination between adjacent peaks [14]. On its part, IEX chromatography separates PEGylated species due to the fact that a net charge change occurs with PEGylation. In fact, cationic interchange is a commonly used method because PEGylated proteins have one less

positive charge for each PEG chain linked to an amino group. However, PEG chains sterically interfere in the interactions of the charged residues producing a charge masking effect [14]. Nowadays, even with a large number of chromatographic approaches to separate PEG-proteins, ion exchange and size exclusion chromatography are the predominant separation methods used. Regardless the approach for the potential separation of the protein-polymer conjugates derived from a PEGylation reaction, a first step involves the initial separation of the unreacted proteins from its conjugates. In this context, aqueous two-phase systems (ATPS) represent an attractive alternative for this purpose.

ATPS is a liquid-liquid fractionation technique that has proved to have potential for the fractionation, recovery and partial purification of biological products, organelles and even whole cells [15-20]. ATPS are formed when two polymers (polyethylene glycol (PEG), dextran, polypropylene glycol, etc.) or one polymer and one salt (phosphate, sulfate, citrate, etc.) are combined over a certain critic concentration forming two phases whose main component is water. The use of polymer-salt systems is preferred over polymer-polymer systems mainly due to economical constraints. The main advantages of ATPS over other primary recovery and partial purification techniques include scaling up feasibility, process integration and intensification, capability and biocompatibility. System parameters such as polymer molecular weight, tie-line length (TLL, which represents the length of the line that connects the composition of the top and bottom phases in a phase diagram [16]) usually have a significant effect on the partition behavior of solutes and particles and thus the need to evaluate their effect in order to optimize partition in the systems [17].

Some works involving PEGylated proteins and their partition in ATPS have been published. Delgado *et al.* [21] compare the results of different studies on the partitioning of three PEGylated proteins (granulocyte-macrophage colony stimulation factor, bovine serum

albumin and immunoglobulin G) using NaCl-enriched PEG-Dextran ATPS. Their findings correlate log K_P values (where K_P, the partition coefficient, is defined as the ratio of the concentration of the molecule at the top and bottom phase) with the number of grafted polymer chains in each protein suggesting that a direct relation exists between log K_P and the polymer weight fraction in each PEGylated species. Sookkumnerd and Hsu [22] report the purification of PEG-lysozyme conjugates in PEG – potassium phosphate countercurrent distribution ATPS showing that it is possible to separate a mixture of unmodified, mono-PEGylated and di-PEGylated lysozymes through this technique.

The objective of the present study was to evaluate the effect of PEG-phosphate ATPS parameters such as PEG molecular weight (PEG MW) and tie-line length (TLL) on the partition behavior of native RNase A and its PEGylated conjugates. Additionally, system parameter conditions that allow a selective partition of native RNase A and its PEGylated conjugates in opposite phases were identified. A potential methodology regarding the recovery and partial purification of the PEGylated species exploiting ATPS is proposed.

2. MATERIALS AND METHODS

2.1 PEGylation reaction of RNase A and conjugate purification by size exclusion chromatography

The PEGylation reaction was done and separated according to the procedure reported by Cisneros-Ruiz *et al* [23]. Briefly, 2.0 ml of an RNase A (Sigma, MO, USA) solution at 3.0 mg/ml in a pH 5.1, 100 mM phosphate buffer with 20 mM sodium cyanoborohydride (Fluka, Switzerland) were added to a flask containing 30 g of 20 kDa mPEG propionaldehyde (Jen Kem Technologies, China). The mixture was vigorously stirred for 17 - 19 hr at 4 °C with a magnetic stirrer and then stored at the same temperature for up to 3

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days before chromatographic separation. PEGylation reaction mixtures were injected into an Äkta Explorer 100 (GE Healthcare, United Kingdom) size exclusion chromatographer equipped with a HiPrep™ 16/60 chromatographic column prepacked with Sephacryl™ S-300 High Resolution resin (GE Healthcare, United Kingdom) in order to separate each conjugate and the unreacted RNase A. Identification of the di-PEGylated, mono-PEGylated and native RNase A species from each SEC peak, obtained under the same chromatographic conditions, was previously conducted and reported by Cisneros-Ruiz *et al.* [23] using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). Fractions collected for each PEGylated conjugate were pooled and concentrated by ultrafiltration under nitrogen atmosphere using an Amicon® ultrafiltration cell 8050 (Amicon INC., MA, USA) with a 10 kDa Diaflo® ultrafiltration membrane (Amicon INC., MA, USA) until 25 ml of each concentrated fraction was obtained. Concentrated fractions were frozen at -80 °C in a REVCO Ultima II ultra low freezer (Thermo Fisher Scientific, MA, USA) and then transferred to a Sentry 2.0 lyophilizer (Virtis, NY, USA) at -80 °C and less than 100 mTorr until all water was removed. Dry purified fractions of mono and di-PEGylated RNase A were stored at -20 °C until used.

2.2 Partition behavior of RNase A, mono-PEGylated RNase A and di-PEGylated RNase A on polyethylene glycol – potassium phosphate Aqueous Two-Phase Systems.

16 PEG-potassium phosphate ATPS (**Table 1**) were constructed in order to study the effect of PEG MW and TLL upon the partition behavior of native RNase A, mono-PEGylated RNase A and di-PEGylated RNase A. Predetermined quantities of stock solutions of PEG (Sigma, MO, USA) of nominal molecular weights of 400, 1 000, 3 350 and 8 000 g/mol (50% w/w, except for PEG 400 which is liquid at room temperature and therefore was used

100% w/w), and a dipotassium hydrogen orthophosphate / potassium di-hydrogen orthophosphate (18:7, 40% w/w pH 7) solution (DEQ, Mexico) were mixed with bi-distilled water and 0.20 g of 10 mg/ml solutions of either RNase A, mono-PEG RNase A or di-PEG RNase A to give a final weight of 2.0 g per system. The partition behavior of RNase A, mono-PEGylated RNase A and di-PEGylated RNase A was characterized by separate. The stock solutions were mixed and the phases were dispersed by mixing for 10 min. Complete phase separation was achieved by centrifugation at 5 000 *g* for 10 min at 25 °C using an Eppendorf 5804R centrifuge (Eppendorf, Germany). TLL values of 15, 25, 35 and 45 % w/w were tested in the systems. V_R (volume ratio, defined as the relation between the volume of top and bottom phase) and pH were kept constant at 1 and 7 respectively. Visual estimates of the volumes of the top and bottom phases were made in graduated tubes. Samples were carefully extracted from the phases for protein quantification. In order to characterize the protein recovery yield for each system, the interphase was considered as part of the bottom phase. Recovery yield at the top and bottom phase was estimated as the amount of native protein or conjugate present in the phase (volume of the phase x protein concentration) and expressed relative to the original amount loaded into the system. Partition coefficient (K_P) was calculated as the protein concentration ratio at the top and bottom phases.

2.3 Quantification of RNase A and its PEGylated conjugates in polyethylene glycol – potassium phosphate Aqueous Two-Phase Systems

Quantification of RNase A and its PEGylated conjugates under ATPS conditions using colorimetric methods has exhibited several difficulties (data not shown). Therefore, quantification of native RNase A, mono-PEGylated RNase A and di-PEGylated RNase A

was carried out spectrophotometrically using an UV absorbance-based method previously developed in our research group [25]. Briefly, phase-specific standard curves for native RNase A (0.25-3.0 mg/ml) were prepared [25]. The effect of grafted PEG chains on UV absorbance (280 nm) of the RNase A PEGylated conjugates was accounted using a correction factor ζ, defined as the ratio between absorbance readings of the PEGylated conjugate and the absorbance readings of native RNase A (ζ = Abs @ 280 nm of PEGylated protein / Abs @ 280 nm of native protein) over a range of known concentrations. The estimated ζ value for mono and di-PEGylated RNase A is 0.32 and 0.46, respectively. Conjugate concentration in each phase was calculated by multiplying the absorbance at 280 nm of a sample by the correction factor ζ, and then dividing that value by the protein fraction of each conjugate (defined as RNase A molecular weight/PEGylated conjugate molecular weight; 0.4062 and 0.2549 for mono and di-PEGylated RNase A, respectively) [25].

2.4 Analytical and statistical procedures

All absorbance measurements were made in a Beckman Coulter DU800 Spectrophotometer (Beckman Instruments, CA, USA) using 1.5 ml PMMA cuvettes (VWR International, PA, USA). Protein was weighted in a Mettler-Toledo X564 analytical balance (Mettler-Toledo International, OH, USA). All experiments were carried out at least by triplicate. Results presented are the mean value of the independent replicas, and the standard error was calculated as the standard deviation divided for the square root of the number of replicas.

3. RESULTS AND DISCUSSION

The partition behavior of RNase A on the selected ATPS is depicted in **Table 2**. It is clear that RNase A exhibited a top phase preference when the lowest molecular weight of PEG (i.e. PEG MW 400) was used. An increase in TLL in PEG 400 – potassium phosphate systems increased the top phase preference of the native RNase A. As a result the top phase protein recovery increases from 69% to 94%. As TLL increases, PEG concentration in the top phase and potassium phosphate concentration in the bottom phase increase. These changes alter the free volume available in each phase for protein partition, alter the surface tension at the interphase and the density of the phases [15]. These effects change the partition behavior of native RNase A specifically promoting it to the top phase.

An increase in PEG MW causes a shift of native RNase A partition towards the bottom phase (see **Table 2** and **Figure 1**). As PEG MW increments, the hydrophobicity of the phase increases. Since RNase A is a highly hydrophilic protein [9], it is then expected to find greater protein concentrations at the bottom phase as PEG MW increases. This behavior may also be explained in terms of the free volume available in each phase. As PEG chain lengths increase, free volume decreases generating steric effects that change protein partition towards the bottom phase. RNase A exhibited bottom phase preference when ATPS of PEG molecular weight of 1 000 g/mol or higher were used (systems 5 to 16 in **Table 2**). In these cases an increase in TLL did not change the phase preference of the protein. In fact, only for the specific case of PEG 400 systems an increment in RNase A recovery yield at the top phase was observed as TLL increased. For the rest of the systems (PEG 1 000, 3 350, and 8 000 g/mol) as TLL increased, a mixed effect was observed probably due to the mixture of hydrophobic, ionic charge and free volume interactions with RNase A. In general, for systems of PEG 1 000, 3 350 and 8 000 g/mol bottom phase

recoveries higher than 59% were obtained. In the particular case of system 8 (PEG 1 000 g/mol, TLL 45%), interestingly, a high top phase recovery yield (75.3%) was obtained. This behavior may indicate that salt concentration at the bottom phase is high enough to counteract the effect of excluded volume in the top phase, shifting protein partition towards such phase.

The partition behavior of mono-PEGylated RNase A is presented in **Table 3**. For all the systems selected, mono-PEGylated RNase A exhibited a bottom phase preference. Regardless of PEG MW and TLL, high bottom phase protein recovery (> 70%) was obtained. Since ATPS are highly hydrophilic, the increment in hydrophobicity granted by the grafted PEG chains promotes the partition of the conjugates towards the bottom phase (particularly towards the interphase, considered part of the bottom phase) [26]. Additionally, the grafted PEG chain of the mono-PEGylated RNase A increases significantly its molecular size when compared with native RNase A. Therefore, the behavior observed may be as well explained on the basis of the available free volume in each phase. PEG MW seems to have no extensive effect on mono-PEG RNase A recovery in the bottom phase. TLL increments in each of the different PEG MW studied did not present a clear effect except for PEG 400 systems where as TLL increases a minor protein partition increment towards the top phase is observed.

Table 4 presents the recovery percentages and the logarithm of the partition coefficients ($\ln K_P$) of di-PEGylated RNase A. As for the mono-PEGylated RNase A most conjugate is recovered in the bottom phase of the system, regardless PEG MW and TLL. However, recovery yields in the top PEG rich phase are slightly higher than those of the mono-PEGylated conjugate. Once again, the observed partition behavior may be explained in terms of phase hydrophobicity and available free volume.

However, as the number of chains linked to the protein increase, it is expected that partition behavior on ATPS may be influenced by free energy considerations that involve net interactions with the polymer rich regions in the system and within the conjugate. These interactions vary depending on the characteristics of the polymer and its concentration [21]. Previous PEGylated protein partition studies in PEG – dextran ATPS show that partition coefficients increase exponentially with the amount of PEG bound to the protein and that a linear relationship between log K_P values and the number of grafted PEG chains is preserved [27]. For these previously reported systems, an increase in partition towards the top phase is observed as the number of PEG chains increases accounting the modified amino groups in each protein. The proteins tested in these previous reports are granulocyte-macrophage colony stimulation factor, bovine serum albumin and immunoglobulin G (all with a large amount of PEGylation sites) and it has been found that the proportionality between log K_P and the number of PEG molecules attached is only compromised for conjugates having relatively large number of grafted PEG molecules in biphasic systems of high concentration of polymers, where a partial precipitation of the conjugates towards the dextran rich phase is observed [27]. This effect cannot be whatsoever compared in PEG – potassium phosphate systems by the study of the increment in TLL values and thus in the increments of the concentrations of the system components. It must also be noted that grafted PEG chains in proteins may interact with one another, with water molecules, ions and might as well form hydrogen bonds [21]. All these possible interactions as the kind of ATPS involved (i.e. polymer – polymer or polymer – salt) may also have an impact on the fractionation of each of the conjugates.

The particular effect of PEG MW on ln K_P of each studied specie at TLL 45% w/w is depicted in **Figure 1**. As it is seen, at PEG MW 400 g/mol, the ln K_P value for native

RNase A has the highest positive value (i.e. 2.5779) indicating its preference for the top phase, while the conjugates present both a negative value and thus showing a preference towards the bottom phase. Therefore, in order to achieve a selective partition of native RNase A from its PEGylated conjugates, it becomes evident that low PEG MW (400 g/mol) systems must be selected. System 4 (PEG 400, TLL 45%) allows the recovery of most of the native RNase A at the top phase (i.e. 95%), while in the bottom phase mono and di-PEGylated conjugates are recovered with yields superior to 84%.

A practical approach to fractionate native RNase A from its mono and di-PEGylated conjugates may involve the use of ATPS of low PEG MW and high TLL (system 4 in **Table 1**). **Figure 2** presents a simplified scheme of the proposed strategy where the expected final top and bottom phase recovery yields of each of the three species are reported. The results reported here, highlight the advantages of the potential application of an ATPS approach to establish the conditions for a single stage separation of RNase A and its conjugates. To date, no previous studies known by the authors have been reported that exploit the potential use of ATPS for this purpose.

4. CONCLUSIONS

PEG MW and TLL have significant effect on the partition behavior of native RNase A and its PEGylated conjugates on polymer – salt ATPS. Native RNase A can be predominantly recovered from the top phase of low PEG MW ATPS while mono and di-PEGylated RNase A have a preference to partition towards the bottom phase regardless of PEG MW and TLL. The characterization of the partition behavior of these species on ATPS resulted in the establishment of a potential strategy to fractionate unreacted native RNase A from its polymer conjugates in a PEGylation reaction mixture. A PEG 400 g/mol, TLL 45%, V_R 1

CONCLUSIONS AND RECOMMENDATIONS

It has been found that in ATPS environments, polyethylene glycol (PEG) and potassium phosphate, at particularly high concentrations, have a significant effect upon RNase A UV absorbance. Furthermore, interactions between the amount of protein dissolved and the concentration of the polymer and the salt in each system have a direct impact on the spectrophotometric readings. Therefore, solvent-specific standard curves are required in order to quantify RNase A on ATPS environments. It was also found that grafted polymer chains on mono and di-PEGylated RNase A induce conformational shifts that affect UV light absorbance on the conjugate. For this reason, a correction factor (ζ, defined as the ratio of absorbance at 280 nm between the PEGylated and native protein), is needed in order to account the effect of grafted PEG molecules. Together with the previously prepared standard curves, the correction factor enabled the correct quantification of the studied species in ATPS with an easy-to implement methodology.

The partition behavior of native RNase A, mono-PEGylated RNase A and di-PEGylated RNase A in selected ATPS was independently studied. Native RNase A can be recovered at the top phase in PEG 400 g/mol systems and it was found for this particular polymer molecular weight that as TLL increases the recovery percentage at the top phase of this enzyme increases as well. For the polymer conjugates, an increase in their hydrophobicity promotes their fractionation towards the bottom phase, but at low PEG MW values, a high TLL percentage promotes an increase in upper phase recovery and vice versa. With these findings, it is concluded that a low PEG MW system (i.e. 400 g/mol) with TLL 45 %, V_R 1 and pH 7, can be used to effectively separate native RNase A from its PEGylated

conjugates at different phases. Theoretically by using this system 94.94 % of the loaded native RNase A can be recovered at the top phase while 87.25% of the mono-PEGylated RNase A and 84.61% of the di-PEGylated RNase A can be recovered at the bottom phase. With this, ATPS have shown to be an effective technique to achieve the primary separation of a native protein from its polymer conjugates.

Further studies involving size exclusion chromatography will allow the validation of the recovery yields reported in this work by reproducing and quantifying potential selected systems trough this technique. This approach would prove that the system chosen to separate native RNase A from its PEGylated conjugates has indeed the best selection of parameters to achieve this. The comparison between the partition behavior in polymer – salt ATPS of RNase A and its polymer conjugates with other PEGylated models will permit a fuller comprehension of the interactions between the system parameters and the solutes in the systems. On the other hand, the potential of using polymer – polymer systems such as PEG – dextran ATPS or countercurrent distribution ATPS in order to fully separate each of the PEGylated species should be studied to achieve better primary separations.

and pH 7 system facilitates the recovery of 94.94% of the total native RNase A from the top phase while showing bottom phase recovery yields of 87.25% and 84.61% for mono and di-PEGylated RNase A, respectively. The potential of PEG – potassium phosphate ATPS for the selective fractionation of native RNase A from its PEGylated conjugates was demonstrated.

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FIGURE CAPTIONS

FIGURE 1. Effect of PEG MW on ln K_P of RNase A, mono-PEG RNase A and di-PEG RNase fractionation in TLL 45% w/w PEG – potassium phosphate Aqueous Two-Phase Systems. ln K_P values for native RNase A (◆), mono-PEG RNase A (■) and di-PEG RNase A (▲) are shown for PEG MW 400, 1 000, 3 350 and 8 000 g/mol systems with TLL 45% w/w. V_R and pH were kept at 1 and 7 respectively.

FIGURE 2. Simplified scheme for the ATPS strategy to selectively fractionate native RNase A from its PEGylated conjugates. Hypothetically, after reaction mixtures of the native RNase A, mono-PEG RNase A and di-PEG RNase A can be separated with the shown recovery yields.

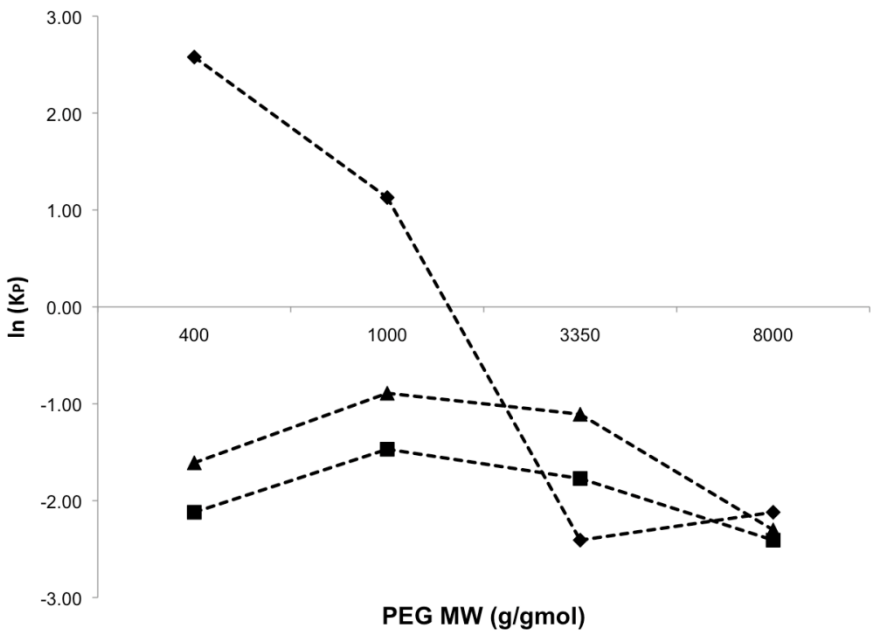
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
ln (Kp)
400
1000
3350
8000
PEG MW (g/gmol)

FIGURE 1

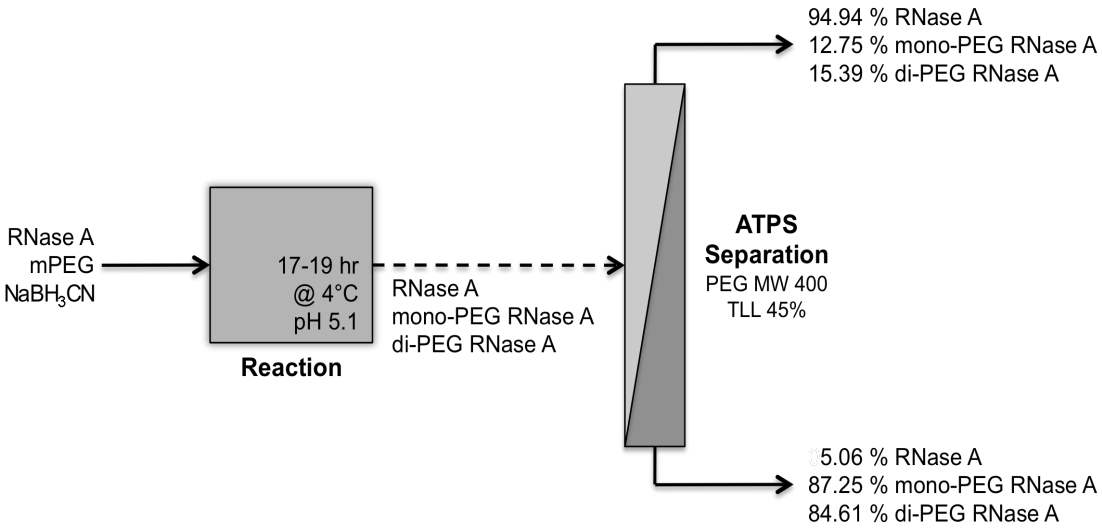
94.94 % RNase A
12.75 % mono-PEG RNase A
15.39 % di-PEG RNase A
RNase A
mPEG
NaBH3CN
17-19 hr
@ 4°C
pH 5.1
Reaction
RNase A
mono-PEG RNase A
di-PEG RNase A
ATPS
Separation
PEG MW 400
TLL 45%
5.06 % RNase A
87.25 % mono-PEG RNase A
84.61 % di-PEG RNase A

FIGURE 2

Table 1. Composition of selected Aqueous Two-Phase Systems polyethylene glycol-potassium phosphate for the fractionation of native RNase A and its PEGylated conjugates.

System	PEG MW (g/mol)	TLL (%, w/w)	PEG (%, w/w)	PO_4 (%, w/w)
1	400	15	16.5	16.0
2		25	17.2	15.5
3		35	21.0	16.9
4		45	22.0	17.0
5	1 000	15	12.0	19.6
6		25	13.3	15.0
7		35	16.0	16.0
8		45	19.2	17.1
9	3 350	15	10.0	10.8
10		25	16.9	11.8
11		35	15.0	13.2
12		45	18.8	15.0
13	8 000	15	10.2	8.8
14		25	13.0	10.4
15		35	16.1	12.3
16		45	20.2	14.8

Systems were designed as described in section 2. For all systems, V_R was kept constant at 1. TLL and the composition of each system were estimated using the binodal curves presented by Zaslavsky [24].

Table 2. Effect of polyethylene glycol molecular weight (PEG MW) and tie-line length (TLL) upon the partition coefficient and recovery yield of RNase A in polyethylene glycol-potassium phosphate Aqueous Two-Phase Systems.

System	PEG MW (g/mol)	TLL (%, w/w)	ln (K_P)	Recovery yield (%, w/w)	
				Top Phase	Bottom Phase
1	400	15	0.5653	69.65 ± 4	30.35 ± 0
2		25	0.5766	72.54 ± 2	27.46 ± 1
3		35	2.4257	94.29 ± 2	5.71 ± 0
4		45	2.5779	94.94 ± 1	5.06 ± 1
5	1 000	15	-0.5621	35.19 ± 1	64.29 ± 3
6		25	-0.4005	40.52 ± 0	59.48 ± 1
7		35	-0.8440	26.46 ± 0	61.71 ± 1
8		45	1.1282	75.30 ± 1	24.38 ± 1
9	3 350	15	-1.7148	12.26 ± 1	75.15 ± 1
10		25	-2.6593	5.32 ± 1	83.20 ± 1
11		35	-1.7148	14.91 ± 0	85.09 ± 1
12		45	-2.4079	7.13 ± 1	83.91 ± 1
13	8 000	15	-2.9957	4.95 ± 1	95.05 ± 2
14		25	-4.6052	0.53 ± 0	98.57 ± 2
15		35	-3.5066	2.93 ± 1	92.56 ± 1
16		45	-2.1203	9.75 ± 2	77.11 ± 7

For all systems, V_R and pH were kept constant at 1.0 and 7.0 respectively.

Table 3. Effect of polyethylene glycol molecular weight (PEG MW) and tie-line length (TLL) upon the partition coefficient and recovery yield of mono-PEGylated RNase A in polyethylene glycol-potassium phosphate Aqueous Two-Phase Systems.

System	PEG MW (g/mol)	TLL (%, w/w)	ln (K_P)	Recovery yield (%, w/w)	
				Top Phase	Bottom Phase
1	400	15	-2.4079	9.48 ± 0	90.52 ± 0
2		25	-2.3026	10.94 ± 0	89.06 ± 0
3		35	-2.1203	12.53 ± 0	87.47 ± 0
4		45	-2.1203	12.75 ± 0	87.25 ± 0
5	1 000	15	-1.6607	14.89 ± 1	85.11 ± 0
6		25	-1.6607	15.32 ± 0	84.68 ± 0
7		35	-2.1203	10.93 ± 1	89.07 ± 0
8		45	-1.4697	19.45 ± 1	80.55 ± 0
9	3 350	15	-1.9661	11.81 ± 1	88.19 ± 0
10		25	-1.7148	13.63 ± 1	86.37 ± 0
11		35	-0.8210	29.08 ± 0	70.92 ± 0
12		45	-1.7720	15.33 ± 0	84.67 ± 0
13	8 000	15	-2.4079	9.21 ± 0	90.79 ± 0
14		25	-1.7720	14.08 ± 1	85.92 ± 0
15		35	-2.3026	8.91 ± 0	91.09 ± 0
16		45	-2.4079	8.96 ± 0	91.04 ± 1

For all systems, V_R and pH were kept constant at 1.0 and 7.0 respectively.

Table 4. Effect of polyethylene glycol molecular weight (PEG MW) and tie-line length (TLL) upon the partition coefficient and recovery yield of di-PEGylated RNase A in polyethylene glycol-potassium phosphate Aqueous Two-Phase Systems.

System	PEG MW (g/mol)	TLL (%, w/w)	ln (K_P)	Recoveryyield (%, w/w)	
				Top Phase	Bottom Phase
1	400	15	-2.0402	10.73 ± 0	89.27 ± 0
2		25	-2.2073	10.04 ± 0	89.96 ± 0
3		35	-1.6607	14.36 ± 0	85.64 ± 0
4		45	-1.6094	15.39 ± 0	84.61 ± 0
5	1 000	15	-1.2379	21.43 ± 0	78.57 ± 0
6		25	-1.1712	23.37 ± 0	76.63 ± 0
7		35	-1.7148	16.87 ± 0	83.13 ± 0
8		45	-0.8916	28.49 ± 0	71.51 ± 0
9	3 350	15	-1.5606	17.36 ± 0	82.64 ± 0
10		25	-1.3093	20.53 ± 1	79.47 ± 0
11		35	-0.1508	46.64 ± 3	53.36 ± 0
12		45	-1.1087	26.22 ± 1	73.78 ± 0
13	8 000	15	-2.0402	13.01 ± 1	86.99 ± 1
14		25	-1.5141	18.34 ± 5	81.66 ± 2
15		35	-1.5141	17.69 ± 0	82.31 ± 1
16		45	-2.3026	9.16 ± 0	90.84 ± 0

For all systems, V_R and pH were kept constant at 1.0 and 7.0 respectively.

CONCLUSIONS AND RECOMMENDATIONS

It has been found that in ATPS environments, polyethylene glycol (PEG) and potassium phosphate, at particularly high concentrations, have a significant effect upon RNase A UV absorbance. Furthermore, interactions between the amount of protein dissolved and the concentration of the polymer and the salt in each system have a direct impact on the spectrophotometric readings. Therefore, solvent-specific standard curves are required in order to quantify RNase A on ATPS environments. It was also found that grafted polymer chains on mono and di-PEGylated RNase A induce conformational shifts that affect UV light absorbance on the conjugate. For this reason, a correction factor (ζ, defined as the ratio of absorbance at 280 nm between the PEGylated and native protein), is needed in order to account the effect of grafted PEG molecules. Together with the previously prepared standard curves, the correction factor enabled the correct quantification of the studied species in ATPS with an easy-to implement methodology.

The partition behavior of native RNase A, mono-PEGylated RNase A and di-PEGylated RNase A in selected ATPS was independently studied. Native RNase A can be recovered at the top phase in PEG 400 g/mol systems and it was found for this particular polymer molecular weight that as TLL increases the recovery percentage at the top phase of this enzyme increases as well. For the polymer conjugates, an increase in their hydrophobicity promotes their fractionation towards the bottom phase, but at low PEG MW values, a high TLL percentage promotes an increase in upper phase recovery and vice versa. With these findings, it is concluded that a low PEG MW system (i.e. 400 g/mol) with TLL 45 %, V_R 1 and pH 7, can be used to effectively separate native RNase A from its PEGylated

conjugates at different phases. Theoretically by using this system 94.94 % of the loaded native RNase A can be recovered at the top phase while 87.25% of the mono-PEGylated RNase A and 84.61% of the di-PEGylated RNase A can be recovered at the bottom phase. With this, ATPS have shown to be an effective technique to achieve the primary separation of a native protein from its polymer conjugates.

Further studies involving size exclusion chromatography will allow the validation of the recovery yields reported in this work by reproducing and quantifying potential selected systems trough this technique. This approach would prove that the system chosen to separate native RNase A from its PEGylated conjugates has indeed the best selection of parameters to achieve this. The comparison between the partition behavior in polymer – salt ATPS of RNase A and its polymer conjugates with other PEGylated models will permit a fuller comprehension of the interactions between the system parameters and the solutes in the systems. On the other hand, the potential of using polymer – polymer systems such as PEG – dextran ATPS or countercurrent distribution ATPS in order to fully separate each of the PEGylated species should be studied to achieve better primary separations.