INSTITUTO TECNOLÓGICO Y DE ESTUDIOS
SUPERIORES DE MONTERREY

CAMPUS MONTERREY

DIVISIÓN DE INGENIERÍA
PROGRAMA DE GRADUADOS EN INGENIERÍA

CHROMATOGRAPHIC SEPARATION OF CONJUGATES POLYMER - PROTEIN

TESIS
PRESENTADA COMO REQUISITO PARCIAL PARA
OBTENER EL GRADO ACADÉMICO DE

DOCTORA EN CIENCIAS DE INGENIERÍA

POR

MAYRA CISNEROS RUIZ

MONTERREY, N.L. DICIEMBRE 2006

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Los miembros del comité de tesis recomendamos que el presente proyecto de tesis presentado por Mayra Cisneros Ruiz sea aceptado como requisito parcial para obtener el grado académico de:

DOCTORA EN CIENCIAS DE INGENIERÍA

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Diciembre 2006

ABSTRACT

The attachment of polyethylene glycol (PEG) molecules, called PEGylation, can improve the therapeutic properties of proteins. The PEGylation product depends on the conditions under which the conjugation reaction takes place. PEGylation reactions often result in a population of conjugate species in terms of the number of attached PEG chains and their locations. As some portion of this population may be biologically inactive the resolution of these protein mixtures represents a challenge to the separation step. Currently, the methods to purify PEGylated proteins have been dominated by size exclusion chromatography (SEC) and ion exchange chromatography (IEX). Research works describing the use of the conjugate hydrophobicity for separation are not very common. It is clear that hydrophobic interaction chromatography (HIC) and reversed phase chromatography (RPC) have not been fully investigated in the past as separation methods for the resolution of PEGylated proteins.

This thesis is focused on the analysis of the chromatographic behavior of PEGylated proteins in RPC and the potential use of a mild hydrophobic support combining HIC and aqueous two phase extraction (ATPE) principles. Two proteins were selected as experimental models: ribonuclease A (RNase A) and apo-αlactalbumin (apo-αLac). Both proteins were reacted with an activated PEG with a nominal molecular weight of 20 kDa and the reaction mixtures were analyzed by SEC and mass spectrometry. The structure of the PEGylated proteins was analyzed, showing that the attachment of PEG molecules did not modify the structure of the proteins.

Reverse phase chromatography (RPC) under neutral pH conditions was used to resolve the populations of PEGylated conjugates. PEG-conjugates were separated with better resolution and in less time using RPC at neutral pH rather than using SEC. RPC also allowed the identification of a tri-PEGylated species produced during the reaction with RNase A; and not identify when SEC was used. The results showed that it is possible to separate PEGylated species by RPC at neutral pH.

Changes of pH (at 2.0, 7.0 and unbuffered) of the mobile phase, showed that the pH does not play a significant role in the chromatographic behavior of PEG-conjugates, when the unmodified protein is not retained. However, when the unmodified protein is retained, the effect of the pH on the PEGylated proteins is similar to that observed for the unmodified species. It was demonstrated that temperature affects the chromatographic separation of PEG-conjugates in a similar manner in which it affects the separation of the neat polymer.

A novel approach to potentially separate PEGylated proteins from the unmodified form using a mild hydrophobic support in which PEG is immobilized in sepharose was addressed. Different behavior retention of the native protein from the PEGylated species was achieved using a gradient elution between 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 and 25 mM potassium phosphate, pH 7.0. Parameters such as pH, salt type and salt concentration had no significant influence on chromatographic behavior of native, mono-PEGylated and di-PEGylated RNase A using this separation system. The proposed approach described here provides a simple and practical chromatographic method to separate unmodified proteins from their PEG conjugates.

ACKNOWLEDGEMENTS

I would like to express my gratitude to many people who made this effort possible.

My husband Luis Carlos, who supported me during my long days of study and lab work. I am eternally indebted to him for being a constant source of love and support not only throughout my graduate studies, but every day since I met him.

My mother, who has taught me how to overcome any obstacle and achieve anything through hard work. She taught me to put a large value on my education. I am in deeply indebted to her for her support and love.

My sister, my brother in law and my nieces, for their love and support every day of my life.

Dr. Marco A. Rito Palomares who introduced me to the great world of research. I am grateful to him for his constant supervision, advice, and guidance from the very early stage of my graduate studies. I am indebted to him for his support and to encourage me to take the internship at CMU.

Dr. Todd M. Przybycien who gave me the chance of being part of his research group. During my internship at CMU, I greatly benefited from his technical expertise and outstanding

research skills. Thank you for the great opportunity to work with an excellent researcher and person.

My committee members, Dr. Manuel Zertuche, Dra. Blanca Lapizco and Dr. Alberto Mendoza, for their time and effort in reviewing this work.

All the people at the ITESM Biotechnology lab who made my journey as a graduate student more enjoyable. Many thanks in particular to Jorge Benavides and Alex Aguilar for the scientific discussions and for letting me have such a pleasant time when working together.

All people at the CMU PTC, specially the members of the Przybycien group who really made my time at CMU PTC more enjoyable. In particular, many thanks to Abby Laurent who helped me in many ways during my stay. Also to Andy Kim, for his friendship and for helping me to improve my English with our little talks in the office.

Francisco M. Vargas, for his friendship during all these years. I still remember our long conversations about why graduate studies were important. Thank you for your advice.

To all my friends who always showed me their support, especially to Gaby Angel, Mayela Villa and Magali Morales.

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Chapter 1.- Introduction

Recent advances in biotechnology have led to the therapeutic use of peptides and proteins for numerous diseases. In general, the main steps in the large-scale production of therapeutic proteins include fermentation (upstream processing), in which the target product is produced and bioseparation (downstream processing), in which the target protein is isolated from cellular and growth media components. The bioseparation part of a biotechnological process can then be subdivided in two stages: primary recovery, including cell separations and concentration (and if the product is intracellular, this stage also involves cell disruption and debris removal); and purification, including primary isolation, high resolution purification and polishing of the final product (Lienqueo and Asenjo, 2000).

Bioseparation is critical for the development of modern biotechnology. The major production costs of many biological products may be attributed to the bioseparations stages of a process (Raghavarao, 2002). These stages can be complicated and the products are typically labile and thus need "gentle" processing conditions (Lightfoot and Moscariello, 2004). However, even the best protein production process cannot guarantee bioefficacy. The *in vivo* effectiveness of therapeutic proteins may be reduced by their susceptibility to destruction by proteolytic enzymes, short circulating half-life, short shelf-life, low solubility, rapid kidney clearance and their ability to generate neutralizing antibodies (Harris and Chess, 2003).

The covalent attachment of polymers, such as polyethylene glycol (PEG) or dextran, to therapeutic proteins, can diminish these challenges. The attachment of PEG chains is most common and can permit the alteration and control of a protein-based drug's biodistribution, pharmacokinetics, and, occasionally, toxicity (Zalipsky, 1995).

1.1 Significance of PEGylation

The term PEGylation has been used since 1977, when Abuchowki, David and collaborators developed a method for the covalent attachment of one or several PEG molecules to peptides and proteins (Greenwald et al, 2003). PEG is a poly-ether with a hydroxyl at each extreme. The most common modification is the use of mono-methoxy PEG (mPEG). Its structure is shown in Figure 1.1 (Roberts M J *et al.,* 2002).

$$CH_3O-(CH_2CH_2O)_n-CH_2CH_2-OH$$

Figure 1.1. mPEG structure (Roberts M J *et al.,* 2002).

mPEG can be dissolved in aqueous solutions and organic solvents. It is nontoxic and is eliminated from the body through a combination of renal and hepatic processes. These properties make it ideal to be used in pharmaceutical applications (Greenwald *et al.*, 2003).

PEGylation increases the protein circulation time in the blood, reduces immunogenicity and antigenicity, and reduces toxicity (Bailon and Berthold, 1998). During the last several years, the FDA has approved PEGylated proteins to treat different diseases in humans, including bovine adenosine deaminase (Adagen manufactured by Enzon Pharmaceuticals, 5,000 Da

methoxyPEG attached) for severe combined immunodeficiency disease, α interferon (PEGasys manufactured by Roche; PEGintron maufactured by Schering;12,000 Da PEG attached) for hepatitis C, L-asparaginase (Oncaspar manufactured by Enzon Pharmacueticals, 5,000 Da methoxy-PEG attached) for acute lymphoblastic leukemia (Greenwald *et al.*, 2003), granulocyte colony-stimulating factor (Neulasta manufactured by Amgen, 20,000 Da methoxy-PEG attached) to stimulate white blood cell production in chemotherapy patients and a growth hormone antagonist (Somavert manufactured by Pfizer, 5,000 Da PEG attached) to treat acromegaly.

There are different chemistries used to attach activated PEG molecules to polypeptides. The most common reactive groups involved in the coupling to proteins are nucleophiles, with the following moieties ranked in decreasing order of reactivity: thiol, alpha amino group, epsilon amino group, carboxylate and hydroxylate. However, this order also depends on the reaction pH and steric accessibility. Furthermore, other residues, such as the imidazole group of histidine, may react under special conditions, (Veronese, 2001). The composition of the product is dependent on the number and local reactivity of the available attachment sites (amino and sometimes other nucleophilic groups), on the starting polypeptide, on the reactivity of the activated PEG reagent and on the conditions of the modification reaction (Zalipsky, 1995). Thus, conjugation may result in the generation of a family of variants characterized by a distribution in number and position of attached PEG groups.

All PEGylated variants are not equal in their efficaciousness (Youn *et al.*, 2004; Lee and Park, 2002). As a result, it is desirable to resolve heterogeneous conjugate mixtures into effective

subpopulations via separation technologies (Koumenis *et al.*, 2000 and Vicentelli *et al.*, 1999) and/or to limit the breadth of the conjugate distribution during the conjugation reaction itself (Deiters *et al.*, 2004). Hybrid approaches such as size exclusion reaction chromatography, which combine reaction and separation unit operations, are also under development (Fee, 2003). It is anticipated that initial solutions at scale will likely be focused on the population resolution alternative.

1.2 Issues in separation of PEGylated proteins

The purification of PEGylated proteins consists in removing all species that are not part of the target product. Purification of PEGylated proteins involves two basic challenges: 1) the separation of PEG-proteins from other reaction products and 2) the sub-fractionation of PEG-proteins on the basis of their degree of PEGylation and positional isomerism. These challenges can be complicated by several factors related to the nature of PEG polymers. Additionally, PEG-proteins are hybrid molecules, and their properties may not necessarily mimic that of either proteins or PEG molecules.

In general, the methods to purify PEGylated proteins are dominated by molecular size-based separations, especially size exclusion chromatography (SEC). SEC has been traditionally used in the separation of PEGylated proteins. Grafted PEG chains increase a protein's hydrodynamic radius, reducing accessibility of the pore space of porous media (with appropriately-sized pores) and altering elution times. However, it has been reported (Pesek *et al.*, 2004) that size differences among the species are insufficient to separate positional conjugate isomers. Additionally, with the addition of successive PEG chains to the protein,

the relative difference in molecular size between the individual species decreases considerably, resulting in lower chromatographic resolution (Snider *et al.*, 1992). Ultrafiltration and diafiltration are used less commonly and mainly for concentration or buffer exchange rather than separation of native and PEGylated forms of proteins (Fee and Van Alstine, 2006).

Another technique commonly used in separation of PEGylated proteins is ion exchange chromatography (IEX). The most common IEX method is cation exchange since the PEGylated proteins contain one less positive charge per each attached PEG molecule when the proteins are PEGylated at amine groups. Additionally, PEG chains can sterically interfere with the interaction of charged residues on the protein with the ion-exchange support (Snider *et al.*, 1992). It is clear that this technique depends on the difference in charge between the unmodified protein and the PEG-conjugates, which is not always sufficient to ensure a predictable separation on basis of charge (Fee and Van Alstine, 2006).

Very little work has been done on utilizing conjugate hydrophobicity for separation. Hydrophobic interaction chromatography (HIC) and reversed phase chromatography (RPC) have not been fully investigated. These methods are of interest given that PEGylation should affect protein hydrophobicity. RPC is an attractive option to the resolution of PEG-protein conjugate populations at scale due to the pendant PEG chains of the conjugates that can function as hydrophobic tags and dominate conjugate interactions with hydrophobic surfaces (Daly *et al.*, 2005). RPC is a powerful separation technique that has been used in the resolution of proteins with small differences in physical properties, including single amino acid variants (Ahrer and Jungbauer, 2006). However, the preparative application of RPC is

limited when protein denaturation in the mobile phase and on the stationary phase appear together with many types of complex behaviors resulting in low recovery of the target product (Sokol *et al.*, 2003).

The effects of the RPC mobile phase on protein structure are not only caused by the organic modifier, but also by the low pH typically used in the process. In general, low pH (~2.0) maintained with trifluoroacetic (TFA) and formic acids are the traditional buffers. Their volatilities make them compatible with mass spectrometry detection and facilitate sample recovery after chromatographic separation. The pH of the mobile phase affects the separations, since the degree of ionization depends on the pH. It seems promising to study the effect of pH on the separation of PEGylated proteins, in order to determine if a less unfolded environment could be used during the separation without affecting the resolution.

Temperature is another parameter that could modify the retention behavior of proteins in reversed phase processes, especially if those proteins have attached PEG molecules. Previous studies have reported that the retention of PEGs increases with increasing column temperatures (Skuland *et al.*, 2003). It is of interest then to study the effect of the temperature on the separation of PEGylated protein, in order to determine if it is possible to improve the resolution of the species through temperatures changes.

The combination of aqueous two-phase extraction (ATPE) and HIC procedures has been used to develop new mild hydrophobic ligands less denaturing to proteins than those utilized in traditional HIC (Diogo *et al.*, 1999). ATPE is a technique extensively described by Albertsson (1986) that exploits mild hydrophobic interactions. It is considered a simple technique,

scalable and biocompatible for the recuperation of biotechnological products, due to its high water content and low interfacial tension. The systems are composed of either two polymer phases or a polymer and a salt phase. ATPE is a liquid-liquid partition method where the separation is based on the differential partitioning of the solutes between the phases. In order to get good separations, it is essential to optimize the partition coefficient of each component (concentration in upper phase divided by concentration in lower phase, *K*) through the appropriate selection of the system composition (Rito-Palomares, 2004).

It seems logical that an approach that involves the principles of the ATPE partition technique in a chromatographic format would be a reasonable separation technique for PEGylation variants in terms of processing stringency and resolution potential. One possibility is the immobilization of phase-forming polymer such as PEG on the chromatographic support, thus resulting in a modification of HIC (Dias-Cabral *et al.*, 2005; Dias-Cabral *et al.,* 2003). Columns with this kind of stationary phase have been used in the separation of enzymes (Mathis *et al.*, 1989; Diogo *et al.*, 1999) and blood cells (Matsumoto and Shibusawa, 1980), showing excellent results and promising to be a good alternative to separate standard and complex mixtures of proteins (Diogo *et al.*, 1999). Additionally, previous studies have shown that the partition coefficient (*K*) of PEGylated proteins in ATPE is related to the number of PEG molecules attached to the protein (Delgado *et al.*, 1997). Columns with PEG-based, mildly hydrophobic ligands could then in principle be used to separate PEG conjugates. Traditional HIC has been used in the separation of these types of conjugates (Seely and Richey, 2001; Clark *et al.*, 1999), but the use of columns with PEG immobilized on a support has not been exploited.

1.3 Dissertation objectives

It is clear that the development of alternative protocols for the purification of PEGylated proteins is needed. From the alternatives previously exploited, it is evident that the success of approaches involving RPC and HIC have not been widely studied nor documented. Thus, the general objective of the present dissertation is focused on the analysis of the chromatographic behavior of PEGylated proteins in RPC and HIC. To address the general objective, the products of model PEGylation reaction systems were characterized and the second and tertiary structures of the proteins were obtained; three particular objectives were defined. Firstly, the behavior of PEGylated proteins in RPC under neutral pH conditions was evaluated. Secondly, the effect of temperature and pH on the behavior of PEGylated proteins in RPC was established. Finally, the potential separation of PEGylated proteins from the unmodified form using a modification of HIC in which PEG is immobilized on the chromatographic support was attempted.

1.4 Experimental models

In order to address the objective defined for this dissertation, two experimental model systems were selected. Ribonuclease A (RNase A) is an interesting protein because it has potential therapeutic application as an aspermatogenic and antitumor agent. Studies have shown an improvement of the therapeutic effect when RNase A is conjugated to PEG (Matousek *et al.*, 2002). RNase A is a small protein (13,686 Da MW) with 124 amino acid residues that lacks tryptophan residues and an isoelectric point at 9.3. Figure 1.2 shows the structure of ribonuclease A in a space filling view. The lysine residues, each of which are potential PEGylation sites in the conjugation reaction employed, are indicated in green (residues 1, 7, 31, 37, 41, 61, 66, 91, 98, 104) (Protein Data Bank structure 1AQP; Berman *et al.*, 2000). Lys

residues 1, 31, 37, 61, 66, 91, 98 are the most exposed to the solvent as assessed by the solvent accessible surface area calculation tool GETAREA (Fracziewicz and Braun, 1997). The N-terminal lysine is indicated as Lys 1 and the three most important residues for the catalysis are indicated as His 12, His 119 and Lys 41 (Raines, 1998).

The second selected model, α-lactalbumin (αLac) is also a small protein, containing 123 amino acids (14,200 Da MW) and an isoelectric point between 4 – 5 (Permyakov and Berliner, 2000). It has been found that some forms of αLac can induce apoptosis in tumor cells, which suggests that the protein can fulfill many important biological functions (Permyakov and Berliner, 2000). Figure 1.3 shows the structure of apo-α–lactalbumin (Protein Data Bank structure 1F6R; Berman *et al.*, 2000) in space filling view; the apo form lacks a bound Ca^{2+} ion. The n-terminal glutamate residue is indicated as Glu 1 and each additional potential PEGylation site is indicated in green (lysine groups 5, 13, 16, 58, 62, 79, 93, 94, 98, 108, 114, 122). The most solvent-exposed Lys residues as assessed by GETAREA are residues 16, 62, 79, 108 and 114.

Both proteins, RNase A and apo-αLac are of interest because they have shown structural changes when in contact with hydrophobic surfaces (Lu *et al.*, 1986; Jones and Fernandez, 2003). These proteins bracket a wide range of protein structural stabilities as determined by the free energy of unfolding under neutral solution conditions and under RPC mobile phase conditions. RNase A is very stable with an unfolding free energy ($\Delta G_{unfolding}$) between 8.46 – 14.8 kcal/mole (Kim *et al.*, 2003, Pace *et al.*, 1998) whereas apo-αLac is marginally stable with a $\Delta G_{unfolding}$ of 3.4 kcal/mol (Canet *et al.*, 2001, Vanderheeren *et al.*, 1996).

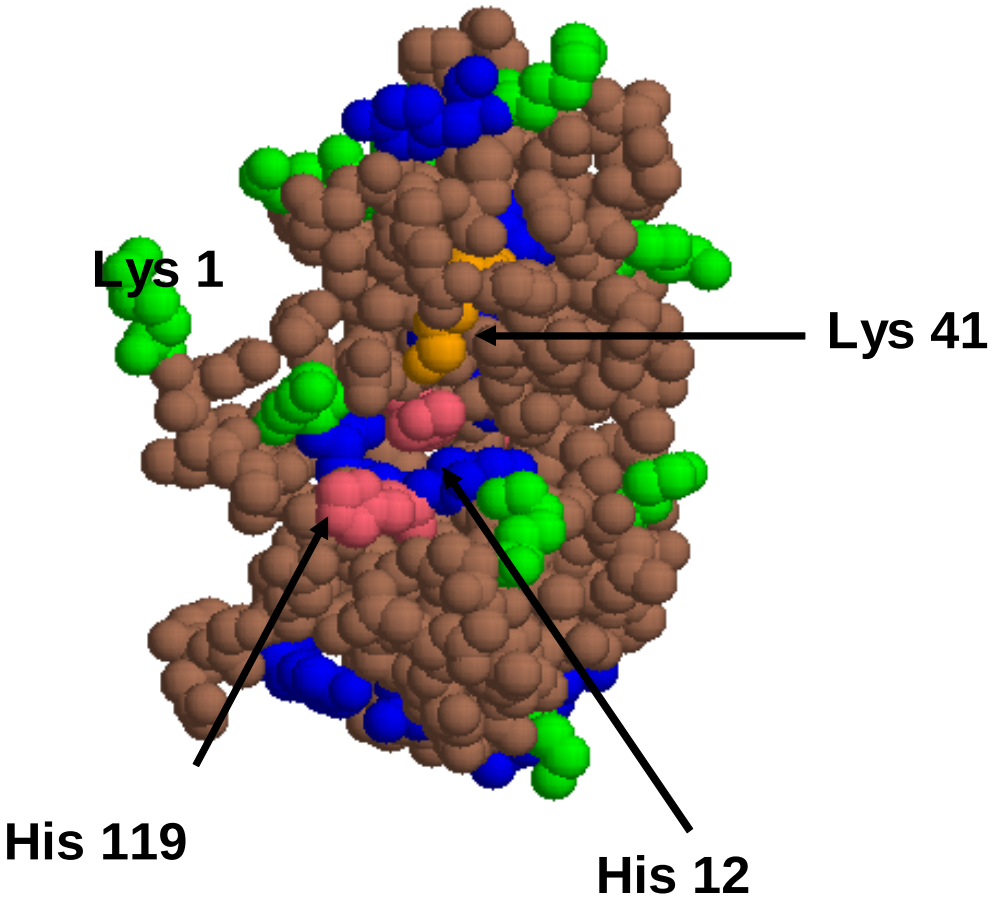

Figure 1.2 Protein structure of Ribonuclease A (1AQP, PDB). The inscriptions refer to the location of the N-terminal Lys 1, and the three residues most important for the catalysis: His 12, His 119 and Lys 41. The lysine groups are shown in green and the hydrophobic (Phe, Tyr and Trp) groups in blue (Protein Data Bank structure 1AQP; Berman *et al.*, 2000).

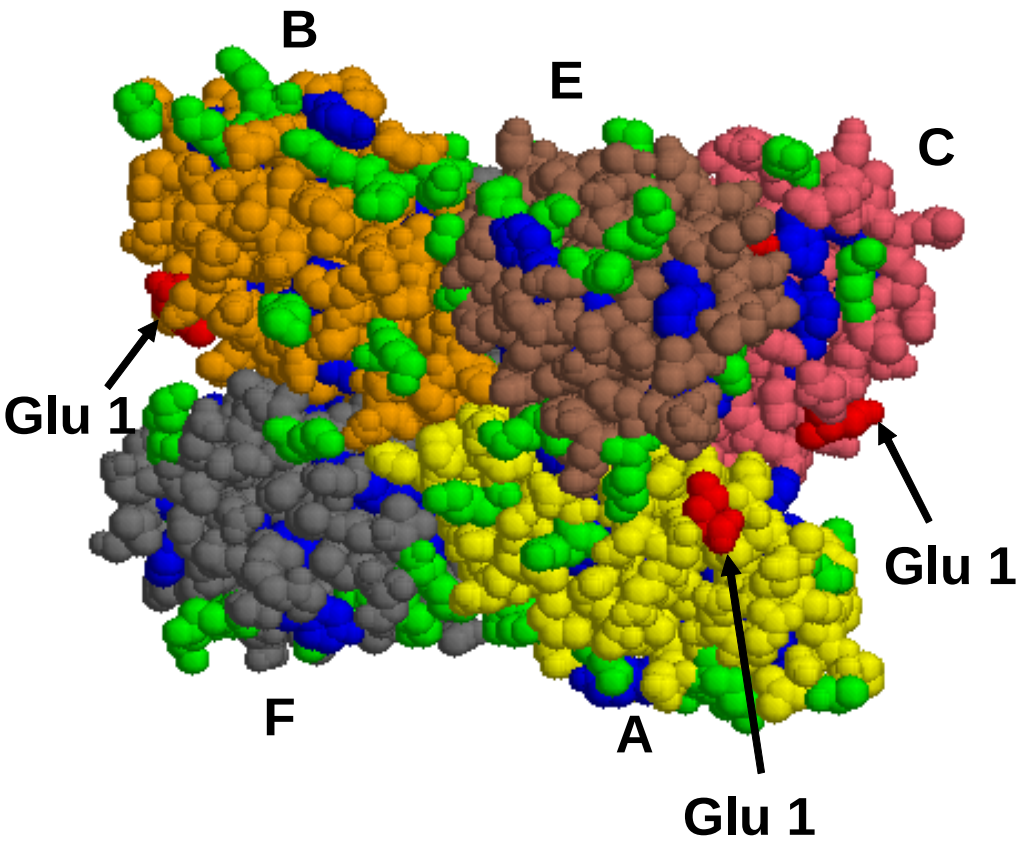

Figure 1.3 Apo-αLactalbumin (1F6R) structure obtained from the RCSB PDB. the figure shows the structure of a hexamer, the inscriptions refer to every chain and their N-terminal Glu 1. The lysine groups are shown in green and the hydrophobic (Phe, Tyr and Trp) groups in blue.

1.5 Dissertation outline

All the materials and methods used during this research are described in Chapter 2, including background information of each technique. The results of the experiments are presented in Chapters 3 to 5. They are reported in "scientific manuscript format", following the instructions for author reported in the journal *Biotechnology and Bioengineering*. Chapter 3 shows the results of the separation of ribonuclease A and apo-α–lactalbumin PEGylated conjugates via reversed phase chromatography at neutral pH conditions. The effect of pH and temperature on the separation of RNase A and apo-αLactalbumin PEG-conjugates by reversed phase chromatography is shown in Chapter 4. The results of the separation of PEGylated RNase A from the unmodified protein using a mild hydrophobic interaction chromatography media comprising polyethylene glycol ligands immobilized on sepharose are presented in Chapter 5. Finally, the conclusions of the research reported here and suggestions for further work are described in Chapter 6.

Chapter 2.- Materials and experimental methods

2.1 Reagents

Bovine pancreatic ribonuclease A (RNase A, cat. No. R5000, Lots 101K1403 and 093K0765) and α-bovine lactalbumin (αLac cat. No. L5385, Lot 063K7009) were purchased from Sigma (St. Louis, MO). Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular mass of 20kDa and methoxy-poly(ethylene glycol)-amine with a nominal molecular mass of 5kDa came from Nektar Therapeutics (Huntsville, AB). Sodium cyanoborohydride was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Activated CH sepharose 4B (cat. No. 17-0490-01, Lot 307571) and the Tricorn 5/100 chromatography column came from Amersham Biosciences (Uppsala, Sweden). Silica based C18 bulk chromatographic media (No. 218TPB1015, Lots E030116-5-1 and E040519-4-5) with 10 – 15 μm particle size, 300 Å pore size, 60 – 110 m^2/g surface area and 8 wt% carbon load was obtained from Vydac (Hesperia, CA). Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific, Pittsburgh, PA), and potassium chloride (Sigma-Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific. Any other salt or solvent used in this research was reagent grade. All reagents were used as received. De-ionized water with a resistance of ~18 Ω/cm was obtained using a Barnstead (Dubuque, IA) Nanopure Diamond water purification system and was used throughout. All buffers used in the chromatography experiments were vacuum filtered using a

general purpose PTFE membrane filter with a pore size of 0.45 μm which was obtained from Millipore (Billerica, MA).

2.2 Enzymatic activity of RNase A and its conjugates

The intrinsic activity of unmodified RNase A, mono-PEG-RNase A, and di-PEG-RNase A was determined using the RNase Alert fluorescence assay (Integrated DNA Technologies, Inc., Coralville, IA). A pool of each sample was collected from at least three chromatographic separations. The substrate is a short RNA oligomer with fluorescein and a quencher on opposite ends of the chain; the quenching is relieved when RNaseA cleaves the oligomer. Fluorescence spectroscopy was measured with a SpectraMax M2 microplate reader (Molecular Devices, Co., CA) spectrofluorimeter. The substrate was suspended at a concentration of 10 to 20 nM in a 50 mM Tris buffer, pH 7.2 by vigorous mixing with a vortex mixer for about 30 min. After this time, the substrate solution was separated into aliquots, in order to prevent contamination. The baseline of the substrate intensity was monitored with excitation and emission wavelengths of λ_{ex} 490 nm and λ_{em} 520 nm, respectively using medium sensitivity. One microliter of the desired protein solution (0.002 mg/mL) was added to 1.0 mL of substrate solution and it was mixed for about 5 seconds, aspirating and expelling the solution from a 200 μL pipette several times. The initial rate of the increase in fluorescence emission was used to estimate the enzyme activity in terms of the specificity constant k_{cat}/K_M (Kelemen *et al.*, 1999) according to the equation 2.1:

$$I = I_o + (I_f - I_o)\left(\frac{k_{cat}}{K_M}\right)[E]t \quad (Eq\ 2.1)$$

where I is the fluorescence intensity measured at a given time during the reaction, I_f is the intensity of the product, I_o is the intensity of the substrate collected prior to the addition of the enzyme and [E] is the enzyme concentration. All vessels used in this assay were cleaned before use with RNaseZap decontamination solution (Ambion, Austin, TX) in order to assure a ribonuclease-free starting condition. A t-test was done in order to determine if there was significant difference between the enzymatic activity of the samples.

2.3 PEGylation reaction

Background and theory

The covalent attachment of polymers such as polyethylene glycol (PEG) or dextran to active biological compounds has become one of the main methods for the alteration and control of biodistribution, pharmacokinetics and toxicity of these compounds. Under certain conditions, the polymer-protein conjugates have demonstrated excellent results (Zalipsky, 1995) with PEG being the most used polymer. The PEGylation term has been used since 1977, when Abuchowski, Davis and collaborators, the founders of Enzon Corporation (a manufacturer of PEGylated protein pharmaceuticals), described a method for the covalent attachment of one or several PEG molecules to a peptide or protein (Greenwald *et al.*, 2003).

Polyethylene glycol is a linear or branched polyether with a hydroxyl group at both termini. The most used modified form in protein PEGylation processes is mono-methoxy PEG (mPEG). Its structure is shown in Figure 2.1.

$$CH_3O-(CH_2CH_2O)_n-CH_2CH_2-OH$$

Figure 2.1 Mono-methoxyPEG structure (Roberts *et al.*, 2002).

Methoxy-PEG is amphiphilic: it dissolves in both organic solvents and water. It is not toxic and is eliminated by the body through the combination of renal and hepatic processes, which make it ideal for use in pharmaceutical applications (Greenwald *et al.*, 2003).

The most commonly used mPEG-based electrophiles, often referred to in the literature as "activated PEGs", are shown in Figure 2.2. They can be divided into three categories (Zalipsky, 1995):

1) Derivatives containing a reactive aryl chloride residue (arylating reagents). The residue is displaced by a nucleophilic amino group upon reaction with peptides or proteins (1 and 2 in Figure 2.2).
2) Derivatives containing acyl groups (acylating reagents). Protein modification with these agents results in acylated amine-containing linkages: amides, derived from active esters (3 – 6 and 11 in Figure 2.1,), or carbamates (7 -10 in Figure 2.2).
3) Alkylating reagents (12 and 13 in Figure 2.2). They react with proteins creating secondary amine attachments from amino-containing residues.

The numbering in Figure 2.2 roughly corresponds to the order in which these activated polymers were obtained. Modifications to the PEGylation reaction have been done in order to avoid unexpected reactions which sometimes result in substantial loss of biological activity. This happens because in some cases the preservation of the biological activity is dependent on

the chemistry of PEG activation and subsequent attachments, as well as on the extent of modification.

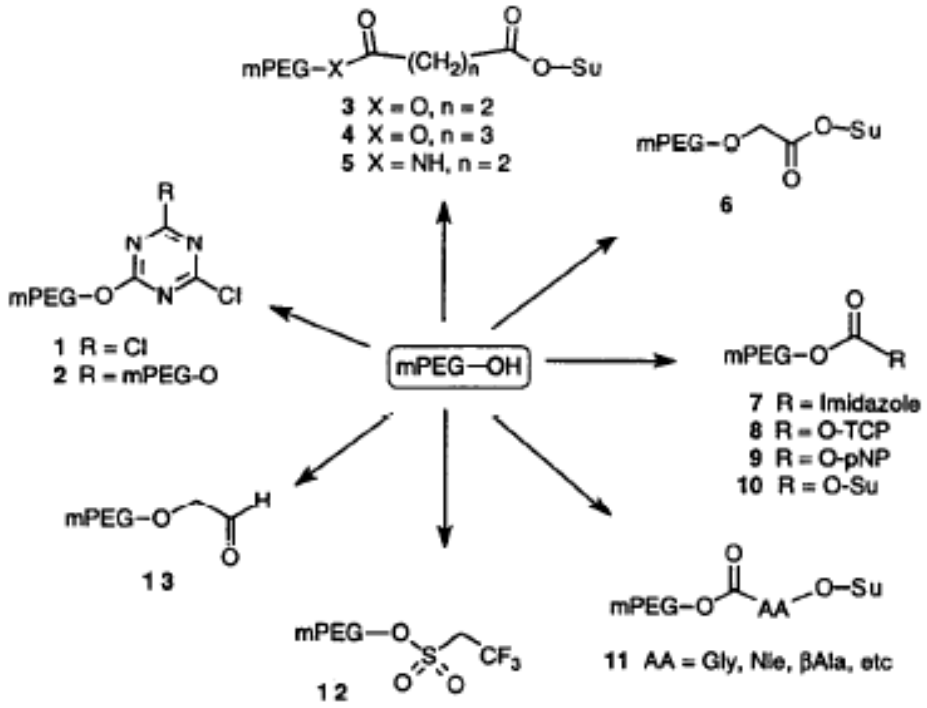

Figure 2.2 Methods used for the preparation of mPEG. mPEG represents $CH_3(OCH_2CH_2)n$- (Zalipsky, 1995).

In general, the most commonly used reactive groups in the PEG conjugation of polypeptides are nucleophilic, and in decreasing order are: thiols, α amino groups, ε amino groups, carboxylates and hydroxylates (Veronese, 2001). However, this order is not absolute. The reaction products depend on the reactions conditions: pH, molar ratio of protein to PEG, protein concentration, reaction time and temperature. By controlling these parameters, the reaction can be managed to produce mono-, di-, tri- and multi-PEG conjugates (Bailon and

Berthold, 1998). Other residues can also be modified under special conditions, generating unexpected products (Veronese, 2001).

Experimental method

The PEGylation reaction was performed under conditions that maximized the modification of the N-terminus. Conjugation at the amino terminus would minimize any undesirable PEG interferences with other biologically important amino acid residues in the protein (Kinstler *et al.*, 2002). N-terminal-specific PEGylation can be obtained by reacting monomethoxy-PEG derivatives at acidic pH conditions. This strategy is based on the fact that primary amine residues in protein have different pKa values: pKa 7.8 for N-terminal α-amino groups and 10.1 for ε-amino groups in lysine residues (Lee *et al.*, 2003). The alkylation of primary amines forms a secondary amine, according to Figure 2.3, and thus preserves the charge on the N-terminal amino group (Kinstler *et al.*, 1996).

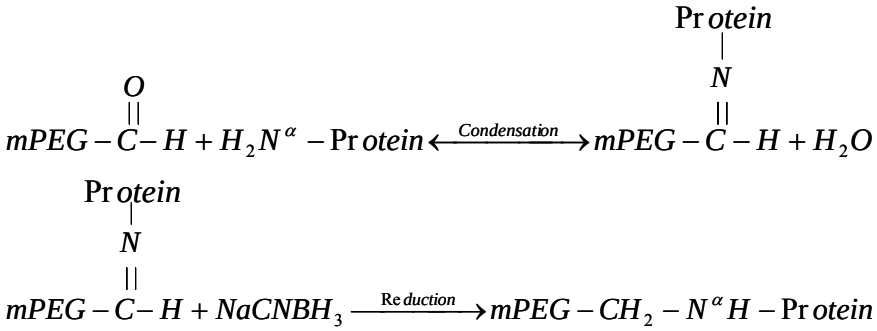

Figure 2.3 PEGylation reaction.

The PEGylation reaction was initiated by placing 2.0 mL of a solution of RNase A or apo-αLac at a concentration of 3 mg/mL in a 100 mM sodium phosphate buffer, pH 5.1 with 20 mM sodium cyanoborohydride in a vial containing 30 mg of the 20 kDa methoxy

poly(ethylene glycol) butyraldehyde. When apo-αLac was reacted, the buffer also contained 2mM EDTA. The reaction mixture was stirred rapidly for 17 – 19 h at 4 °C using a magnetic stirrer. After this time, the reaction mixture was filtered with an acrodisc 0.45 μm syringe filter and stored less than 3 days at 4°C prior to analysis and separation. During this time, no significant further reaction was observed according analysis by SEC (see section 2.6).

In order to maintain the reactivity of the mPEG, it was stored in several pre-weighed aliquots (30 mg) at -86°C under an inert atmosphere (filling the container with nitrogen) and in the dark. Before use, after storage, the container was allowed to come to room temperature.

2.4 Circular Dichroism (CD)

Background and theory

Circular dichroism is sensitive to the overall conformation and folding of polypeptides. It is an absorptive phenomenon represented as the difference in the absorption of left-handed circularly polarized light (A_L) and right-handed circularly polarized light (A_R) (see equation 2.2),

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \frac{\Delta A}{lc} = \frac{A_L - A_R}{lc} \quad (Eq\ 2.2)$$

where Δε is the differential molar extinction coefficient, per moles per centimeter ($M^{-1}cm^{-1}$), of left-handed circularly polarized light (ε_L) and right-handed circularly polarized light (ε_R), ΔA is the differential absorbance of left-handed circularly polarized light (A_L) and right-

handed circularly polarized light (A_R), l is the path-length in centimeters and c is the concentration in moles per liter (mol/L). This difference in absorption is converted to ellipticity Θ, a common unit for reporting the intensity of CD bands using equation 2.3 (Manning, 1989):

$$\Theta = 3298\Delta\varepsilon \quad (Eq\ 2.3)$$

CD instruments measure the ellipticity Θ of a solution. However, the ellipticities are most often reported on a molar basis using a mean residue weight (MRW; equation 2.4), $[\Theta]_{MRW,}$ where the units are degrees centimeters squared per decimole (Mulkerrin, 1996).

$$[\Theta]_{MRW} = \frac{\Theta_{obs} MRW}{10 l c} \quad (Eq\ 2.4)$$

In order to exhibit CD signal, a system must possess either local or global chirality. Achiral molecules will no CD spectum. As CD is an electronic absorption process, the bands are characteristic of the electronic excited states of the molecule, with distinctive intensities and frequencies. Since it is a difference method, the bands also posses either a negative or positive sign (Manning, 1989).

CD in the far ultraviolet

The far-UV CD spectrum of a protein reflects its secondary structure content. The chromophore in the far-UV is the amide bond. There is a restricted rotation about the O=C-

NH bond of the peptide amide because of the double bond character that results in the coplanarity of the CONH atoms. There are, therefore, two bonds in which rotation is allowed: these are the RCα-NH (known as the ϕ bond) and the CO-CαR (the ψ bond). The specific dihedral angles about the ϕ and ψ bonds are responsible for CD spectra peculiar to specific secondary structural elements α-helix, β-sheet, various turns, non-ordered or random structures (Mulkerrin, 1996).

Various empirical methods have been developed for analyzing protein CD spectra for quantitative estimation of the secondary structure content. During this work, the CDPro software was used (Sreerama and Woody, 2000). CDPro uses the secondary structure assignments from DSSP (Kabsch and Sander, 1983) to determine the secondary structure fractions of the globular proteins in the reference set. The α-helix and β-strand structures are split into regular and distorted classes, considering four residues per α-helix (two residues from each end) and two residues per β-strand (one residue from each end) distorted in view of their incomplete backbone hydrogen bonding. The grouping used gives six secondary structural classes: regular α-helix, distorted α-helix, regular β-strand, distorted β-stand, turns and unordered.

CD in the near ultraviolet

In the near-UV, the aromatic residues, trypthophan, tyrosine, phenylalanine, and the cysteine disulfide are the chromophores. The near ultraviolet region extends from 250 to 350 nm for proteins without prosthetic groups. The aromatic residues in the protein absorb in this portion of the spectrum. If these aromatic residues are held rigidly in an asymmetric environment,

they will exhibit CD bands. In addition to the aromatic chromophores, the cysteine disulfide bonds can make a major contribution to the near-UV CD spectrum. Because the CD spectra of these species depends upon both their specific environment and the freedom or lack of freedom of rotation about C_α, C_β or $C\gamma$ bonds, their optical activity will be a function of the folded structure of the protein. Unlike the far-UV CD spectrum of proteins, where the potential exists for calculating the secondary structural content, there is little interpretable structural information in the near-UV CD spectrum. The utility of the near-UV CD spectrum is often in the determination of the difference between the folded and unfolded state of the protein and in the different participation of each of the chromophores in the transition (Mulkerrin, 1996).

The phenylalanine side chain has a high degree of symmetry, leading to very weak adsorption. The spectrum of phenylalanine has four vibronic bands at 254, 256, 262 and 267 nm. The tyrosine bands in the absorption spectrum are observed in the CD spectrum. In a folded protein, the tyrosine absorption spectrum is an envelope with broad overlapping bands, and the tyrosine main peak is at approximately 276 nm with a shoulder at 283 nm.

The disulfide chromophore is the result of two cysteines in the primary structure of a protein forming a covalent bond. Although the disulfide absorption is weak, the intensity of the CD band may be quite strong. However, the CD contribution of the disulfide is often buried under the aromatic chromophores. The contribution of the disulfide to the near UV is a broad band with a peak, either positive or negative, from 250 to 300 nm with no distinguishable vibronic bands in the spectrum.

CD instrumentation and experimental protocol

Far-UV CD spectra of the samples collected from SEC and RPC experiments were recorded using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). Spectra were collected in the 190-310 nm wavelength range at room temperature using a 1 mm path length cell. The secondary structures of the protein samples were estimated with the CDPro software (Sreerama and Woody, 2000). A mean residue molecular weight of 110 was determined for RNase A from the molecular mass, 13,686 Da, and the number of residues, 124 (Raines, 1998). The mean residue molecular weight of apo-αLac was determined to be 115, based on a molecular mass of 14,200 Da and 123 amino acids (Permyakov and Berliner, 2000). Near-UV spectra for tertiary structure characterizations were similarly collected from 250 to 350 nm using a 1 cm path length cell. In both types of spectral acquisitions, the scan speed was 100 nm/min, the slit width was 1 nm, and at least 10 scans were accumulated per spectrum. A protein-free control spectrum was recorded for each condition and subtracted from the protein spectra. All the samples were equilibrated at room temperature for 1 h before the measurements were taken. The results were expressed as a mean residue weight (MRW) ellipticity, $[\Theta]_{MRW}$.

2.5 Size exclusion chromatography (SEC)

Background and theory

Size exclusion chromatography (SEC) is a general name for the process of separation of molecules according to their size when a solution flows through a packed bed of porous packing media. Within SEC, gel permeation chromatography (GPC) is the chromatographic separation of synthetic macromolecules with the use of porous gels or rigid inorganic packing

particles, while gel filtration chromatography (GFC) or simply gel filtration is a similar process of separation for biological macromolecules (biopolymers) (Kostanski *et al.*, 2004).

A volume of sample solution is introduced into to a column containing packing material with pores that are of comparable size to the molecules to be separated. Mobile phase is then allowed to pass through the column. The molecular size of a solute molecule determines the degree to which it can penetrate the pores. Separation occurs by a molecular sieving effect where the larger molecule explores less of the intraparticle void fraction (i.e., pores) than does a smaller molecule. Larger molecules elute first because they spend less time inside the stationary phase than smaller molecules. Separation can be achieved if the porosity of the stationary phase is properly selected, and there is a significant difference in size of the molecules being separated (as measured by their hydrodynamic ratio) (Ladisch, 2001).

Molecules that are completely excluded from the stationary phase emerge at the void volume, V_o. This represents the volume of the interstitial space (outside the support particles) and is determined by the elution of very large molecules. Molecules that can freely enter the pores have full access to an additional space, the internal pore volume, V_i. Such molecules emerge at V_t, the total volume available to the mobile phase or total liquid volume,

$$Vt = Vo + Vi \quad (Eq\ 2.5)$$

which corresponds to the elution volume of molecules that are small enough to be distributed freely both inside and outside the pores (Irvine, 1997).

A solute molecule that is partially restricted from the pores will emerge with an elution volume, V_e, between the two extremes, V_o, and V_t,

$$V_e = V_o + K_d V_i \quad (Eq\ 2.6)$$

The distribution coefficient, K_d, for such a molecule represents the fraction of V_i available to it for diffusion.

SEC instrumentation and experimental protocol

The PEGylation reaction mixture (2.0 mL) was analyzed via size-exclusion chromatography (Akta Explorer, Amersham Pharmacia, Uppsala, Sweden) using a Sephacryl S-300 column (1.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and buffer exchanged with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2. RNase A concentration was measured spectrophotometrically using absorbance at 280 nm and an extinction coefficient of 8,045 $M^{-1}cm^{-1}$. Apo-αLac concentrations were similarly determined using an extinction coefficient of 23,556 $M^{-1}cm^{-1}$. All mobile phases and samples were filtered through 0.45 μm membrane before use.

2.6 Reversed phase chromatography (RPC)

Background and theory

Approximately 75% of the total amount of liquid chromatographic (LC) analysis world-wide is performed using the reversed phase mode. The broad range of compounds that can be analyzed, non-ionic, ionizable and ionic, has caused this widespread applicability. Other main contributions are the short equilibrium times, the possibility to perform gradient analysis and the possibility to use water-rich eluents and samples (Vervoort *et al.*, 2000)

Reversed-phase chromatography (RPC) is a powerful tool for separating and purifying proteins. It has been shown that proteins with as little as a single amino acid difference can be effectively separated by RPC (Ahrer and Jungbauer, 2006). However, the value of preparative RPC can be limited by several factors, including safety and environmental problems associated with the use of organic solvents, low recoveries from the denaturing mobile and stationary phases involved, and the appearance of several kinks of complex chromatographic behavior. One class of complex behavior is the elution of broad, shouldered, or multiple peaks, even when a pure protein sample has been injected (Sokol *et al.*, 2003)

Liquid chromatography is based on the differential affinity of various soluble molecules for specific type of solids. Reversed phase chromatography (RPC) employs a hydrophobic phase bonded to the surface of the resin. The hydrophobic phases that are bonded to silica supports are typically octyl (C_8), octyldecyl (C_{18}), phenyl and methyl (C_1); these media bind hydrophobic solutes more strongly than hydrophilic solutes. Solutes are typically introduced into reversed-phase columns in water, or with minimal amounts of organic solvent, so that

most solutes partition to the stationary phase. The organic content of the mobile phase is slowly increased, decreasing the polarity of the mobile phase (Harrison *et al.*, 2003).

pH and mobile phase composition are the two relevant optimization parameters: pH to vary the dissociation extent of residual silanols on the silica support and mobile phase composition to compensate for the diminution of retention when the solute ionization increases. In fact, the influence of these two parameters in the retention process is somewhat more complex since the variation of the mobile phase composition (type of organic modifier, organic modifier content, type of buffer, ionic strength) induces a variation of the degree of ionization of the analytes as well (Heinisch and Rocca, 2004)

Packing RPC column

The chromatographic column packing method was carried out with empty Tricorn 5/100 columns of 4.6mm inner diameter and overall 150mm column length. These columns were packed with a stationary phase using a slurry packing technique (Tricorn empty columns, user manual, Amersham Biosciences). The bottom filter was attached to the bottom adaptor, which was then connected to the glass column. A 20% v/v solution of ethanol was added to the bottom of the column and marked at 10 cm, the target column height. Then, 2.0 g of C18 (Vydac, Hesperi, CA) reversed phase media were weighed out and suspended in 4.0 mL of 50% v/v ethanol. The slurry was aspirated with a disposable glass pipette and slowly expelled into the column with a rotary motion, attention was paid to avoid the entrainment of air bubbles within the media bed. The column was packed between 1.0 and 2.0 cm above the 10 cm mark. Extra 20% v/v ethanol was added to achieve a smooth surface at the top of the

media bed. The top filter and the top adaptor assembly were then attached to the column. The media bed was compressed for 30 minutes with the Akta Explorer system, using a flow rate of 0.8 mL/min, which is about 130% of the regular flow rate. Finally, the adaptor lock was put in the column.

Once the column was packed, the integrity of the packed bed was tested by the elution behavior of a 1% v/v acetone solution. The column was equilibrated for 15 minutes with a 20% v/v ethanol mobile phase applied at a flow rate of 0.6 mL/min. One hundred microliters of acetone sample were loaded onto the column using a 100 μL loop. Acetone was used because it is a non-retained component which elutes at the dead volume of the column. Retention volume data was used to obtain the column dead volume, while peak shape data was used to estimate the column efficiency and the number of theoretical plates (see Table 2.1). Those parameters were obtained using the UNICORN software (UNICORN user's manual, Amersham Biosciences).

Table 2.1 RPC Column efficiency data.

Column	Dead volume (V_0, mL)	Asymmetry	Theoretical plates
C_{18} (at CMU)	2.04 ± 0.01	1.6 ± 0.002	236 ± 3
C_{18} (at ITESM)	2.92 ± .004	1.2 ± 0.007	165 ± 36

RPC instrumentation and experimental protocol

Chromatographic separations were carried out with an Akta Explorer 100 integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). PEG conjugate

mixtures were applied to a Tricon 5/100 column packed with Vydac C18 media and 236 theoretical plates (Amersham Biosciences) at room temperature. Gradient elution was carried out at a flow rate of 0.6 mL/min with solvent A (aqueous phase: 6 mM sodium phosphate, pH 7.2 with 12 mM sodium chloride or 0.1% TFA) and solvent B (20% v/v phase A in acetonitrile). The following segmented gradient profile was chosen: 43.8% B over 5 min, 43.8 – 55% B over 24.4 min, 55% B – 100% B over 5 min. The UV response was measured at 215 nm, 254 nm and 280 nm. Fractions corresponding to each respective protein peak were collected separately and stored at 4°C for structure analysis.

2.7 Mass Spectrometry by MALDI-TOF

Background and theory

Mass spectrometry is a powerful instrumental technique for protein identification and characterization. Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOFMS) is widely used mode for the analysis of proteins and peptide mixtures.

Mass spectrometry using lasers for ion generation dates back to the 1960s and was initially used for elemental analysis and later for biomolecular analysis. The MALDI process utilizes a specific matrix material mixed with the analyte. It has the unique characteristic of providing soft ionization, which allows the analysis of large nonvolatile molecules with molecular masses in excess of 500,000 Da with minimum fragmentation. However, it should be kept in mind that doubly and triply charged species are often observed as dimers and trimers (Muddiman *et al.*, 1995).

The matrix is the key component in the MALDI technique. The essential matrix properties are: high extinction coefficient at the specific laser wavelength; solubility in the same solvent as the analyte material; physical properties such as lattice structure and heat of sublimation to induce efficient desorption; promotion of ionization; and good vacuum stability. Ionization of the biomolecule is assumed to occur in the condensed phase or just above the surface in the volume of solid that is vaporized by a laser pulse (Muddiman *et al.*, 1995).

TOF (time of flight) mass analyzers have several advantages like simplicity, high transmission, theoretically unlimited mass range, simultaneous detection of all ions to yield the multiplex advantage, high speed of analysis, and direct compatibility with pulsed ionization sources. Mass-to-charge ratios are determined by measuring the time it takes for ions to move through a field-free region. Given a constant acceleration voltage, the flight time for an ion is related to its mass-to-charge or m/z ratio. The flight path for an ion can be increased, without increasing the size of the flight tube, by incorporating an ion mirror or reflector at the end of the flight tube. Ion direction is reversed to send the ions back down the same vacuum chamber at a slightly different angle so the flight path of the reflected ions does not cross with the ions entering the reflector. Most importantly, a reflector can also correct for minor kinetic energy differences among ions of the same m/z value, minimizing variations in flight times (Yates, 1998).

MALDI-TOF instrumentation and experimental protocol

Each protein peak collected from SEC and RPC experiments was analyzed by mass spectrometry. Prior to analysis, the samples were desalted using centrifugal filters (Microcon

YM-3, Millipore, 3 kDa nominal molecular weight cut-off). MALDI-TOF/MS was performed with a PerSeptive Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared mixing 1.0 μL sample and 1.0 μL of matrix.

2.8 Hydrophobic Interaction Chromatography (HIC)

Background and theory

Hydrophobic interaction chromatography (HIC) is a purification technique used to separate proteins on the basis of the surface hydrophobicity of the solute molecule. It consists of injecting a protein sample into a column with a hydrophobic packing under conditions of high salt concentration. Hydrophobic ligands, such as n-alkyl or phenyl groups, are chemically attached to the matrix and the separations are based on differences in the surface hydrophobicity of proteins. Typically the extent of support modification with hydrophobic groups and the types of hydrophobic groups used give media that are significantly less hydrophobic than those used in RPC. Bound proteins are eluted by lowering the salt concentration or by the use of a chaotropic agent or an organic modifier such as ethylene glycol. During this process, the hydrophobic ligands of the resin interact with the exposed hydrophobic zones of the protein to reversibly bind the protein to the resin (Mahn and Asenjo, 2005).

The main system characteristics affecting protein retention in HIC are concentration and type of salt and density and type of hydrophobic ligand attached to the matrix. The main physicochemical property of proteins that determines chromatographic behavior in HIC is hydrophobicity (Mahn and Asenjo, 2005). The influence of different salts on hydrophobic interactions follows the Hofmeister series (or lyotropic series, see Figure 2.4) for the precipitation of proteins from aqueous solutions: The salts at the beginning of the series promote hydrophobic interactions and protein precipitation (salting-out effect) and are called antichaotropic or kosmotropic. They are considered to be water structuring, whereas salts at the end of the series (salting-in or chaotropic ions) randomize the structure of the liquid water and thus tend to decrease the strength of hydrophobic interactions (Queiroz *et al.*, 2001).

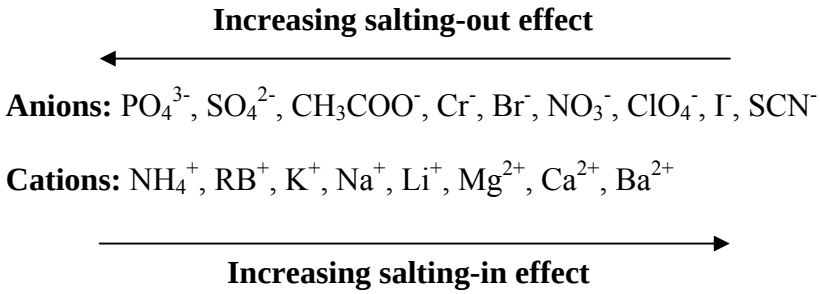

Figure 2.4 Hofmeister series (Queiroz *et al.*, 2001).

The structural damage to the biomolecules and biological activity losses experienced by proteins during processing via HIC is typically less than that experienced during processing via RPC. HIC is an alternative way of exploiting the hydrophobic properties of proteins, working in a more polar and less denaturing environment than RPC, since this technique requires the use of non-polar solvents for the protein elution due to strong binding to adsorbent (Queiroz *et al.*, 2001). However, strong hydrophobic interactions sometimes result in almost

irreversible adsorption or denaturation during the elution with often harsh mobile phase conditions (e.g. low pH. chaotropic agents, detergents, organic solvents, etc.) (Hubert *et al.*, 1991). Thus, the use of ligands with intermediate hydrophobicity can be of interest, as they would provide an adequate binding strength without the above drawbacks.

Aqueous two-phase extraction (ATPE) is a separation technique that exploits mild hydrophobic interactions that has been extensively described by Albertsson. ATPE is considered a simple technique, scalable and biocompatible for the recovery of biotechnological products, since the aqueous solutions comprising the two-phase system have high water content and low interfacial tensions. The systems are composed of either two polymer phases or a polymer and a salt phase. ATPE is a liquid-liquid partition method where the separation is based on the differential partitioning of the solutes between the phases. In order to get good separations, it is essential to optimize the partition coefficient (K) of each component through the appropriate selection of the system's composition (Shibusawa, 1996).

In the present research, the principles of the partition technique were transposed to a chromatographic procedure using one of the polymers (PEG) immobilized on the chromatographic support (Dias-Cabral *et al.*, 2005; Dias-Cabral *et al.*, 2003; Hubert *et al.*, 1991), thus a modification of HIC could be achieved. Columns with this kind of support have been used in the separation of enzymes (Mathis *et al.*, 1989; Diogo *et al.*, 1999) and blood cells (Matsumoto and Shibusawa, 1980), showing excellent results and promising to be a good alternative to separate standard and complex mixtures of proteins (Diogo *et al.*, 1999). This kind of column could be used to separate PEG conjugates. Even when HIC has been used in

the separation of these proteins, the use of columns with PEG immobilized on the support has not been exploited.

Coupling the ligand

PEG immobilized on sepharose was used as a mild hydrophobic support. Activated CH Sepharose 4B (Amersham Biosciences, Uppsala, Sweden Lot: 307571) was used as the support matrix and methoxy-polyethylene glycol amine (Nektar Therapeutics, Lot PT-04G-16, mPEG-NH_2) was used as the hydrophobic ligand. The mPEG-NH_2 was coupled to the matrix according the supplier instructions (instructions CH Sepharose 4B, Amersham Biosciences). Briefly, the mPEG-NH_2 (0.65 g) was dissolved in the coupling buffer (0.1M $NaHCO_3$, pH 8 containing 0.5 M NaCl). Separately, 1.3 g of Sepharose was washed with 250 mL of 1 mM HCl and mixed with the ligand solution over 24 h at 4°C. After this time, the mixture was allowed to settle and a sample of the supernatant was taken for future analysis. The excess ligand was washed away with ~250 mL of coupling buffer. In order to block the remaining active groups, the medium was transferred to 0.1 M Tris-HCl buffer, pH 8. After 1 hour, the Sepharose was washed with 3 cycles of alternating pH (0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris-HCl buffer, pH 8 containing 0.5 M NaCl).

A colorimetric method was used to estimate the amount of mPEG-NH_2 coupled to the matrix (Nag *et al.*, 1996). Ammonium ferrothiocyanate reagent was prepared by dissolving 16.2 g anhydrous ferric chloride ($FeCl_3$) and 30.4 g ammonium thiocyanate (NH_4SCN) in distilled water. The final volume was adjusted to 1 L. A calibration curve was prepared with mPEG-NH_2 standards containing different concentrations. Two milliliters of ammonium

ferrothiocyanate and 2 mL of chloroform were mixed in a glass tube containing 200 μL of the mPEG-NH_2 standard solution. The biphasic systems were vigorously mixed for 30 min and then the tubes were centrifuged at 2,000 rpm for 20 min. The lower chloroform layers were removed and their absorbances were recorded at 510 nm using a quartz cuvette with 1 cm optical path length in a Beckman Spectrophotometer. The amount of mPEG-NH_2 (~30 μmol) on the matrix was calculated as a difference between the initial amount added to the coupling buffer and the remaining amount of mPEG-NH_2 in solution after the coupling reaction.

Packing HIC column

The methodology used for packing the HIC was similar to that described in section 2.6 (Packing RPC column). However, the support was compressed using the chromatographic equipment and the column was filled until a length of ~10 cm was achieved. The top cap of the column was attached to the Akta Explorer and a 0.8 mL/min flow was set up. Once the support was compressed, the top cap was carefully detached from the column, without stopping the flow. Additional support was then put into the column. The top cap was put back on the column in order to compress the support again. The efficiency data obtained for the column, based on the elution behavior of a 100 μL pulse of 1 % v/v acetone, is shown in Table 2.2.

Table 2.2 HIC Column efficiency data.

Column	Dead volume (V_0, mL)	Asymmetry	Theoretical plates
Sepharose-PEG	3.37 ± 0.00	0.8 ± 0.003	65 ± 6

Separation of Native protein from PEGylated conjugates via a modified HIC Packing

Chromatographic experiments were carried out with an Akta Explorer 100 integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). Protein samples, unmodified RNase A, monoPEG- RNase A and diPEG-RNase A (PEGylated proteins were collected from SEC separation, see section 2.5) were applied to a Tricorn 5/100 column (Amersham Biosciences) packed with Sepharose-mPEG-NH_2 media at 25°C. Gradient elutions were carried out at a flow rate of 1 mL/min with a solvent A: 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 or 2 M potassium phosphate, pH 7.0 or 3 M ammonium sulfate in 25 mM acetate buffer, pH 5.0 and a solvent B: 25 mM potassium phosphate, pH 7.0 or 25 mM acetate buffer, pH 5. The gradient profile used was: 0% B for 6.5 mL, 0 – 100% B over 29.5 mL, 100% B for 15 mL. The UV response of the column effluent was monitored at 215 nm, 254 nm and 280 nm.

Separation of Ribonuclease A and apo-αLactalbumin PEGylated conjugates via reversed phase chromatography at neutral pH conditions

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Abstract

The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins – called PEGylation- is becoming more commonplace due to improved therapeutic efficacy. Protein PEGylation reactions often result in a population of conjugate species both in terms of the number of attached PEG chains and their locations. As some portion of this population may be biologically inactive, a challenging separation problem arises. In this work, reverse phase chromatography (RPC) under neutral pH conditions was used to resolve heterogeneous populations of PEGylated conjugates of ribonuclease A (RNase A) and apo-αlactalbumin (apo-αLac). Also, the possibility that conjugated PEG chains can play dual roles as both group-specific separation tags and as structural stabilizers in RPC was explored. Both proteins were reacted with an activated PEG species with a nominal molecular weight of 20 kDa and the reaction mixtures were analyzed by size exclusion chromatography and mass spectrometry. In both cases, the products were characterized as di-PEGylated, mono-PEGylated and unmodified protein. The mixtures were separated via RPC with a C18 column using a gradient elution profile comprising a phosphate buffer pH 7.2 and acetonitrile. RPC also allowed the identification of a tri-PEGylated species produced during the reaction with RNase A; this additional PEGylation probably reflects the greater accessibility of lysine residues in this protein as compared to apo-αLac. In the cases studied the attachment of PEG molecules did not modify the structures of the proteins. This was confirmed by measuring the secondary and tertiary structure of each species before and after RPC processing. The results showed that it is possible to separate PEGylated species by RPC at neutral pH without structural degradation.

Key words: polyethylene glycol, PEGylated conjugates, protein separation, protein structure

Introduction

Polypeptide drugs show great potential to be used as therapeutic agents. However, their *in vivo* effectiveness may be reduced by their susceptibility to destruction by proteolytic enzymes, short circulating half-life, short shelf-life, low solubility, rapid kidney clearance and their propensity to generate neutralizing antibodies (Harris and Chess, 2003). These factors can occasionally be mitigated by modifying the drugs via covalent attachment of polyethylene glycol (PEG) chains, through a process called "PEGylation". Additional benefits of PEGylated proteins can be reduced immunogenicity and antigenicity, as well as reduced toxicity (Bailon and Berthold, 1998). A variety of PEG conjugated proteins such as human growth hormone (5,000 Da PEG attached) (Clark *et al.*, 1996), insulin (750 and 2000 Da methoxy-PEG attached) (Hinds *et al.*, 2000), and salmon calcitonin (5,000 Da PEG attached) (Lee *et al.*, 1999) have shown superior pharmacological properties relative to the unmodified forms. Additionally, during the last several years, the FDA has approved PEGylated proteins to treat different diseases in humans, including bovine adenosine deaminase (Adagen manufactured by Enzon Pharmaceuticals, 5,000 Da methoxyPEG attached) for severe combined immunodeficiency disease, α interferon (PEGasys manufactured by Roche; PEGintron manufactured by Schering; 12,000 Da PEG attached) for hepatitis C, L-asparaginase (Oncaspar manufactured by Enzon Pharmaceuticals, 5,000 Da methoxy-PEG attached) for acute lymphoblastic leukemia (Greenwald *et al.*, 2003), granulocyte colony-stimulating factor (Neulasta manufactured by Amgen, 20,000 Da methoxy-PEG attached) to stimulate white blood cell production in chemotherapy patients and a growth hormone antagonist (Somavert manufactured by Pfizer, 5,000 Da PEG attached) to treat acromegaly.

There are different chemistries used to attach activated PEG molecule to polypeptides. The most common protein reactive groups involved in coupling are nucleophiles with the following moieties ranked in decreasing order of reactivity: thiol, alpha amino groups, epsilon amino groups, carboxylates and hydroxylates. However, this order depends on the reaction pH and steric accessibility. Furthermore, other residues may react under special conditions, such as the imidazole group of histidine (Veronese, 2001). The composition of the product is dependent on the number and local reactivity of the available attachment sites (amino and sometimes other nucleophilic groups) on the starting polypeptide, the reactivity of the activated PEG reagent and the conditions of the modification reaction (Zalipsky, 1995). Thus, conjugation may result in the generation of a family of species characterized by a distribution in number and position of attached PEG groups.

PEGylated variants are not equal in their effectiveness (Lee and Park, 2002; Youn *et al.*, 2004). As a result, it is desirable to resolve heterogeneous conjugate mixtures into effective subpopulations using adequate separation strategies (Vicentelli *et al.*, 1999) and/or to limit the breadth of the conjugate distribution during the conjugation reaction itself (Deiters *et al.*, 2004; Koumenis *et al.*, 2000). Hybrid approaches such as size exclusion reaction chromatography, which combine reaction and separation unit operations, are also under development (Fee, 2003). Near-term solutions at scale will likely be concentrated on the population resolution route.

Size exclusion chromatography (SEC) and ion exchange (IEX) are the most popular routes to the separation of conjugate species (Fee and Van Alstine, 2006). However, SEC is not sufficient to resolve micro-heterogeneity, or species that vary only in the location in which

PEG chains are attached. For SEC, the relative difference in molecular size between individual species decrease gradually with the addition of the successive PEG chains to the protein, lowering chromatographic resolution (Snider *et al.*, 1992). Additionally, processing time is long, buffer consumption is high and product dilution is significant. For IEX, a change in the charge properties of the protein is required. The attachment of PEG chains may remove a charged group at the site of covalent modification, sterically protect surface charges, and alter the pKa of ionizable surface residues via hydrogen bonding between PEG and the residues. However, in practice the pI of various PEGylated species does not vary significantly, preventing their separation (Fee and Van Alstine, 2006).

An attractive route to the resolution of PEG-protein conjugate populations at scale involves the use of reversed phase chromatography (RPC), due to the pendant PEG chains of the conjugates that can function as hydrophobic tags and dominate conjugate interactions with hydrophobic surfaces (Daly *et al.*, 2005). RPC is a powerful separation technique that has been used in the resolution of proteins with small differences in physical properties, including single amino acid variants. Additionally, it has been used to separate PEG-conjugates for analytical purposes. Youn et al (2004) separated positional isomers of PEG-grown hormone realizing factor (1-29). However, the preparative application of RPC is limited when protein denaturation in the mobile phase and on the stationary phase, appear together with many types of complex behaviors resulting in low recovery of the target product (Sokol *et al.*, 2003). In this context, ribonuclease A (RNase A) and apo-α-lactalbumin (apo-αLac) are two interesting proteins, which have shown structural changes when in contact with hydrophobic surfaces (Lu *et al.*, 1986; Jones and Fernandez, 2003). These proteins bracket a wide range of protein

structural stabilities as determined by the free energy of unfolding under neutral solution conditions and under RPC mobile phase conditions. RNase A is very stable with an unfolding free energy ($\Delta G_{unfolding}$) between 8.46 – 14.8 kcal/mole (Kim *et al.*, 2003, Pace at al., 1998) whereas apo-αLac is marginally stable with a $\Delta G_{unfolding}$ of 3.4 kcal/mol (Canet *et al.*, 2001, Vanderheeren *et al.*, 1996).

RNase A is a protein of interest because it has potential therapeutic application as an aspermatogenic and antitumor agent. Studies have shown an improvement in the therapeutic effect when RNase A is conjugated to PEG (Matousek J *et al.*, 2002). RNase A is a small protein (13,686 Da MW) with 124 amino acid residues that lacks tryptophan residues and has an isoelectric point at 9.3. Figure 1A shows the structure of ribonuclease A (Protein Data Bank structure 1AQP; Berman *et al.*, 2000) in space filling view. The lysine residues, each of the potential PEGylation sites, are indicated in green (residues 1, 7, 31, 37, 41, 61, 66, 91, 98, 104). Lys residues 1, 31, 37, 61, 66, 91, 98 are the most exposed to the solvent as assessed by the solvent accessible surface area calculation tool GETAREA (Fracziewicz and Braun, 1997). The n-terminal lysine is indicated as Lys 1 and the three most important residues for the catalysis are indicated as His 12, His 119 and Lys 41 (Raines, 1998).

α-Lactalbumin is also a small protein, containing 123 amino acids (14,200 Da MW) and has an isoelectric point between 4 and 5. It has been found that some forms of α-Lac can induce apoptosis in tumor cells, which suggests that the protein can fulfill many important biological functions (Permyakov and Berliner, 2000). Figure 1B shows the structure of apo-αlactalbumin (Protein Data Bank structure 1F6R, Berman *et al.*, 2000) in space filling view;

the apo form lacks a bound Ca^{2+} ion. The n-terminal glutamate residue is indicated as Glu 1 and each additional potential PEGylation site is indicated in green (lysine groups 5, 13, 16, 58, 62, 79, 93, 94, 98, 108, 114, 122). The most solvent-exposed Lys residues as assessed by GETAREA are residues 16, 62, 79, 108 and 114.

In this study both proteins, RNase A and apo-αLac, were conjugated with 20 kDa PEG chains. The PEGylation reaction was found to produce a mixture of mono-PEGylated, di-PEGylated and tri-PEGylated species, in addition to residual unconjugated species, even when the PEGylation conditions used favored the N-terminal PEGylation (Lee *et al.*, 2003). This molecular weight was selected, because current therapeutic studies focus on PEG with high molecular weight. Above 20 kDa the polymer is slowly cleared in the urine and feces, increasing its half life (Harris and Chess, 2003). The resulting PEG-conjugates for both proteins were separated via RPC under neutral pH conditions. The secondary and tertiary structures as well as the enzymatic activity (RNase A) of each species were assessed before and after the RPC processing in order to identify structural changes.

The present work explores the possibility that conjugated PEG chains can play dual roles as both group-specific separation tags and as structural stabilizers in RPC-based protein purification processes, allowing active PEG-conjugates to be resolved and recovered after processing via RPC. The PEG portion of PEG-conjugates is expected to dominate the conjugate-media interaction, whether the conjugate adopts a dumbbell-like structure consisting of a protein domain and (a) PEG domain(s) (Daly *et al.*, 2005) or a core-shell structure consisting of (a) PEG chain(s) wrapped around the surface of the protein (Fee and Van

Alstine, 2004). This should reduce the extent to which the protein portion of the conjugate can interact with the hydrophobic surface, thereby reducing protein unfolding on the surface of RPC media. While both C4 and C18 RPC media were explored, this work focuses on the use of C18 media as C4 media could not resolve the studied conjugates. In the same way, ethanol and acetonitrile were studied as organic modifiers; this study focuses on the use of acetonitrile as ethanol-containing mobile phases could not resolve the studied conjugates.

Materials and Methods

Materials

Bovine pancreatic ribonuclease A (cat. No. R5000 Lot 101K1403 and 093K0765) and α-bovine lactalbumin (cat. No. L5385 Lot 063K7009) were purchased from Sigma (St. Louis, MO). Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular mass of 20 kDa was obtained from Nektar Therapeutics (Huntsville, AB) and sodium cyanoborohydride from ICN Biomedicals Inc. (Costa Mesa, CA). All reagents were used as received. Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific), and potassium chloride (Sigma-Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific (Pittsburgh, PA). Silica-based C18 bulk chromatographic media (No. 218TPB1015, Lot. E030116-5-1) with 10 – 15 μm particle size, 300 Å pore size, 60 – 110 m^2/g surface area and 8 wt% carbon load was obtained from Vydac (Hesperia, CA).

PEGylation Reaction

A solution of RNase A or apo-αLac (6 mg) at 3 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 30 mg of the nominal 20 kDa molecular mass methoxy poly(ethylene glycol) butyraldehyde. When apo-αLac was reacted, the buffer contained 2mM EDTA. The reaction mixture was stirred rapidly for 17 – 19 h at 4 °C. After this time, the reaction mixture was stored no longer than 3 days at 4°C prior to analysis and separation. During this time, no significant further reaction was observed as shown in Figure 2 (A and B).

Analysis of PEGylated protein mixture by Size Exclusion Chromatography

The reaction mixture (2 mL) was analyzed via size-exclusion chromatography (Akta Explorer, Amersham Pharmacia, Uppsala, Sweden) using a Sephacryl S-300 column (1.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. When PEG-α-lactalbumin species were separated, the buffer contained 2 mM EDTA. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and diafiltered with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2. RNase A concentration was measured spectrophotometrically using absorbance at 280 nm and an extinction coefficient of 8,045 $AU \bullet M^{-1} \bullet cm^{-1}$. Apo-αLac concentrations were similarly determined using an extinction coefficient of 23,556 $AU \bullet M^{-1} \bullet cm^{-1}$.

Mass Spectrometry Analysis by MALDI-TOF

Each peak collected from SEC experiments was analyzed by mass spectrometry. Prior to analysis the samples were desalted using centrifugal filters (Microcon YM-3, Millipore). MALDI-TOF/MS was performed with a PerSeptive Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared by mixing 1 μL of sample and 1 μL of matrix.

Separation of PEG Conjugates by Reversed Phase Chromatography

Chromatographic separations were carried out with an Akta Explorer 100 integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). PEG conjugate mixtures were applied at room temperature to a Tricorn HR 5/10 column (Amersham Biosciences, 4.6 x 150 mm) packed with Vydac C18 media with 236 theoretical plates and a 1.6 peak symmetry factor, as assessed via the elution behavior of 100 μL pulses of 1% (v/v) acetone solution (the data represents the average of three replicate). Gradient elution was carried out at a flow rate of 0.6 mL/min with solvent A (6 mM sodium phosphate, pH 7.2) and solvent B (20% v/v phase A in acetonitrile). The following segmented gradient profile was chosen: 43.8% B over 5 min, 43.8 – 55% B over 24.4 min, 55% B – 100% B over 5 min. The UV response was measured at 215 nm, 254 nm and 280 nm. Fractions corresponding to each respective peak were collected separately and stored at 4°C for structural analysis.

Protein secondary and tertiary structure

Far-UV CD spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). Spectra were collected in the 190-310 nm wavelength range at room temperature using a 1 mm path length cell. The secondary structures of the protein samples were estimated with the CDPro software (Sreerama and Woody, 2000). A mean residue molecular weight of 110 was determined for RNase A from the molecular mass, 13,686 Da, and the number of residues, 124 (Raines, 1998). The mean residue molecular weight of apo-αLac was determined to be 115, based on a molecular mass of 14,200 Da and 123 amino acids (Permyakov and Berliner, 2000). Near-UV spectra for tertiary structure characterization were similarly collected from 250 to 350 nm using a 1 cm path length cell. In both types of spectral acquisitions the scan speed was 100 nm/min, the bandwidth was 1 nm, and at least 10 scans were accumulated per spectrum. A protein-free control spectrum was recorded for each condition and subtracted from the protein spectra.

Enzymatic Activity of RNase A conjugates

The intrinsic activity of unmodified RNase A, mono-PEG-RNase A, and di-PEG-RNase A was determined using the RNase Alert fluorescence assay (Integrated DNA Technologies, Inc., Coralville, IA). The substrate is a short RNA oligomer with fluorescein and a quencher on opposite ends of the chain. Fluorescence spectroscopy was measured with a SpectraMax M2 microplate reader (Molecular Devices, Co., CA) spectrofluorometer. The baseline of the substrate intensity was monitored at excitation and emission wavelengths of 490 nm and 520 nm, respectively. One microliter of the desired protein solution (0.002 mg/mL) was added to 1 mL of substrate solution in buffer Tris 50 mM pH 7.2 (10 - 20 nM) and it was mixed for

about 5 s. The initial rate of the increase in fluorescence emission was used to estimate the enzyme activity in terms of the specificity constant k_{cat}/K_M (Kelemen *et al.*, 1999). A t-test was done in order to determine if there was significant difference between the measured enzymatic activities of the samples.

Results and Discussion

Analysis by Size Exclusion Chromatography

Size exclusion chromatography has been used to separate PEGylated proteins, since the PEG conjugates have a larger hydrodynamic volume than globular proteins of the same mass. However, this technique has shown insufficient resolving power at production scale and has failed to separate other PEGylation variants (Seely and Richey, 2001; Youn *et al.*, 2004). In the present research, SEC was used to characterize the products of the PEGylation reaction. Figures 2A and 2B show the SEC behavior of RNase A/PEG and Apo-αLac/PEG reaction mixtures, respectively. It is clear that at least two PEGylated species were produced in both cases, even though the conditions of the reaction favor N-terminal modification. The first peak in Figure 2A elutes at 48 mL which represents ~30% of the reaction products. The second, representing ~70% of the products, elutes at 58 mL and the third at 96 mL. Similar behavior was observed in Figure 2B, where the first peak elutes at 50 mL (~16% of the reaction products), the second at 60 mL (~38% of the reaction products) and the third at 89 mL (corresponding to the ~45% of the reaction products). Completion of the reaction was not achieved, despite the use of excess activated PEG: the third peak comprises unmodified protein from the PEGylation reaction mixture as determined by the overlaps with the peak obtained when unmodified protein is loaded on the SEC column. After storage (~42 h), we

observed an increment in the area ratio of di-PEG/mono-PEG species, providing evidence that even when the conditions favor the N-terminal modification, other reactions take place.

Each peak was collected from the FPLC and subjected to MALDI-TOF-MS. The PEG and the native proteins used in the reaction were also analyzed by mass spectrometry. The average mass/charge ratio of the PEG was 22,000 (*m/z*), and the unmodified RNase A and apo-αLac were 13,686 (*m/z*) and 14,200 (*m/z*), respectively. The MALDI-TOF spectra correspond to di-PEGylated species in the first peak fraction (*m/z*: 57,686 di-PEG RNase A and *m/z*: 58,200 di-PEG apo-αLac), mono-PEGylated species in the second peak fraction (*m/z*: 35,686 mono-PEG RNase A and *m/z*: 36,200 mono-PEG apo-αLac) and unmodified protein in the third peak (*m/z* = 13,686 unmodified RNase A and *m/z*: 14,200 unmodified apo-αLac). The presence of the PEG molecule in the conjugates was identified from the corresponding mass spectra detected by MALDI-TOF MS, due to the appearance of each species as a broad peak that matched the polydispersity of the starting activated PEG material.

Separation by Reversed Phase Chromatography

Reverse phase chromatography was used in order to improve the separation of the conjugates and to reduce the processing time as compared to SEC. Previous studies have shown that it is possible to separate different PEGylated species using as mobile phases water with 0.1% trifluoroacetic acid (TFA) and acetonitrile as a modifier containing 0.1% TFA (Lee and Park, 2002; Seely and Richey, 2001; Youn *et al.*, 2004). In general, TFA and formic acids are the traditional buffers for low pH work since their volatility makes them compatible with mass spectrometry detection and facilitates sample recovery after chromatographic separation. In

this study the separation was obtained using a 6 mM phosphate buffer, pH 7.2 and acetonitrile as a modifier (Figure 3), in order to maintain a gentle environment for the proteins.

For the RPC chromatograms shown in Figure 3A, the initial mobile phase conditions were set so that unmodified RNase A is not retained and therefore, retention is due solely to the attached PEG chains. PEG is generally considered a hydrophilic polymer, although under reverse phase conditions the effect of the addition of PEG to a protein is to increase its hydrophobicity and, in turn, its retention time relative to the unmodified protein (Snider *et al.*, 1992). PEG can assume configurations with differing polarities, allowing it to adapt to the polarity of a solvent or to a hydrophobic surface. In polar solvents, the PEG chain prefers polar conformations and the gauche conformation around the C-C bond is favored, and in nonpolar solvents, nonpolar structures are favored (Karlsström and Engkvist, 1997). In the same way, in the vicinity of hydrophobic surfaces, the polymer will adopt more nonpolar conformations, since those are stabilized by the hydrophobic media; far from hydrophobic stationary phases, the polymer adopts a more polar conformation (Karlsström and Engkvist, 1997). As a consequence, in RPC, the separation of PEGylated species depends on the hydrophobicity of the protein relative to the PEG (Seely and Richey, 2001). Under the experimental conditions used, the first retained peak that elutes is expected to represent the monoPEG-RNase A species followed by the diPEG-RNase A species and higher levels of modification thereafter. The previous behavior was confirmed by mass spectrometry analysis. The third peak observed in Figure 3A corresponds to a tri-PEGylated species, triPEG-RNase A; this species was not identified in the SEC separations due to low resolution of this technique. These results are in agreement with the reaction conditions used as the largest peak area obtained corresponds to a mono-PEGylated species; the reaction strongly favored N-

terminal modification. The total protein recovered from the column (unmodified, monoPEG, diPEG and triPEG) was 73% of the total amount of protein injected; subsequent regeneration steps with 100% buffer B (80% acetonitrile – 20% 6 mM phosphate buffer) yielded the remaining protein which had been tightly bound.

When the apo-αLac/PEG reaction mixture was applied to the RPC column under the same conditions (Figure 3B), the unmodified protein was retained. This behavior could be expected since apo-αLac is a more hydrophobic protein with a Bigelow hydrophobicity of 1,107 cal/res compared with 880 cal/res for RNase A (Bigelow, 1967). Two peaks (at ~ 4 and ~ 6 mL) corresponding to the unmodified protein are observed, suggesting a surface induced unfolding of the protein (Benedek *et al.*, 1984). To determine if this behavior was independent of the presence of PEG, pure apo-αLac was applied to RPC under the same conditions (Figure 4). It is clear that at least two peaks at the same elution volumes (at ~4 and ~6 mL) are observed, as in the case of Figure 3B, showing that this behavior is not caused by the presence of PEG. In Figure 3B, as expected, the third and forth peaks correspond to the monoPEG-apo-αLac and diPEG-apo-αLac species, respectively. The total protein recovery from the column with those conditions was ~72% (unmodified, monoPEG and diPEG protein) of the protein injected. As in the case of RNase A proteins, subsequent regeneration steps with 100% buffer B (80% acetonitrile – 20% 6 mM phosphate buffer) yielded the remaining protein which had been tightly bound. It is interesting to observe that PEGylated species for this protein are eluted as one peak (at ~14 mL and ~16.5 mL). It is also possible to observe that the peak corresponding to the monoPEGylated-apo-αLac species is shifted towards higher elution volume, compared with the corresponding monoPEG RNase A species, while the volume elution of the

diPEGylated-apo-αLac species is maintained. This suggests that, when the protein is retained under the tested conditions, the behavior of the species containing just one attached PEG molecule is a combination of the properties of the PEG and the protein, while for the proteins containing two molecules of PEG, the elution behavior is dominated by the PEG. Similar results were obtained with SEC retention: as more PEG is grafted to a protein, the SEC behavior shifts from the native protein's position towards that of the PEG (Fee and Van Alstine, 2004). The difference presented in the retention volume retention of the unmodified apo-αLac could be caused by small variations in temperature, since its behavior is sensitive to temperature and no changes were observed when only unmodified protein was subjected to RPC under the same conditions (Figure 4).

In Figures 2 and 3 it is possible to observe that the SEC separation consumes more buffer (~100 mL) than does the RPC separation (~25 mL). Thus, SEC technique requires more processing time, ~200 min as compared to RPC, which requires ~40 min at the flow rates used. Additionally, in the case of the PEG-RNase A reaction mixture separation, it was possible to achieve a better resolution of the mixture using the RPC technique.

Analysis of second and tertiary structure by circular dichroism

The effect of PEGylation on the structure of the protein was evaluated by acquiring CD spectra of the unmodified and modified protein collected from the SEC analysis in both the near- and far-UV regions. Both the secondary and tertiary structure contents of the PEG conjugates as isolated via SEC are similar to those of the unmodified proteins. The CD spectra in the far UV (Figure 5A and 5B, upper) and the secondary structure contents (symbol

◆ in Figure 6A and 6B), as estimated using CD Pro (Sreerama and Woody, 2000), for the PEGylated proteins are in good agreement with those obtained from the unmodified protein in buffer. In the same way, the tertiary structure of PEG-RNAse A (diPEG-RNase A ●, monoPEG-RNase A ▲ and unmodified RNase A ◇ in Figure 7A) and PEG-apo-αLac (diPEG-apo-αLac ●, monoPEG-apo-αLac ▲ and unmodified apo-αLac ◇ in Figure 7B) conjugates is intact for each sample as demonstrated by the large negative mean residue ellipticity at ~275 nm and at ~272 nm, respectively, representing the presence of optically active cysteine and aromatic chromophores (Strickland, 1972; Robbins and Holmes, 1970).

The effect of the hydrophobic mobile phases was analyzed by recording the second and tertiary structure of the proteins in solutions with acetonitrile concentrations similar to those of the mobile phase during elution. The CD spectra in the far UV are shown for both proteins in Figure 5A and B (middle). It is possible to observe differences between the proteins in contact with the solvent and the corresponding unmodified protein in buffer. These differences are reflected in the secondary structure content shown in Figure 6A and 6B (symbol ■) for RNase A and apo-αLac, respectively. There is a slight difference between the proteins in buffer and those in the solutions containing acetonitrile. For the RNase A species, the difference appeared in the ordered and distorted helix structure content, while in the apo-αLac species it appeared in both the helix structures and in the regular β-strand structure. Those changes can be caused by the exposure of hydrophobic residues to the environment; organic solvents are known to destabilize protein structure.

The CD spectra of the unmodified and PEGylated RNase A (DiPEG-RNase A ○, monoPEG-RNase A △ unmodified RNase A ◇ in Figure 7A) in RPC mobile phases in the near-UV region show that the proteins exhibited no significant change when they are exposed to the solvent. RNase A structure is unperturbed in the RPC solvent, suggesting that PEG molecules just serve as a hydrophobic retention tag. In contrast, when unmodified and PEG conjugated apo-αLac species were exposed to the mobile phase, the tertiary structure was lost as shown by the near dissappearance of the negative band at 260-290 nm (diPEG-apo-αLac (_ _ _.), monoPEG-apo-αLac (-.-.-.) unmodified apo-αLac (____) in Figure 7B). PEGylation improves apo-αLac structure retention, but not nearly enough to avoid denaturation in the RPC mobile phase, at least under the conditions studied. The loss of the tertiary structure of both unmodified and PEGylated apo-αLac species seems to be accompanied by a small increase in the ordered and distorted helix (Hr and Hd) content and a small decrease in the regular β-strand (Sr) and the unordered content (Unrd) (symbol ■ in Figure 6B).

The secondary and tertiary structures of the proteins were also taken after RPC processing in order to see any structural changes arising from exposure to the hydrophobic support. The CD spectra in the far UV are shown in Figure 5A for RNase A and in Figure 5B for apo-αLac (lower). It is possible to observe differences between the unmodified protein in buffer and the proteins after RPC process. The secondary structure estimates after RPC processing of PEG-conjugated RNase A presented small changes in the helix (Hr and Hd) and strand β sheet (Sr) content (symbol △ Figure 6A). The near UV CD spectra of the RNase A species after RPC had a somewhat more negative molar ellipticity peak around 275 nm than that of the unprocessed protein (diPEG-RNase A (_ _ _.), monoPEG-RNase A (-.-.-.), unmodified

RNase A (____) in Figure 7A). The more negative molar ellipticity peak intensities around 275 nm, unaccompanied by shifts in band positions, indicates that the microenvironment of at least one of the Tyr residues becomes more hydrophobic in the average conformations of the native-state ensemble on exposure to the RPC stationary phase (Kim *et al.*, 2003). In the case of the secondary structure of the unmodified and PEGylated apo-αLac after RPC (symbol △ in Figure 6B), the proteins present a small increase of the regular and distorted helix (Hr and Hd) content, while a small decrease of regular and distorted β-strand (Sr and Sd) content is observed. Even when the changes are small, it is possible to observe that the unmodified apo-αLac suffered the largest change. The tertiary structure of these proteins was not recorded because it was lost by the contact with the organic solvent before RPC process.

Enzymatic activity of RNase A conjugates

The enzymatic activity of each RNase A conjugate was evaluated before and after the reverse phase separation. The estimated k_{cat}/K_m values in solution of the triPEG, diPEG, monoPEG and unmodified protein are shown in Table 1. The intrinsic activity values reported in this study for the unmodified RNase A (Park and Raines, 2000; Kelemen *et al.*, 1999; James and Woolley, 1998) and PEGylated RNase A (Daly *et al.*, 2005), collected from SEC experiments are of the same order of magnitude of those reported in the literature for similar substrates. The results show that the PEGylated conjugates are active. A t-test indicated that there was no significant difference between the activity of the unmodified protein and the monoPEG-RNase A collected from the SEC separation. However, a significant difference between the activities of the unmodified protein and the diPEG-RNase A species was noted.

A significant decrease in the intrinsic activity of the unmodified protein obtained after RPC processing is observed. This decrease could be caused by structural perturbations caused by the RPC process. This is interesting in that the unmodified RNase A has at most only transient interaction with the stationary phase since it elutes in the flow-through fraction; the initial mobile phase modifier concentration may be responsible in this case. The intrinsic activity of mono-PEG, obtained from SEC and RPC experiments, is within the standard error of that of the unmodified protein. The mono-PEG species had a greater intrinsic activity than the unmodified protein, despite the fact that it was retained longer within the RPC column and was exposed to higher acetonitrile concentrations in the mobile phase. The di-PEG RNase A obtained from SEC experiments had significantly lower intrinsic activity than the unmodified protein in buffer. However, there was no significant difference between the activities of the unmodified protein in buffer and the di-PEG protein obtained after RPC processing. This is likely due to the presence of the lower activity tri-PEGylated species that co-elutes with the diPEG conjugate, decreasing the overall intrinsic activity of the sample. The catalytic efficiency, or specificity constant, of a mixture tri/diPEG-RNase A, taking into account the relative amounts of these species from RPC analysis, should be $(4.74 \pm 0.69)\times10^{-7}$ $M^{-1}\bullet s^{-1}$. This value is higher than the corresponding specificity constant of $(3.4 \pm 0.32) \times10^{-7}$ $M^{-1}\bullet s^{-1}$ obtained from the diPEG protein fraction with SEC analysis. However, it is important to keep in mind that the fraction collection strategy for SEC may have reduced the relative amount of diPEG protein present in the sample. The results obtained with tri-PEG conjugate collected after RPC processing suggests that there is steric interference between the third PEG molecule and the substrate binding site. These enzymatic activity results confirm that PEG molecules did not modify the structure of the RNase A protein, that the active site of mono- and di-

PEGylated protein remained accessible to the substrate and that PEGylation may provide some degree of protection against activity loss during processing with RPC media.

Conclusions

This study has shown that PEG-conjugates can be separated with better resolution and in less time using RPC at neutral pH rather than SEC. RPC allowed the separation of the three species (mono, di and tri PEG-RNase A) whereas only two conjugate species were identified using SEC. In the case of RNase A species, since the unmodified protein was not retained, it was possible to adjust the chromatographic conditions so that the separation was dominated by the attached polymer and not by the properties of the protein. The findings reported here suggest either that PEGylation confers some structural stability to the protein or that the PEG chain(s) shield(s) the protein from denaturing interactions with the hydrophobic support. PEG modification was not sufficient to protect the structure in RPC mobile phases in the case of apo-αLac. This opens the possibility that RPC may be useful for the separation of PEG conjugates in cases where the intrinsic stability of the target protein is sufficient.

Acknowledgements

The authors wish to acknowledge the financial support of the National Science Foundation (Grant No. BES-0214183) and the Biotechnology Research Chair of Tecnológico de Monterrey (Grant CAT005). The assistance of Mark Bier and the Center for Molecular Analysis in making MALDI mass spectrometry measurements is also gratefully acknowledged.

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Figure 1 Protein structures obtained from the RCSB PDB. A) **Ribonuclease A (1AQP)** the inscriptions refer to the location of the N-terminal residue, Lys 1, and the three residues most important for the catalysis: His 12, His 119 and Lys 41. B) **Apo-αLac (1F6R)** the figure shows the structure of a hexamer, the inscriptions refer to each chain and their N-terminal residue, Glu 1. The lysine groups are shown in green and the hydrophobic (Phe, Tyr and Trp) groups in blue.

Figure 2 A) Size exclusion chromatography profile of Native RNase A (dotted line) and PEG/RNase A reaction mixture (solid line) after 17.5 and 43 h. B) Size exclusion chromatography profile of Native Apo-αLac (dotted line) and PEG-Apo-αLac reaction mixture (solid line) after 17 and 42 h. The vertical lines show where the fractions were collected. Flow rate 0.5 mL/min.

Figure 3 RPC behavior of PEG-protein reaction mixture as a function of sample size: 50 μL (▩), 100 μL (◆), 200 μL (▲). A) PEG-RNase A and **B)** PEG-apo-αLac. Profile elution using 0.6 mL/min: mobile phases, phosphate buffer 6mM pH 7.2 (A) and 80% Acetonitrile-20% phase A (B); stay 5 min at 43.8% B, gradient from 43.8% to 55% B in 24.4 min, gradient from 55% B to 100% B. Dead volume 2.1 mL.

Figure 4 RPC behavior of unmodified Apo-α-lactalbumin as a function of sample size: 50 μL (▩), 100 μL (◆), 200 μL (▲). Profile elution using 0.6 mL/min: mobile phases, phosphate buffer 6mM pH 7.2 (A) and 80% Acetonitrile-20% phase A (B); stay 5 min at

43.8% B, gradient from 43.8% to 55% B in 24.4 min, gradient from 55% B to 100% B. Dead volume 2.1 mL.

Figure 5 CD Spectra in the far UV of unmodified and PEGylated proteins A) RNase A and B) apo-αLac. In 25 mM sodium phosphate buffer pH 7.2 (upper): unmodified protein ●, monoPEGylated ■, diPEGylated ◆. In solution 60% 6 mM phosphate buffer pH 7.2 – 40% acetonitrile (middle): unmodified protein ○, monoPEGylated □, diPEGylated ◇. After RPC (lower): unmodified (X), monoPEGylated (-), diPEGylated (+), unmodified apo-αLac second peak (✳). In each graphic the line of the corresponding unmodified protein in phosphate buffer is included as comparison.

Figure 6 Secondary structure of unmodified and PEGylated proteins in 25 mM sodium phosphate buffer pH 7.2 ◆, in solution 60% 6 mM phosphate buffer pH 7.2 – 40% acetonitrile ■, and after RPC △. A) Unmodified RNase A, monoPEG-RNase A and diPEG-RNase A. **B)** Unmodified apo-αLac, monoPEG- apo-αLac and diPEG-apo-αLac, the unmodified apo-αLac corresponds to the second peak obtained from RPC experiments. Far UV CD spectra of each protein in solution were deconvoluted into secondary structure fractions using the CD Pro (Sreerama and Woody, 2000) software package. Hr represents ordered helix content; Hd, distorted helix; Sr, regular β-strand; Sd, distorted β-strand; Trn, turns; and Unrd, unordered. Error bars represent the standard deviation of three repetitions. The samples in phosphate buffer and in phosphate buffer – acetonitrile were taken from SEC experiments.

Figure 7 Tertiary structure of unmodified and PEGylated proteins. A) Near UV CD spectra of diPEG-RNase A ●, monoPEG-RNase A ▲ and unmodified RNase A ◆ in 25mM sodium phosphate buffer, pH 7. DiPEG-RNase A ○, monoPEG-RNase A △ unmodified RNase A ◇ in solution 60% 6 mM phosphate buffer pH 7.2 – 40% acetonitrile. DiPEG-RNase A (_ _ _), monoPEG-RNase A (. . . . .), unmodified RNase A (_____) after RPC. **B)** Near UV CD spectra of diPEG-apo-αLac ●, monoPEG-apo-αLac ▲ and unmodified apo-αLac ◆ in 25mM sodium phosphate buffer, pH 7. DiPEG-apo-αLac (_ _ _), monoPEG-apo-αLac (. . . . .) unmodified apo-αLac (_____) in solution 60% 6 mM phosphate buffer pH 7.2 – 40% acetonitrile. The samples in phosphate buffer and in phosphate buffer – acetonitrile were taken from SEC experiments.

Figure 8 Enzymatic activity of the RNase A conjugates. Results are reported in terms of the mean measured catalytic efficiency or specificity constant k_{cat}/K_M. SEC indicates size exclusion chromatography samples collected in 25 mM phosphate buffer, pH 7.2. RPC indicates reverse phase chromatography samples collected using the 25 mM phosphate buffer, pH 7.2 as mobile phase A and 80% acetonitrile – 20% phase A as a mobile phase B; samples were diluted the phosphate buffer prior to enzymatic assay. Error bars indicate one standard deviation for multiple ($n \geq 3$) assays on pooled samples. An asterisk indicates statistical significance relative to the unmodified SEC sample ($p \leq 0.05$; Student's *t*-test).

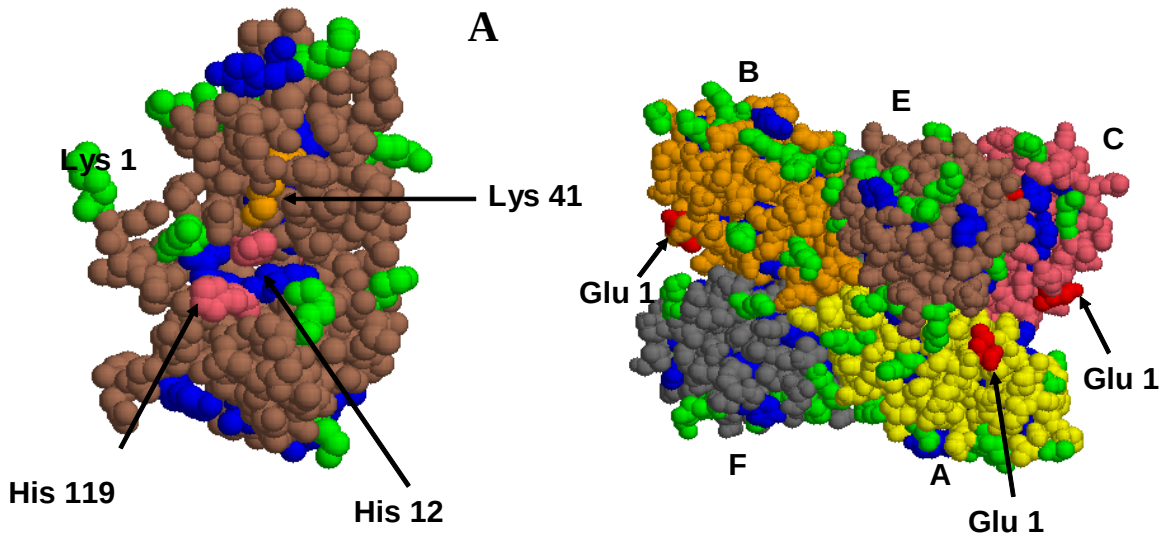


Figure 1

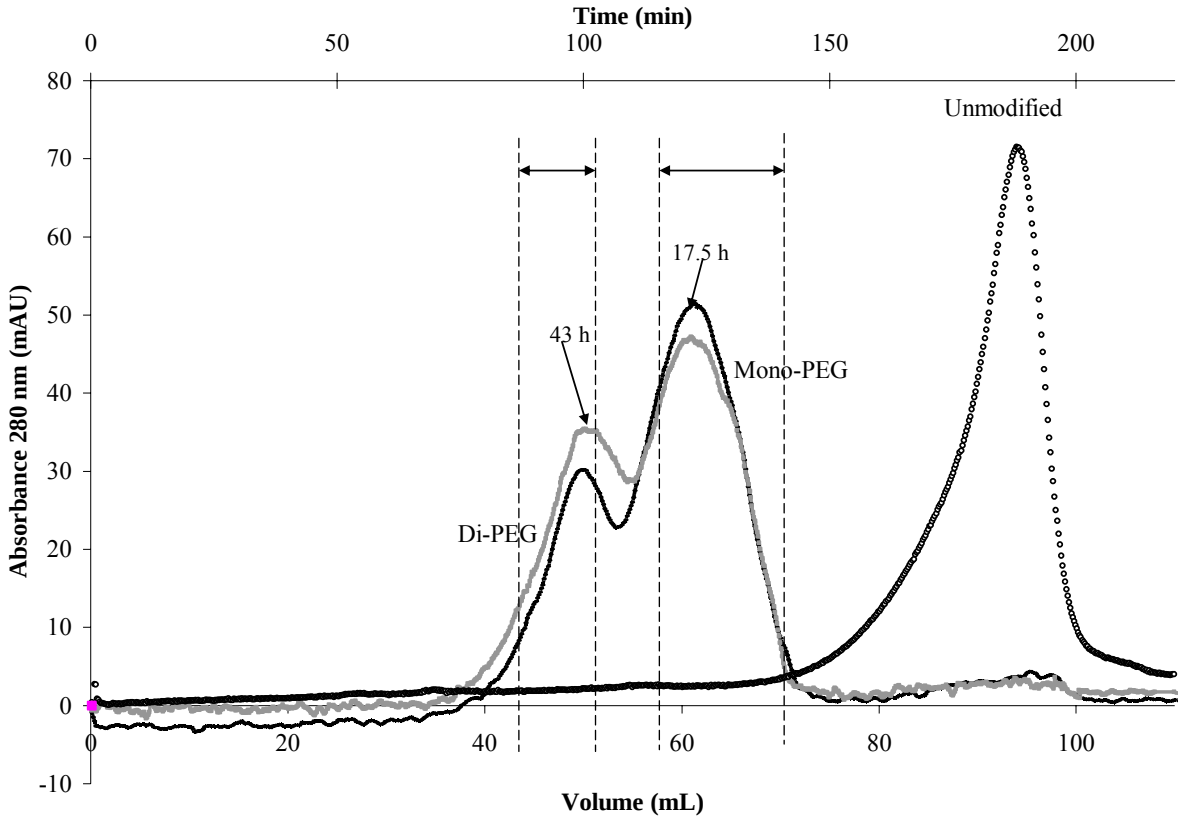
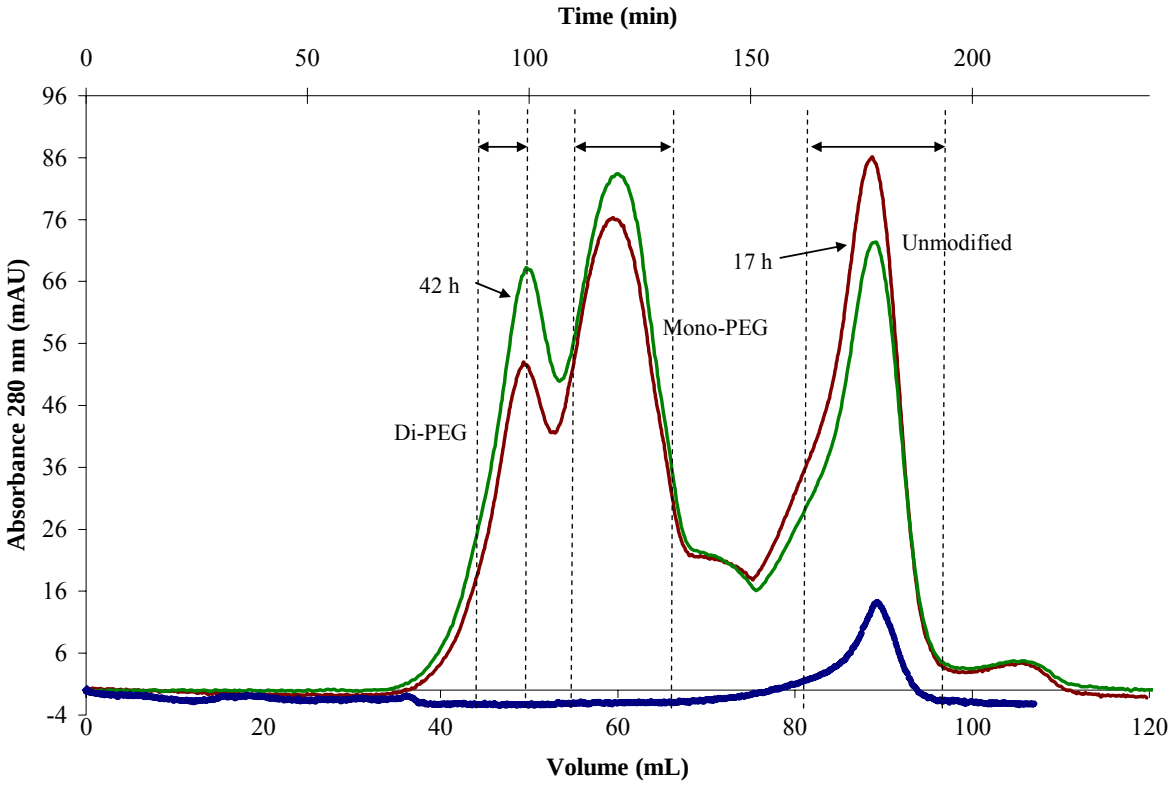

Time (min)
0
50
100
150
200
80
70
60
50
40
30
20
10
0
-10
Absorbance 280 nm (mAU)
Unmodified
17.5 h
43 h
Mono-PEG
Di-PEG
0
20
40
60
80
100
Volume (mL)

Figure 2 A)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100
150
200
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86
76
66
56
46
36
26
16
6
-4
Absorbance 280 nm (mAU)
42 h
17 h
Unmodified
Mono-PEG
Di-PEG
0
20
40
60
80
100
120
Volume (mL)

Figure 2 B)

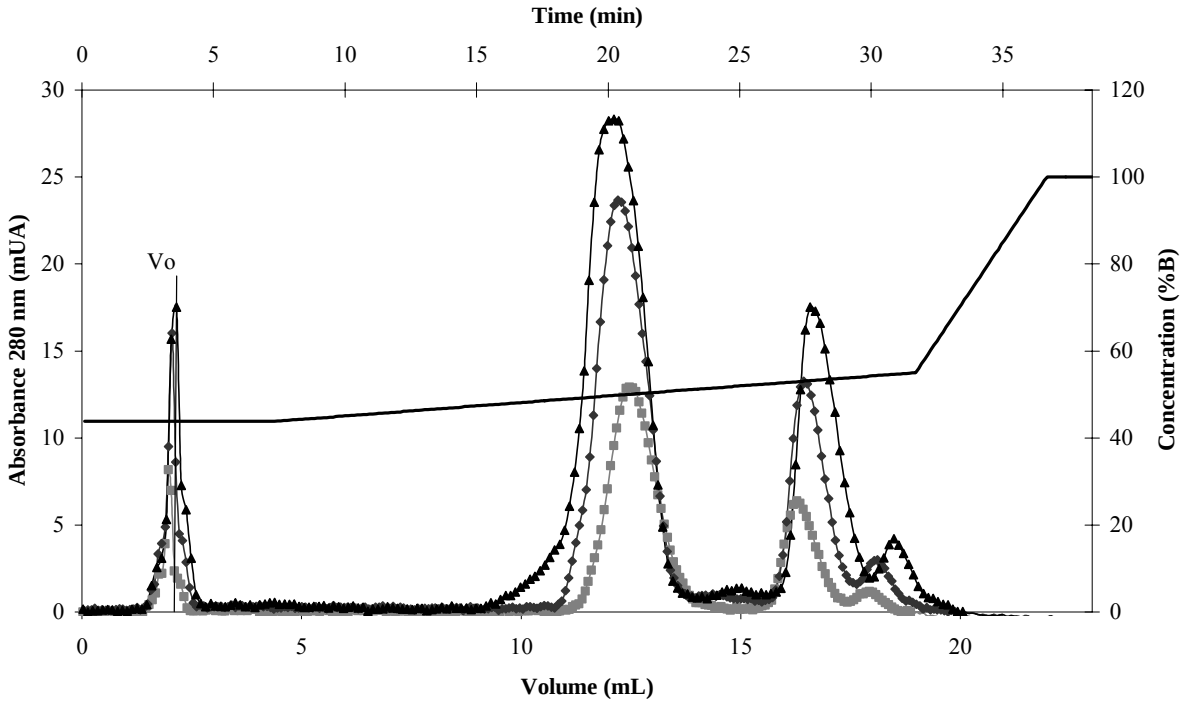
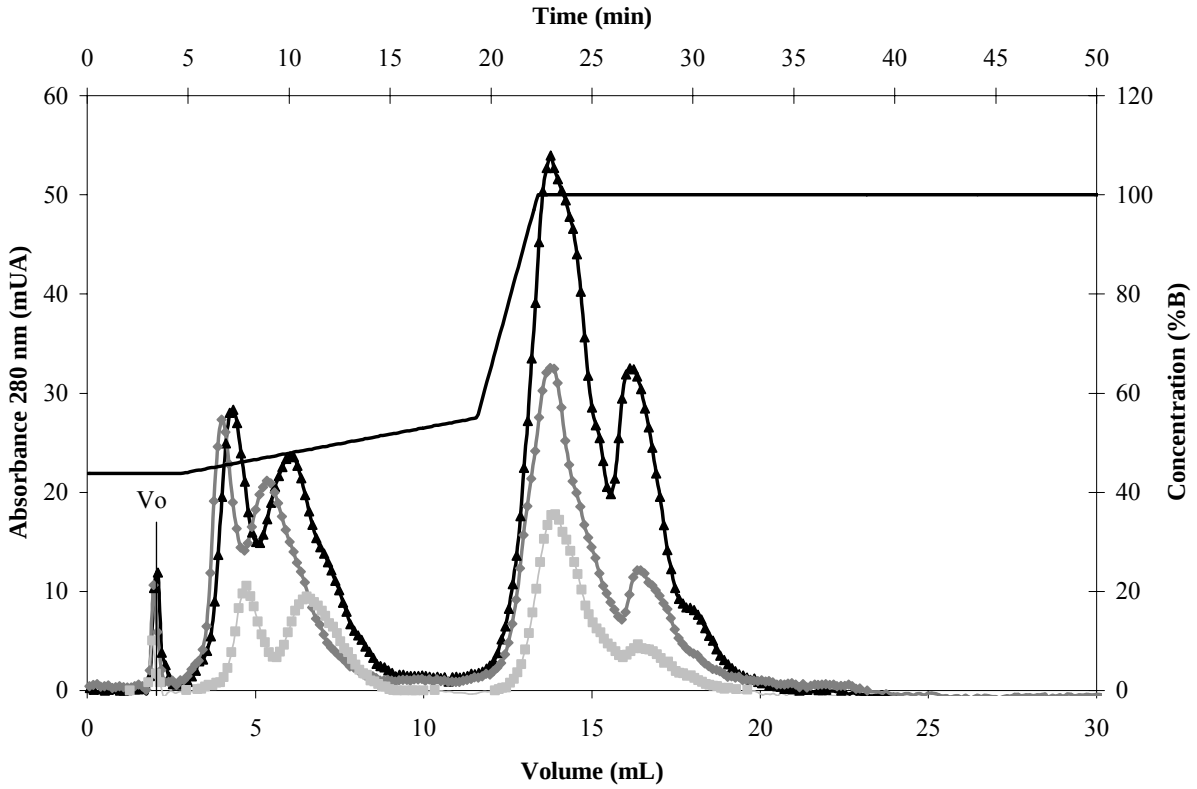

Time (min)
0
5
10
15
20
25
30
35
Absorbance 280 nm (mUA)
Concentration (%B)
Vo
Volume (mL)
0
5
10
15
20
0
5
10
15
20
25
30
0
20
40
60
80
100
120

Figure 3 A)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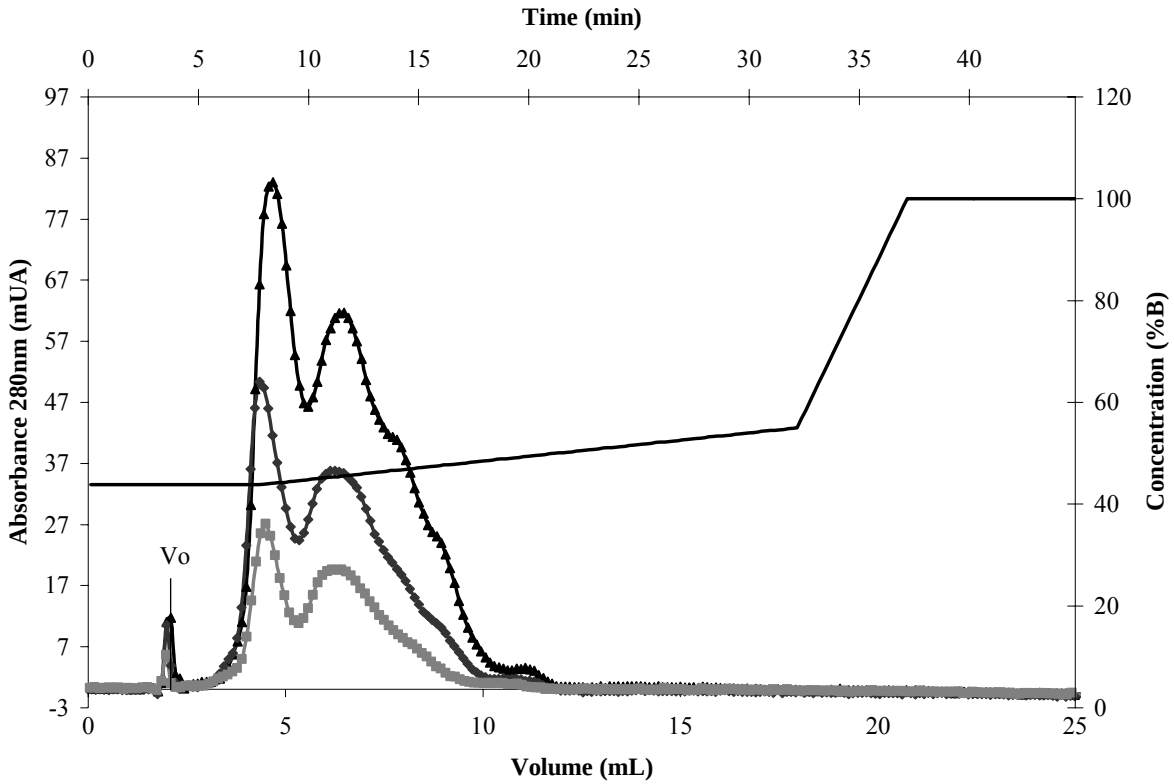
10
15
20
25
30
35
40
45
50
60
50
40
30
20
10
0
120
100
80
60
40
20
0
Absorbance 280 nm (mUA)
Concentration (%B)
Vo
0
5
10
15
20
25
30
Volume (mL)

Figure 3 B)


Time (min)
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5
10
15
20
25
30
35
40
Absorbance 280nm (mUA)
97
87
77
67
57
47
37
27
17
7
-3
Concentration (%B)
120
100
80
60
40
20
0
Vo
0
5
10
15
20
25
Volume (mL)

Figure 4

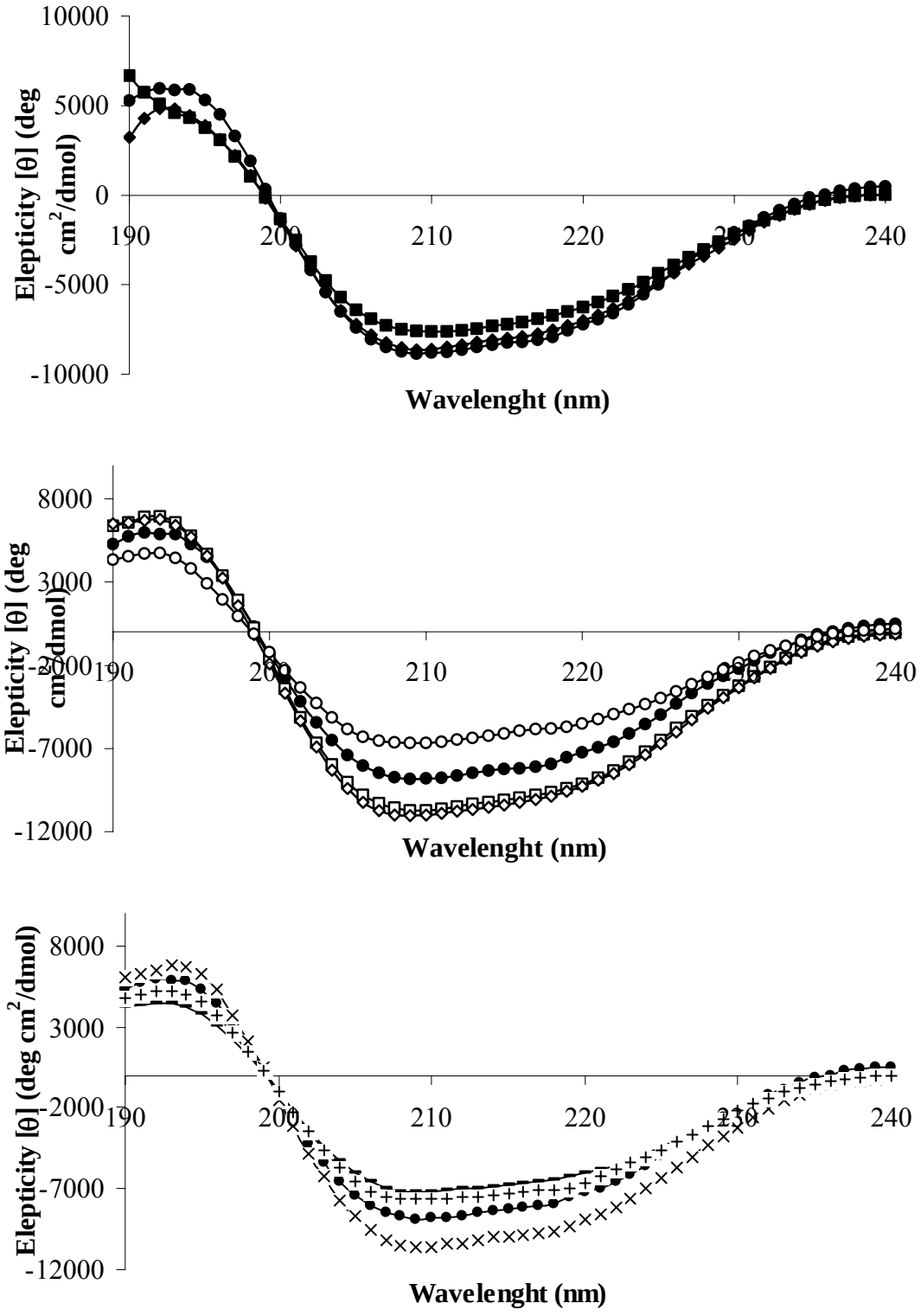

10000
5000
0
-5000
-10000
190
200
210
220
230
240
Elepticity [θ] (deg cm²/dmol)
Wavelenght (nm)
8000
3000
-2000
-7000
-12000
190
200
210
220
240
Elepticity [θ] (deg cm²/dmol)
Wavelenght (nm)
8000
3000
-2000
-7000
-12000
190
200
210
220
230
240
Elepticity [θ] (deg cm²/dmol)
Wavelenght (nm)

Figure 5 A)

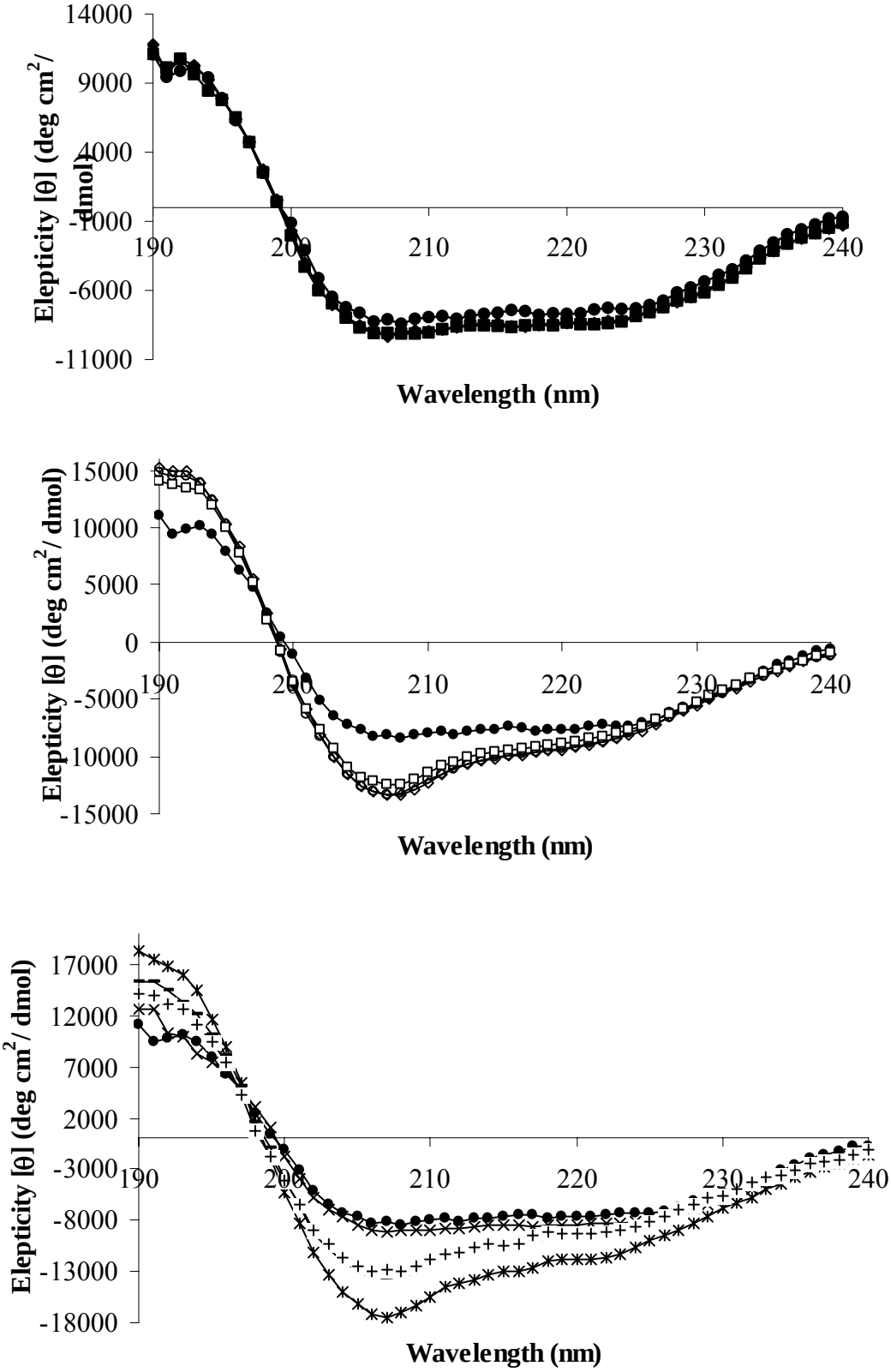

14000
9000
4000
-1000
-6000
-11000
190
200
210
220
230
240
Elepticity [θ] (deg cm2/ dmol)
Wavelength (nm)
15000
10000
5000
0
-5000
-10000
-15000
Elepticity [θ] (deg cm2/ dmol)
Wavelength (nm)
17000
12000
7000
2000
-3000
-8000
-13000
-18000
Elepticity [θ] (deg cm2/ dmol)
Wavelength (nm)

Figure 5 B)

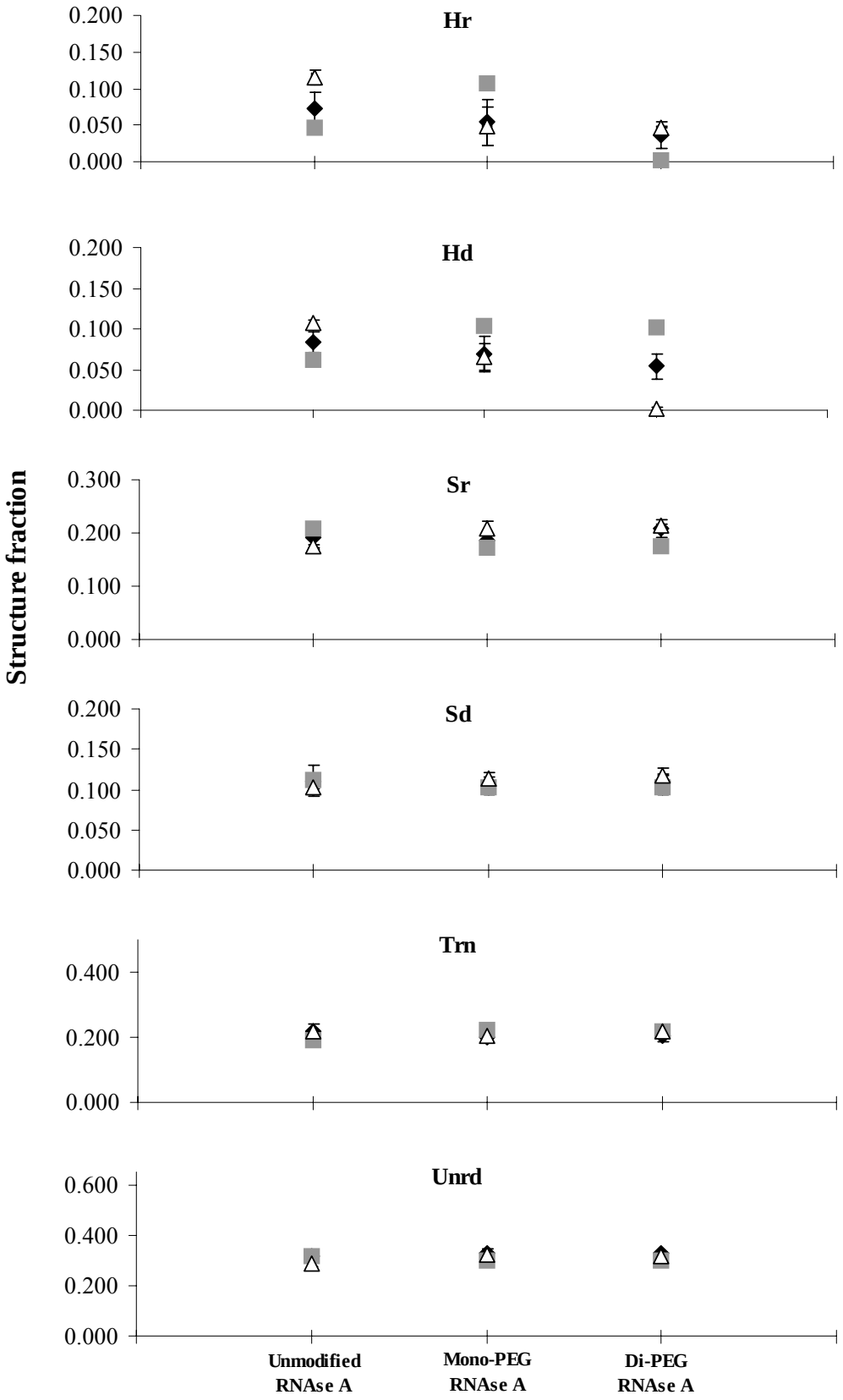

Structure fraction
Hr
0.200
0.150
0.100
0.050
0.000
Hd
0.200
0.150
0.100
0.050
0.000
Sr
0.300
0.200
0.100
0.000
Sd
0.200
0.150
0.100
0.050
0.000
Trn
0.400
0.200
0.000
Unrd
0.600
0.400
0.200
0.000
Unmodified RNAse A
Mono-PEG RNAse A
Di-PEG RNAse A

Figure 6 A)

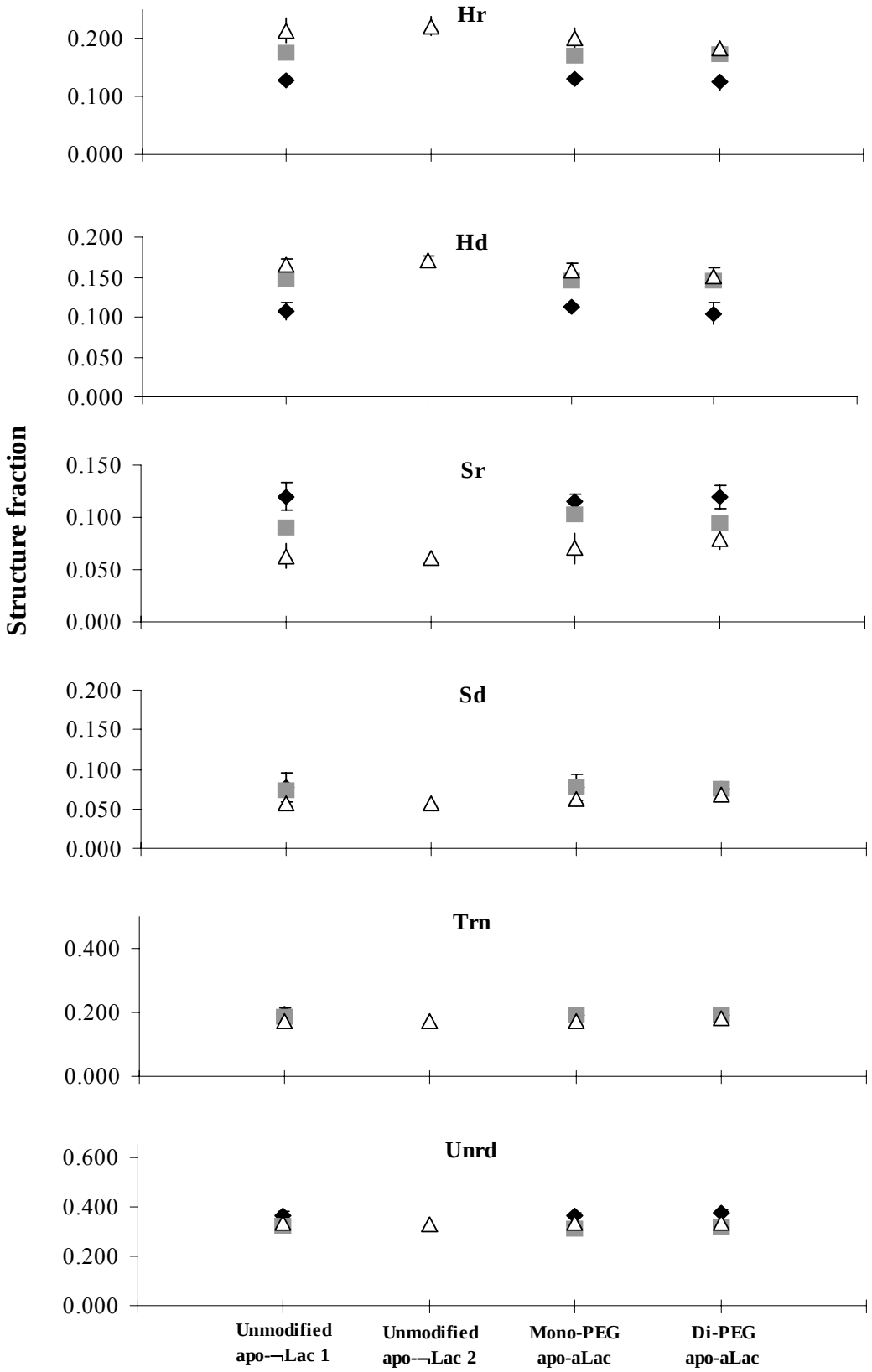

Hr
Hd
Sr
Sd
Trn
Unrd
Structure fraction
0.200
0.100
0.000
0.150
0.050
0.400
0.600
Unmodified apo-¬Lac 1
Unmodified apo-¬Lac 2
Mono-PEG apo-aLac
Di-PEG apo-aLac

Figure 6 B)

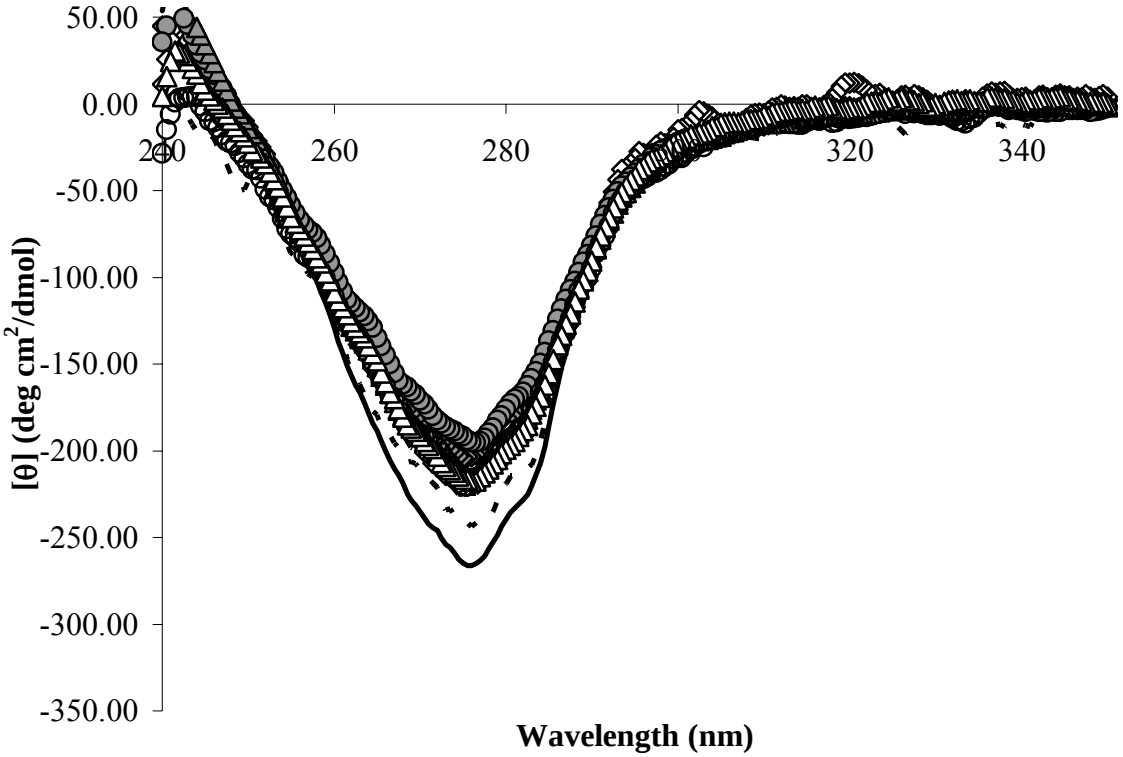
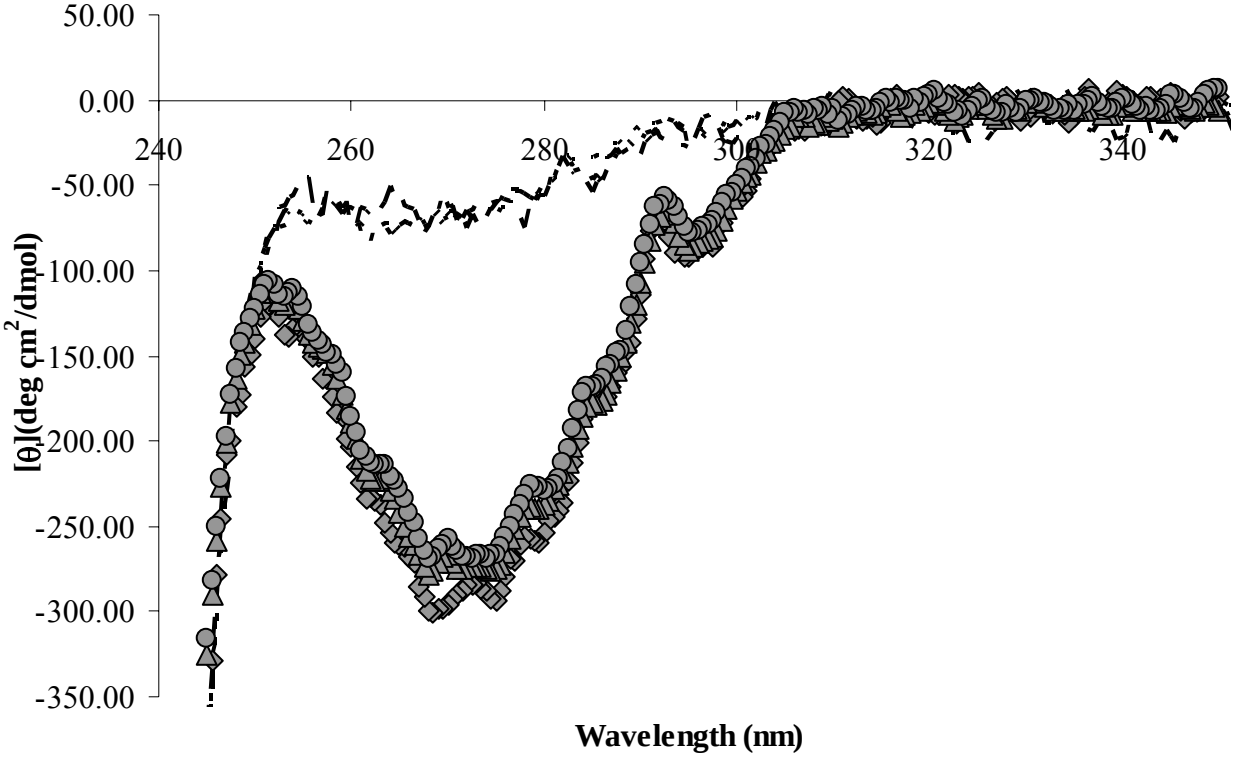

50.00
0.00
-50.00
-100.00
-150.00
-200.00
-250.00
-300.00
-350.00
240
260
280
320
340
[θ] (deg cm2/dmol)
Wavelength (nm)

Figure 7 A)


50.00
0.00
240
260
280
300
320
340
-50.00
-100.00
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-200.00
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[θ](deg cm2/dmol)
Wavelength (nm)

Figure 7 B)

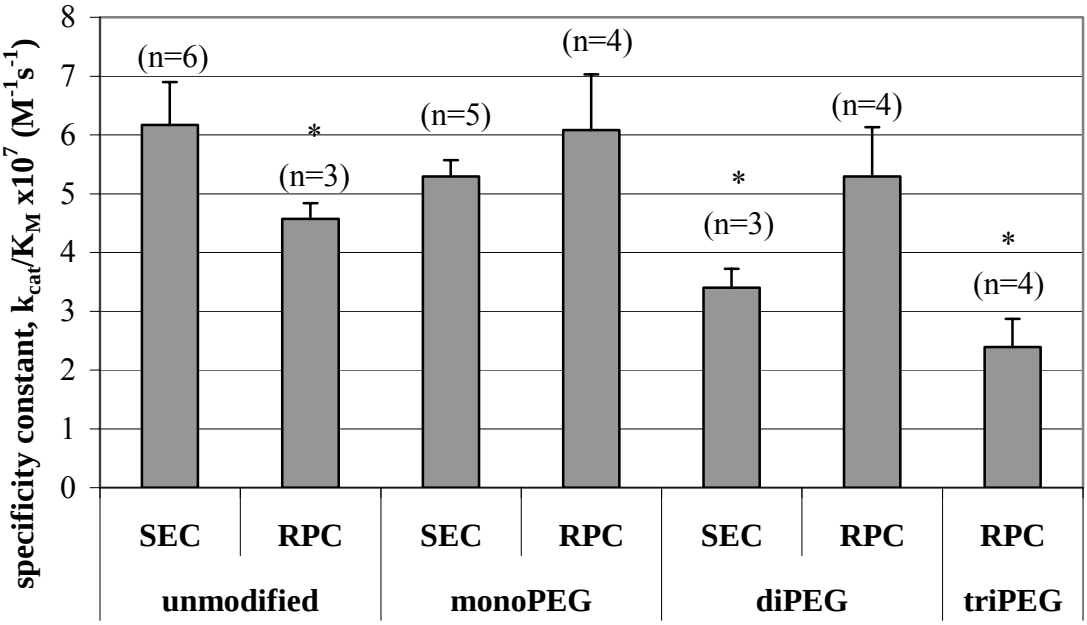
specificity constant, k_{cat}/K_M x10^7 ($M^{-1}s^{-1}$)
8
7
6
5
4
3
2
1
0
(n=6)
*
(n=3)
(n=5)
(n=4)
*
(n=3)
(n=4)
*
(n=4)
SEC
RPC
SEC
RPC
SEC
RPC
RPC
unmodified
monoPEG
diPEG
triPEG

Figure 8

Effect of pH and temperature on the separation of Ribonuclease A and apo-αLactalbumin PEG-conjugates by reversed phase chromatography

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Abstract

The PEGylation of pharmaceutical proteins improves their therapeutic efficacy. The PEGylation product obtained depends on the conditions under which the conjugation reaction takes place. Typically, PEGylation products are a family of species characterized by a distribution in the number and position of attached polyethylene glycol (PEG) groups. The resolution of these protein mixtures represents a challenge to the separation step. An attractive alternative to the resolution of PEG-protein conjugate populations at scale involves the use of reversed phase chromatography (RPC). However, in some cases, RPC results in low recoveries due to protein denaturation in the mobile phase and on the stationary phase. The effects of the mobile phase could be minimized if less stringent conditions such as neutral pH instead of low pH could be used without affecting resolution. Temperature is another parameter that could modify the retention behavior of PEGylated proteins in reversed phase processes since previous studies have reported that the retention of PEG increases with increasing column temperatures. In this work, the effect of pH (at 2.0, 7.0 and unbuffered) and temperature (15, 25 and 35°C) on the separation of PEG-conjugates using RPC separation was evaluated. PEGylated conjugates of ribonuclease A (RNase A) and apo-αLactalbumin (apo-αLac) were selected as model protein systems. Conjugate mixtures were separated with a C18 column using a gradient elution profile with acetonitrile as an organic modifier. It was observed that the pH of the mobile phase does not play a significant role in the chromatographic behavior of PEG-conjugates, when the unmodified protein is not retained. However, when the unmodified protein is retained, the effect of the pH on the PEGylated proteins is similar to that observed for the unmodified species. It was demonstrated that temperature affects the chromatographic separation of PEG-conjugates in a similar way in

which it affects the separation of the neat polymer. This information could be useful in the optimization of separation processes of other PEG-conjugate species.

Key words: PEGylated proteins, polyethylene glycol, protein separation, conjugate heterogeneity

Introduction

Polypeptides show great potential to be used as therapeutic agents. However, their effectiveness is occasionally reduced by their susceptibility to enzymatic degradation, short circulation time, low solubility and other factors. (Harris and Chess, 2003). The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins – a process known as "PEGylation"- mitigates these problems and improves their therapeutic properties (Bailon and Berthold, 1998). During the last several years, the FDA has approved PEGylated proteins to treat different diseases in humans, including bovine adenosine deaminase (PEG Mw 5,000 Da attached) for severe combined immunodeficiency disease, α interferon (PEG Mw 12,000 Da attached) for hepatitis C and L-asparaginase (PEG Mw 5,000 Da attached) for acute lymphoblastic leukemia (Greenwald *et al.*, 2003). Additionally, a variety of PEG conjugated proteins have shown superior pharmacological properties relative to the unmodified forms (Clark *et al.*, 1996; Hinds *et al.*, 2000; Lee *et al.*, 1999).

The PEGylation products obtained strongly depends on the conditions under which the reaction takes place and is based on both the protein and the attachment chemistry. A family of species may result from PEGylation owing to the multiple sites that are typically available for modification leading to a distribution of species both in number and position of attached PEG (Zalipsky, 1995). The separation and characterization of number and position variants have gained importance because only few of the PEGylated species may provide optimal biological properties (Lee and Park, 2002; Youn *et al.*, 2004). As a result, it is desirable to resolve heterogeneous conjugate mixtures into effective subpopulations using appropriate separation strategies (Vicentelli *et al.*, 1999) and/or to limit the breadth of the conjugate

distribution during the conjugation reaction itself (Deiters *et al.*, 2004; Koumenis *et al.*, 2000). Near-term solutions at scale will likely be concentrated on the population resolution approach.

Size Exclusion Chromatography (SEC) is the natural choice to resolve PEGylation variants, since the PEG conjugates have larger molecular sizes than their unmodified counterparts. But this technique is not sufficient to resolve micro-heterogeneity. For SEC, the relative difference in molecular size between individual species decreases gradually with the addition of successive PEG chains to the protein, lowering chromatographic resolution (Snider *et al.*, 1992). Ion exchange (IEX) is another popular alternative to the separation of conjugate species. For IEX, a difference in the charge properties of the proteins to be separated is required. PEGylation may indeed affect charge properties: the attached PEG may protect surface charges, conjugation chemistries targeting charged amino acid residues will remove charges from the protein surface, and attaches PEG chains may alter the micro-environment and in turn alter local residue pKas and hydrogen bonding patterns. However, in practice it is found that the pI of various PEGylated species does not vary significantly, preventing their separation via IEX (Fee and Van Alstine, 2006).

An attractive alternative to the resolution of PEG-protein conjugate populations at scale involves the use of reversed phase chromatography (RPC). RPC is a powerful separation technique that has been used in the resolution of proteins with small differences in physical properties, including single amino acid variants. However, in some situations, RPC results in low recoveries caused by protein denaturation in the mobile phase and on the stationary phase, and the appearance of many types of complex behaviors (Sokol *et al.*, 2003). The effects of the mobile phase on the protein structure are not only caused by the organic modifier, but also

by the low pH traditionally used in the process. Typically, low pH (~2.0) mobile phases, maintained with trifluoroacetic acid (TFA) or formic acid, are used. The volatility of these acids makes them compatible with mass spectrometry detection and facilitates sample recovery after chromatographic separation. The pH of the mobile phase affects the separations, since the degree of ionization of the both the proteins to be separated and the residual silanol groups of silica-based RPC media depends on the pH. Studies have reported that acids induced a wide range of effects on protein structure: from initial complete unfolding to a complete molten globule folding (Fink *et al.*, 1994; Goto *et al.*, 1990). It seems promising to study the effect of pH on the separation of PEGylated protein, in order to determine if a less stringent environment could be used during the separation without affecting the resolution.

Temperature is another parameter that could modify the retention behavior of proteins in reversed phase processes, especially if those proteins have attached PEG molecules. High temperatures induce protein thermal unfolding and the retention of PEG itself on hydrophobic media increases with increasing column temperatures (Andersen *et al.*, 2001). Temperature causes conformation changes in chain PEG at room temperature they are coiled in solution, as the temperature increases the degree coiling will be reduced and the molecular surface area will increase (Skuland *et al.*, 2003). It seems interesting to study the effect of the temperature on the separation of PEGylated protein in order to determine if it is possible to improve the resolution of the species through temperatures changes without causing increased denaturation.

Two proteins were chosen as experimental model systems, ribonuclease A (RNase A) and apo-α-lactalbumin (apo-αLac). RNase A has potential therapeutic application as an

aspermatogenic and antitumor agent. Studies have shown an improvement of the therapeutic effect when RNase A is conjugated to PEG (Matousek J *et al.*, 2002). RNase A is a small and very stable protein with an unfolding free energy ($\Delta G_{unfolding}$) between 8.46 – 14.8 kcal/mole (Kim et al., 2003, Pace at al., 1998) with 124 amino acid residues (13,686 Da MW) that lacks tryptophan residues and an isoelectric point at 9.3. α-Lactalbumin is a small protein with marginal stability with a $\Delta G_{unfolding}$ of 3.4 kcal/mol (Canet et al., 2001, Vanderheeren et al., 1996), containing 123 amino acids (14,200 Da MW) and an isoelectric point between 4 – 5. It has been found that some forms of α-Lac can induce apoptosis in tumor cells, which suggests that the protein can fulfill many important biological functions (Permyakov and Berliner, 2000).

During this research, both proteins were conjugated with 20 kDa PEG chains. The PEGylation reactions produced a mixture of mono-PEGylated and di-PEGylated species in the case of apo-αLac; an additional tri-PEGylated species was obtained only in the case of RNase A. In addition, the PEGylation reaction products contain residual unconjugated species. Although most of the current FDA approved PEGylated drugs have PEG 5 kDa chains attached, the 20 kDa molecular weight was selected because therapeutic studies suggest the use of PEG with high molecular weight is beneficial: above 20 kDa, the polymer is slowly cleared in the urine and feces, increasing its half life (Harris and Chess, 2003).

The effect of the pH and temperature on the separation of PEG-conjugates using RPC was evaluated comparing the chromatographic behavior under different conditions. Three mobile phases: pH 2.0 (containing 0.1% TFA), pH 7.0 (phosphate buffer) and un-buffered (12 mM

NaCl) solutions were tested. When testing the effect of the temperature on the resolution of the PEG-conjugates, three particular values (15, 25 and 35°C) were selected. The obtained results should be useful in determining the optimal conditions for the resolution of PEGylation variants.

Materials and Methods

Materials

Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular mass of 20kDa was purchased from Nektar Therapeutics (Huntsville, AB). Sodium cyanoborohydride was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Silica-based C18 bulk chromatographic media (No. 218TPB1015, Lots E030116-5-1 and E040519-4-5) with 10 – 15 μm particle size, 300 Å pore size, 60 – 110 m^2/g surface area and 8 wt% carbon load was obtained form Vydac (Hesperia, CA). Bovine pancreatic ribonuclease A (cat. No. R5000, Lot 093K0765) and α-bovine lactalbumin (cat. No. L5385, Lot 063K7009) were purchased from Sigma Aldrich (St. Louis, MO). All reagents were used as received. Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific, Pittsburgh, PA), and potassium chloride (Sigma-Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific and WVR.

PEGylation Reaction and analysis

An amount of protein (RNase A or apo-αLac) was dissolved in a 100 mM sodium phosphate buffer, pH 5.1 with 20 mM sodium cyanoborohydride in order to obtain 3 mg/mL solution. Two milliliters of the protein solution were added to a vial containing 30 mg of the nominal 20

kDa molecular mass methoxy poly(ethylene glycol) butyraldehyde. When apo-αLac was reacted, the buffer contained 2mM EDTA, in order to maintain the calcium ion-free apo form of the protein. The reaction mixture was mixed during for 17 – 19 h at 4 °C using a magnetic stirrer. After this time, the reaction mixture was stored at 4°C prior and used within 3 days of preparation.

The PEGylation reaction products were analyzed by size exclusion chromatography and mass spectrometry. During the storage time, no significant further reaction was observed, according to the size exclusion chromatography analysis (SEC, Akta Explorer, Amersham Pharmacia, Uppsala, Sweden). Two milliliters of the reaction mixture were applied to a Sephacryl S-300 column (Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2 containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. When PEG-α-lactalbumin species were separated, the buffer contained 2 mM EDTA. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and buffer-exchanged with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2.

Each peak collected from SEC experiments was analyzed by mass spectrometry. Prior to analysis, the samples were desalted using centrifugal microconcentrators (Microcon YM-3, Milipore). MALDI-TOF/MS was performed with a PerSeptive (Framingham, MA) Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid

(Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared mixing 1 μL of sample with 1 μL of matrix.

Separation of PEG Conjugates by Reversed Phase Chromatography

Chromatographic experiments were carried out with an Akta Explorer 100 integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). PEG conjugate mixtures were applied to a Tricorn 5/100 column packed with Vydac C18 media (Amersham Biosciences, 4.6 x 150 mm); column efficiency characterization experiments with 1% acetone pulses gave 236 theoretical plates.

The effect of pH on the chromatographic separation of the PEG-conjugates was tested using three different mobile phases. A 0.1% TFA solution was the starting point as this represents the typical mobile phase employed in analytical RPC. Low pH (2.0) is used to ensure that residual silanols on silica-based media are protonated to avoid secondary retention effects from electrostatic interactions. The second aqueous phase employed was 12 mM sodium chloride, to keep the ionic strength constant but without pH buffering. The neutral pH was achieved using 6 mM phosphate buffer, pH 7.0. In order to ensure the apo conformation of αLac was maintained, 2mM EDTA was added to the mobile phases A.

Gradient elution was carried out at a flow rate of 0.6 mL/min with the different solvents A and solvent B (20% v/v phase A in acetonitrile). The following segmented gradient profile was chosen: 43.8% B over 5 min, 43.8 – 55% B over 24.4 min, 55% B – 100% B over 5 min. The UV absorbance of the column effluent was monitored at 215 nm, 254 nm and 280 nm.

Experiments using the 6 mM phosphate buffer, pH 7.0 solvent A and 20% phase A – 80% acetonitrile solvent B gradients were done at different temperatures (15, 25 and 35°C). The temperature was controlled by placing the mobile phase reservoirs and the column in a water bath at the desired temperature. The samples were equilibrated to the desired temperature for 30 min prior to injection.

Protein secondary and tertiary structure of RNase A conjugates

The effects of the pH on protein secondary and tertiary structure were analyzed only for the RNase A species, since previous studies have shown that apo-αLac species lose their tertiary structure when they are exposed to the solvent (acetonitrile) at neutral pH while RNase A species do not (Cisneros-Ruiz *et al.*, 2006). Far-UV CD spectra were recorded using a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). Spectra were collected in the 190-310 nm wavelength range at room temperature using a 1 mm path length cell. The secondary structures of the protein samples, monoPEG, diPEG and unmodified RNase A, were estimated with the CDPro software (Sreerama and Woody, 2000). A mean residue molecular weight of 110 was determined for RNase A from the molecular mass, 13,686 Da, and from the number of residues, 124 (Raines, 1998). Near-UV spectra for tertiary structure characterization were similarly collected from 250 to 350 nm using a 1 cm path length cell. In both types of spectral acquisitions, the scan speed was 100 nm/min, the slit width was 1 nm, and at least 10 scans were accumulated per spectrum. A protein-free control spectrum was recorded for each condition and subtracted from the protein spectra.

Enzymatic Activity of RNase A conjugates

The intrinsic activity of unmodified RNase A, mono-PEG-RNase A, and di-PEG-RNase A collected from RPC experiments was determined using the RNase Alert fluorescence assay (Integrated DNA Technologies, Inc., Coralville, IA). The substrate is a short RNA oligomer with fluorescein and a quencher on opposite ends of the chain; as RNase A cleaves the oligomer, the fluorophore quenching is relieved and fluorescence increases. Fluorescence emission was measured with a SpectraMax M2 microplate reader (Molecular Devices, Co., CA) spectrofluorometer. The baseline of the substrate intensity was monitored at excitation and emission wavelengths of 490 nm and 520 nm, respectively. One microliter of the desired protein solution (0.002 mg/mL) was added to 1 mL of substrate solution in 50 mM Tris buffer, pH 7.2 (yield a substrate concentration of 10 or 20 nM) and it was mixed for about 5 seconds. The initial rate of the increase in fluorescence emission was used along with the enzyme concentration to estimate the enzymatic activity in terms of the specificity constant k_{cat}/K_M (Kelemen *et al.*, 1999).

Results and Discussion

Effect of pH on the chromatographic behavior of the PEGylated proteins

Figure 1A shows the chromatographic behavior of the PEG-RNase A conjugates using different aqueous mobile phases at different pHs. In Figure 1A, the first retained peak that elutes (at ~ 12 mL) represents the monoPEG-RNase A species followed by diPEG- RNase A (at ~16 mL) species and triPEG-RNase A (at ~ 18 mL), respectively. The behavior was confirmed by mass spectrometry analysis. These results showed little difference in the

retention volumes of the PEGylated RNase A species, showing that the pH plays an insignificant role in the retention of the PEG-RNase A species.

The behavior of the PEGylation reaction mixture and unmodified apo-αLac (Figure 1B) was studied under the same chromatographic conditions as RNase A. It is possible to observe that the behavior of the PEG-apo-αLac conjugates was affected by the pH of the mobile phase. The proteins were eluted later in the gradient, towards higher concentrations of organic modifier, using mobile phases with low pH (0.1% TFA) or un-buffered solutions (12 mM NaCl). These results can be explained because of structural modifications that are caused by the mobile phases, exposing the hydrophobic residues thus causing stronger retention of the proteins. Additionally, when a mobile phase with neutral pH was used, the unmodified protein eluted as more than one peak (see Figure 1B, middle), characteristic behavior for proteins that exist in both folded and unfolded states during processing with RPC.

In order to prove that the changes presented in the chromatographic behavior of PEG-apo-αLac were caused by structural changes in the protein domain and not in changes in the polymer domain, the chromatographic behavior of the corresponding unmodified proteins was studied. It has been reported that acidic pH can result in so-called acid-denatured molten globule states (Goto Y *et al.*, 1990), changing the protein structure so that hydrophobic residues become more exposed at the surface of the protein. If the hydrophobic residues are more exposed using 0.1% TFA as a mobile phase, longer retention time would be expected during RPC.

The behavior of the unmodified RNase A was analyzed (Figure 2A) under the same conditions. Since this protein elutes at the dead volume of the column in the gradient used for the PEG conjugates, the gradient was changed to observe the retention behavior with the mobile phases. Using a mobile phase A with low pH (0.1% TFA), the unmodified protein is more strongly retained than with the other mobile phases. Also, this mobile phase gives a more symmetric peak shape. In reverse phase chromatography, the retention of ionizable compounds is both mobile phase and buffer pH dependent (Espada and Rivera-Sagredo, 2003). These results suggest that the unmodified protein suffers structural changes during the RPC process. However, because the unmodified protein is not retained under these conditions, the results suggest that it is possible to adjust the mobile phase conditions so that the separation is dominated by the attached polymer and not by the properties of the protein. Because of this, changes in the conditions that affect the chromatographic behavior of the protein may not affect the chromatographic behavior of the conjugates.

The case of PEG-apo-αLac is different, since the unmodified protein is retained under the conditions under which the conjugate separation is done. Figure 2B shows the chromatographic behavior of the unmodified protein. It is possible to observe a similar tendency to that of the corresponding PEG-conjugates: larger retention volumes using 0.1% TFA or 12 mM NaCl as mobile phases and multiple peaks with the 6 mM phosphate buffer, pH 7.0. These results suggest that the protein suffers structural changes during the RPC process, regardless of whether the conjugated PEG chains are present. So, conjugate retention behavior as a function of mobile phase pH depends on the impact of pH on the retention of the protein domain of the conjugate. For the case of RNase A, the protein itself is unretained

under the conjugate separation conditions studied and changes in pH do not affect conjugate retention. For the case of apo-αLac, where both unmodified and modified forms are retained, pH has a significant impact on unmodified protein retention and also impacts the conjugate retention.

The best resolution between the PEGylated species of apo-αLac, corresponding to the last two peaks in Figure 1B (middle), was obtained with the mobile phase containing 6mM phosphate buffer pH 7.2. This mobile phase also presents the lowest volume retention for the unmodified protein. These results suggest that the weaker the retention of the unmodified protein, the more the attached PEG domains the chromatographic behavior. At this point it is possible to obtain the separation of the PEGylated species.

Analysis of second and tertiary structure by Circular Dichroism (CD)

RNase A is stable in the RPC solvent at neutral pH, as we have shown previously (Cisneros-Ruiz *et al.*, 2006), which suggests that PEG molecules just serve as a hydrophobic retention tag in this case. However, there is a considerable loss of tertiary structure (Figure 3) of both unmodified and PEGylated protein when exposed to the mobile phase with low pH (0.1% TFA). These results match with the secondary structure results which show an increase in the unordered fraction (Unrd) (Figure 4), characteristic of unfolded protein. They also support the chromatographic behavior of the unmodified protein at low pH (Figure 2), which suggests that under this condition the protein is unfolded. The results show that the change in the structure for RNase A is caused by the low pH and not by the solvent used as the mobile phase

modifier. The structural changes suggest that under the conditions studies it is clear that attached PEG confers no additional stability to the protein.

Enzymatic activity of RNase A conjugates

The effect of different pH conditions on the enzymatic activity of the unmodified and PEGylated RNase A after the RPC process was evaluated. It has been reported that RNase A undergoes reversible denaturation in solution under the chromatographic conditions, low pH and mixed aqueous/organic solvents (Cohen *et al.*, 1985) and it presents a molten globule state, when it is treated with acid (Kumar *et al.*, 1994)

The unmodified protein recovered from RPC using the 6mM phosphate, pH 7.2 mobile phase previously reported (Cisneros-Ruiz *et al.*, 2006) and the protein recovered using 12mM NaCl both exhibited a slight decrease in activity compared with the protein recovered using 0.1%TFA as a mobile phase (Table 1). A potential explanation involves the fact that the protein suffers random unfolding when it is in contact with the hydrophobic media, presented as a broad peak during RPC elution (Figure 2A) and some of the protein is miss-folded when the conditions are changed in order to measure the enzymatic activity. In contrast, when the protein is eluted using 0.1%TFA as a mobile phase, it is already unfolded, and once the protein is under stable conditions, the folding process follows a specific order.

Temperature effect on the chromatographic behavior of PEGylated proteins

The temperature effect was evaluated at 15, 25 and 35 °C using the pH 7.0 mobile phase, since a better separation of the PEG-apo-αLac proteins was obtained at this pH. Figures 5A and 5B show the effect of the temperature on the chromatographic behavior of the PEGylation

mixture reaction of RNase A and apo-αLac, respectively. In both cases, it is possible to observe that lower temperature results in a weaker hydrophobic interaction between the PEG-conjugates and the stationary phase; conjugates elute at lower concentrations of mobile phase modifier B and unretained, unmodified protein (first peak) elution is unaffected. Previous reports have shown that PEG-molecules at different temperatures present different conformations. They can present a compact dihedral helical structure (called the meander form) or an extended open coil (called the zigzag form), each having intrinsically different retention characteristics. At lower temperatures, the PEG molecules present a compact structure (meander) decreasing contact surface area, making the chromatographic behavior more similar to the unmodified protein. In contrast, the zigzag form has a larger molecular surface area than the corresponding meander form, which makes stronger the interaction between the C18 ligands of the stationary phase and the PEG molecules (Escott and Mortimer, 1991). Furthermore, the solubility of PEGs in aqueous solutions decreases with increased temperature and this correlates with the increase in retention as the temperature increases (Skuland, *et al.*, 2003). This behavior at different temperatures of PEG-molecules might explain the chromatographic behavior of the PEGylated proteins.

Figure 5B also shows a shift in the elution volume of the peak corresponding to the unmodified apo-αLac (first peak). At higher temperatures, the protein presents higher elution volume. This shift could be attributed to stronger hydrophobic interactions, which increases with increasing temperature.

Knowing the effect of the pH and temperature on the PEGylated and unmodified proteins, it is possible to use these effects in order to obtain good resolution between PEGylated species. According to the properties of the protein, these parameters could be useful in order to optimize not only the separation process, but also to define better conditions to preserve the protein structure.

Conclusions

It was observed that the pH of the mobile phase does not have a significant effect on the chromatographic behavior of PEGylated RNase A, under conditions where the unmodified protein is not retained and the PEG molecules dominate the elution behavior. However, when the protein is retained and its chromatographic behavior is affected by the pH of the mobile phase, as in the case of apo-αLac the behavior of both PEGylated and unmodified proteins is affected in a similar manner. This information is useful in order to optimize separation conditions to obtain better resolution between conjugates species and allow the use of less unfolding environments.

The PEG-molecules presents two different conformations, one of these is a compact dihedral helical structure (called meander form) and the other is an extended open coil (called the zigzag form). At higher temperature the zigzag conformation is the most predominant, which has a larger molecular surface area. This polymer form has better interaction between the polymer and the hydrophobic support, due to higher retention volume of the PEGylated proteins. These conformational changes and the fact that increased temperature strengthen hydrophobic interactions might explain the observed effect. This parameter could be used in

order to improve the resolution between PEG-conjugates, especially if the protein is thermostable.

Acknowledgements

The authors wish to acknowledge the financial support of the National Science Foundation (Grant No. BES-0214183) and the Biotechnology Research Chair of Tecnológico de Monterrey (Grant CAT005).

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Figure 1 Effect of pH on the separation of PEG-conjugates isomers by RPC: A) PEG-RNase A and **B)** PEG-apo-αLac. Mobile phases: phase A, 0.1% TFA (upper), 6 mM phosphate buffer pH 7.0 (middle) and 12 mM sodium chloride (lower), in every case the phase B was 80% acetonitrile – 20% phase A. Profile elution using 0.6 mL/min: stay 5 min at 43.8% B, gradient from 43.8% to 55% B in 24.4 min, gradient from 55% B to 100% B. The volume injection was 200 μL for the case of RNase A and 100 μL in the case of apo-αLac.

Figure 2 Effect of pH on the elution behavior of unmodified proteins by RPC: A) RNase A and **B)** apo-αLac**.** Mobile phases: phase A, 0.1% TFA (upper), 6 mM sodium phosphate buffer pH 7.2 (middle) and 12 mM sodium chloride (lower), in every case the phase B was 80% acetonitrile – 20% phase A. Profile elution using 0.6 mL/min: stay 5 min at 20% B, gradient from 20% to 45% B in 54 min, gradient from 45% B to 100% B in 10 min. The volume injection was 200 μL in the case of RNase A and 100 μL in the case of apo-αLac.

Figure 3 Effect of pH on the tertiary structure of unmodified and PEGylated RNase A proteins. Near UV CD spectra of diPEG-RNase A ●, monoPEG-RNase A ▲ unmodified RNase A ◆ in 25mM sodium phosphate buffer, pH 7. DiPEG-RNase A (+), monoPEG-RNase A (x), unmodified RNase A (-) in solution 60% 0.1% TFA – 40% acetonitrile.

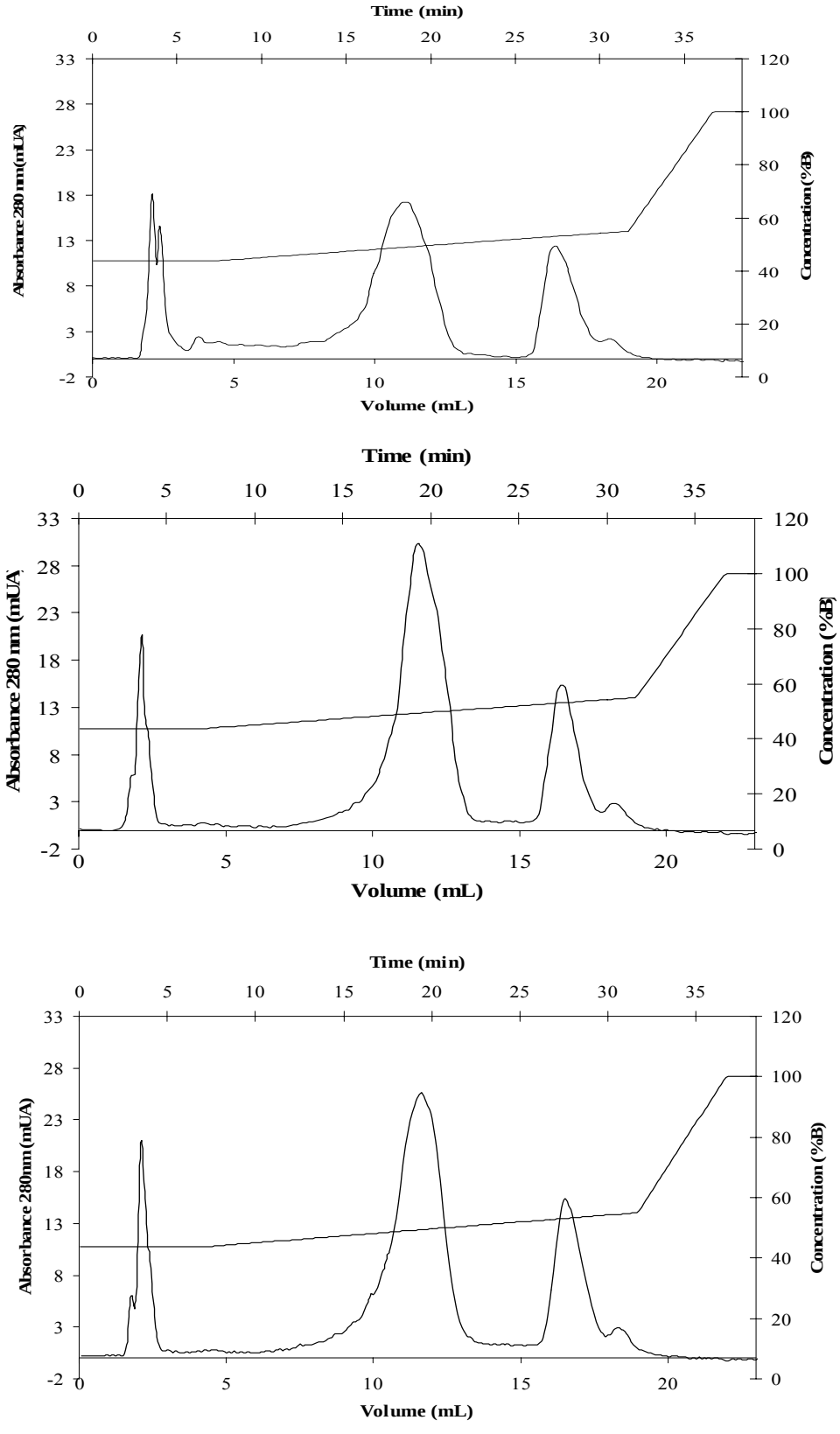
Figure 4 Effect of pH on the secondary structure of unmodified and PEGylated RNase A proteins in 25mM sodium phosphate buffer, pH 7 ◆, and in 60% 0.1% TFA – 40% acetonitrile ○. Far UV CD spectra of each protein in solution were deconvoluted into secondary structure fractions using the CD Pro (Sreerama and Woody, 2000) software package. Hr represents ordered helix content; Hd, distorted helix; Sr, regular β-strand; Sd, distorted β-strand; Trn, turns; and Unrd, unordered. Error bars represent the standard deviation of three repetitions.

Figure 5 Effect of temperature on the separation of PEG-conjugates isomers by RPC: ■ at 15°C, ⬭ at 25°C and △ at 35 °C. A) RNase A and **B)** apo-aLac. The mobile phase A was 6 mM sodium phosphate buffer pH 7.2 and mobile phase B was 80% acetonitrile – 20% phase A. Profile elution: stay 5 min at 43.8% B, gradient from 43.8% to 55% B in 24.4 min, gradient from 55% B to 100% B. The volume injection was 100 μL in the case of RNase A and 500 μL in the case of α-apoLac.

Table 1 Enzymatic activity of the RNase A conjugates after RPC separation with two different mobile phases.

	Unmodified k_{cat}/K_m **(10^7 $M^{-1}s^{-1}$)**	**Mono-PEG** k_{cat}/K_m **(10^7 $M^{-1}s^{-1}$)**	**Di-PEG** k_{cat}/K_m **(10^7 $M^{-1}s^{-1}$)**	**Tri-PEG** k_{cat}/K_m **(10^7 $M^{-1}s^{-1}$)**
From FPLC 12mM NaCl[a]	4.40 ± 0.49	5.58 ± .77	4.95 ± 0.44	ND
From FPLC 0.1% TFA[a]	7.21 ± 0.52	6.67 ± 0.29	6.99 ± 0.20	2.20 ± 0.39

a Mobile phase A used during the RPC process.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Concentration (%B)
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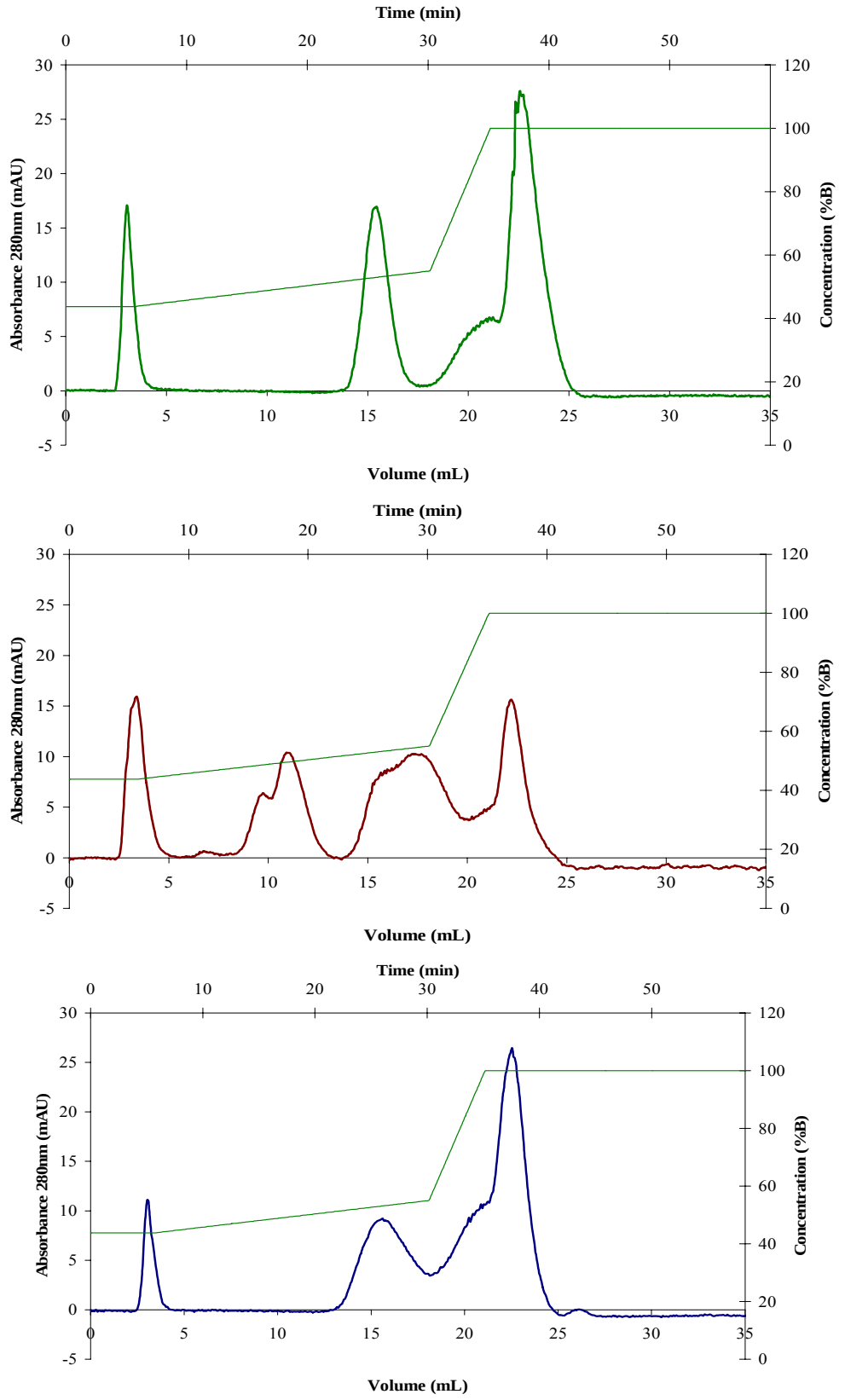
Volume (mL)
0 5 10 15 20
Time (min)
0 5 10 15 20 25 30 35
Absorbance 280 nm (mUA)
33 28 23 18 13 8 3 -2
Concentration (%B)
120 100 80 60 40 20 0
Volume (mL)
0 5 10 15 20
Time (min)
0 5 10 15 20 25 30 35
Absorbance 280nm (mUA)
33 28 23 18 13 8 3 -2
Concentration (%B)
120 100 80 60 40 20 0
Volume (mL)
0 5 10 15 20

Figure 1 A)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Volume (mL)
Time (min)
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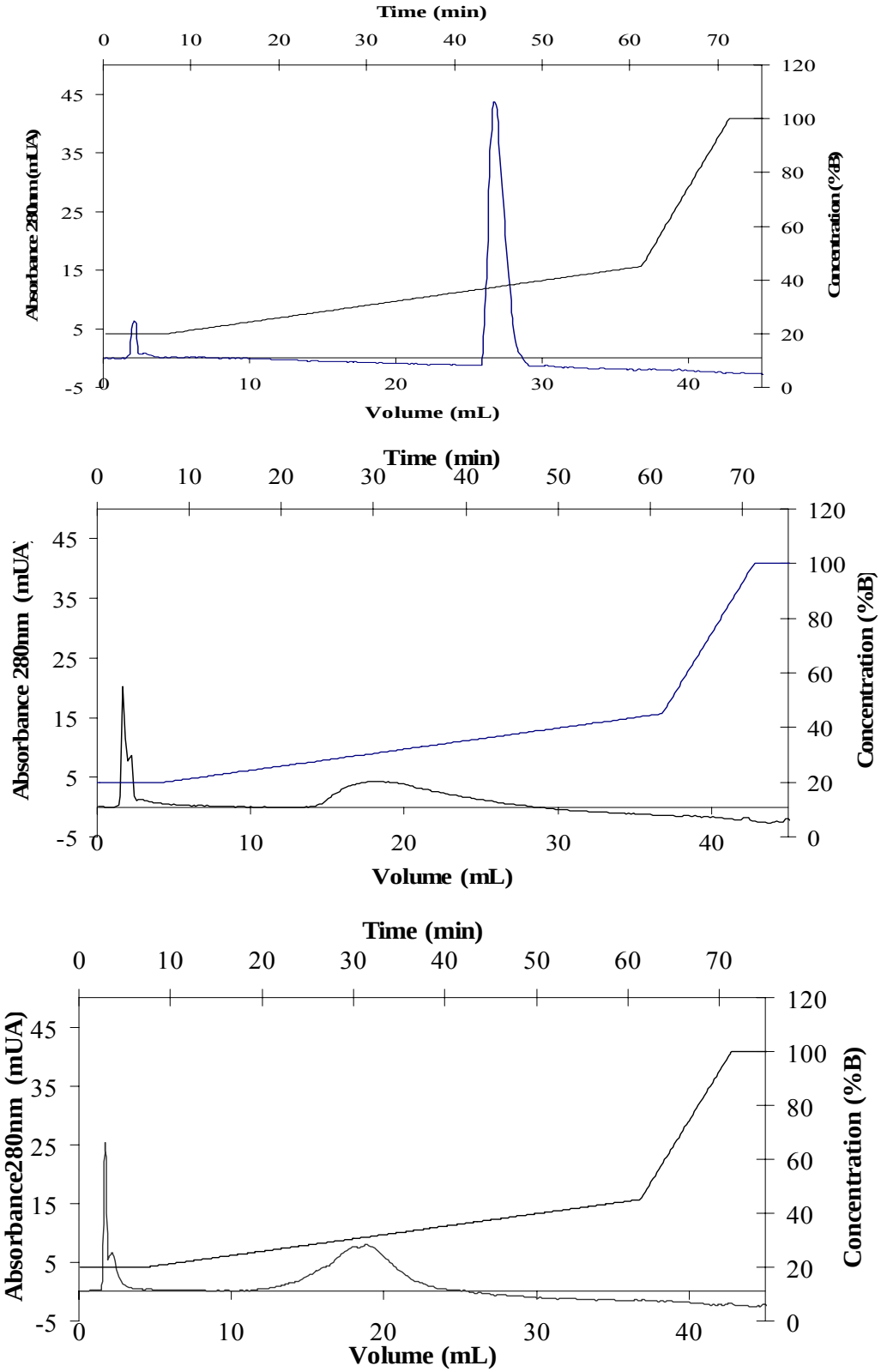
Concentration (%B)
Volume (mL)

Figure 1 B)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0 10 20 30 40
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Time (min)
0 10 20 30 40 50 60 70
Absorbance 280nm (mUA)
45 35 25 15 5 -5
Concentration (%B)
120 100 80 60 40 20 0
0 10 20 30 40
Volume (mL)
Time (min)
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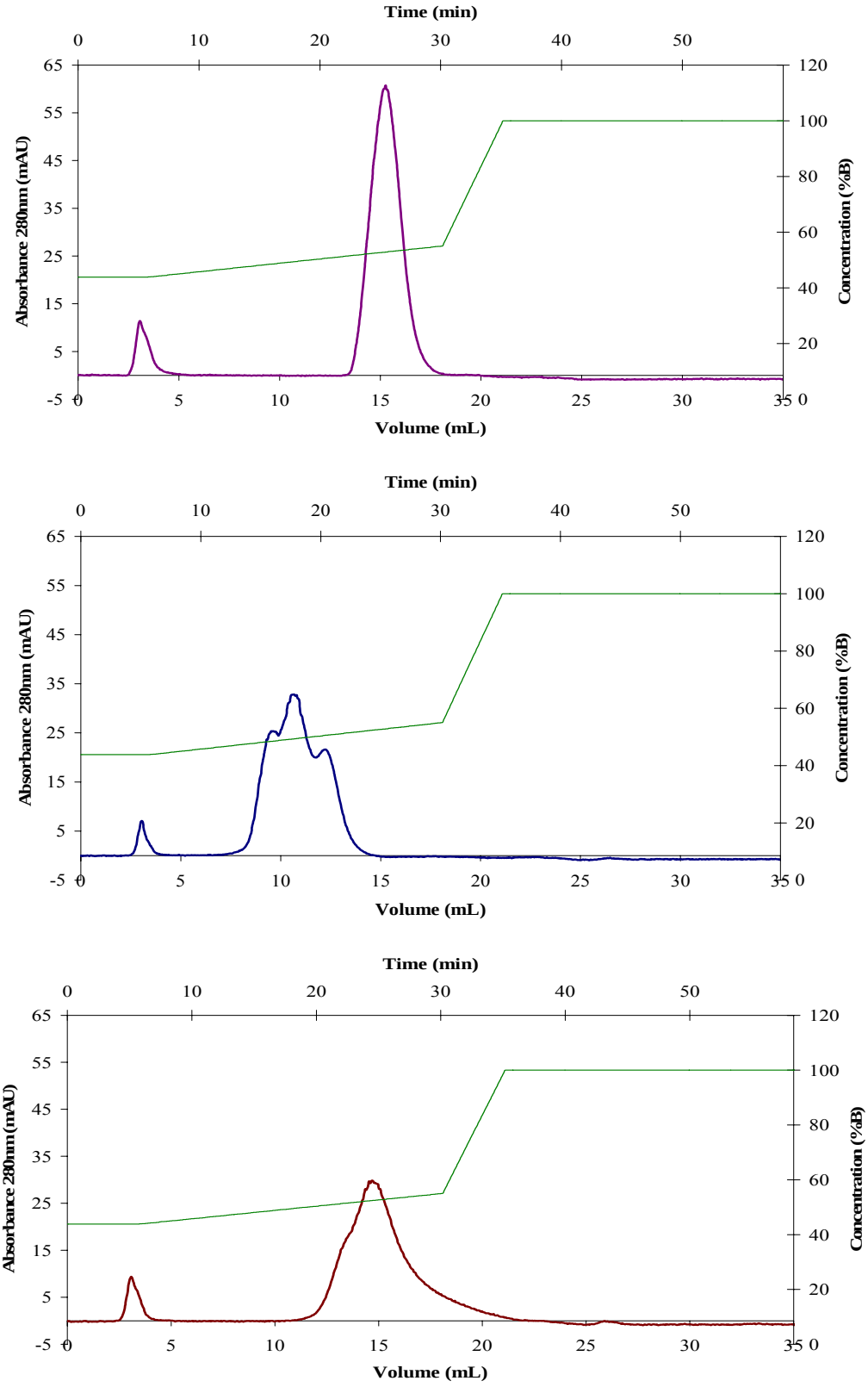
0 10 20 30 40
Volume (mL)

Figure 2 A)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20
30
40
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55
45
35
25
15
5
-5
Concentration (%B)
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60
40
20
0
Volume (mL)
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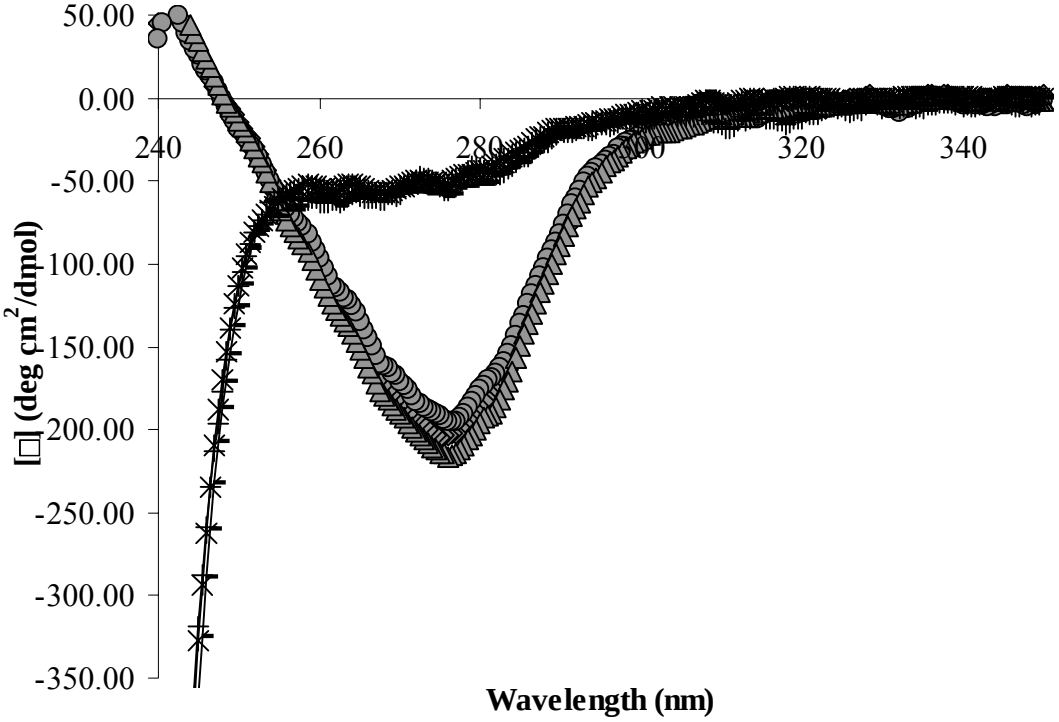
5
10
15
20
25
30
35

Figure 2 B)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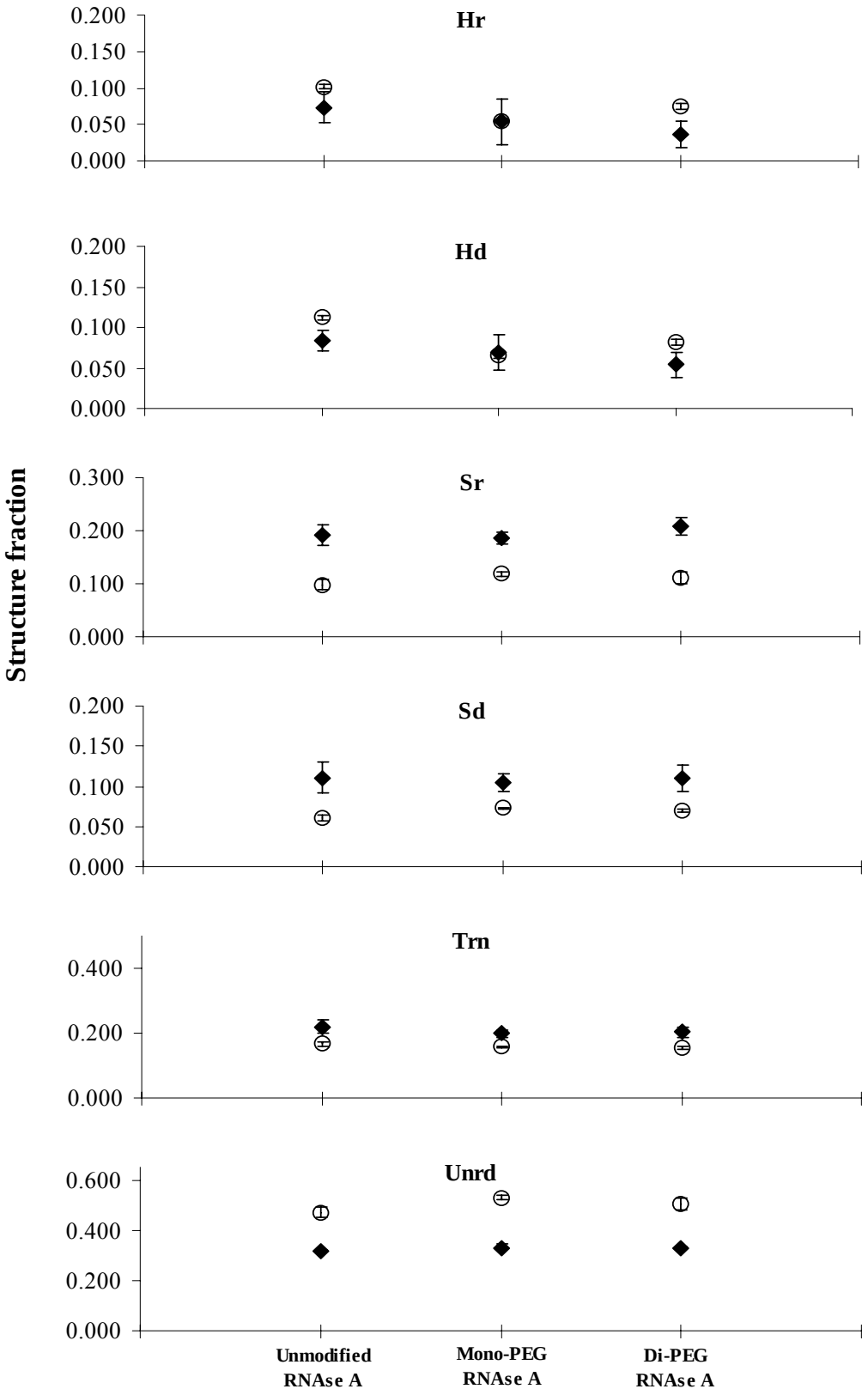
[□] (deg cm2/dmol)
Wavelength (nm)

Figure 3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Hd
Sr
Sd
Trn
Unrd
Structure fraction
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0.100
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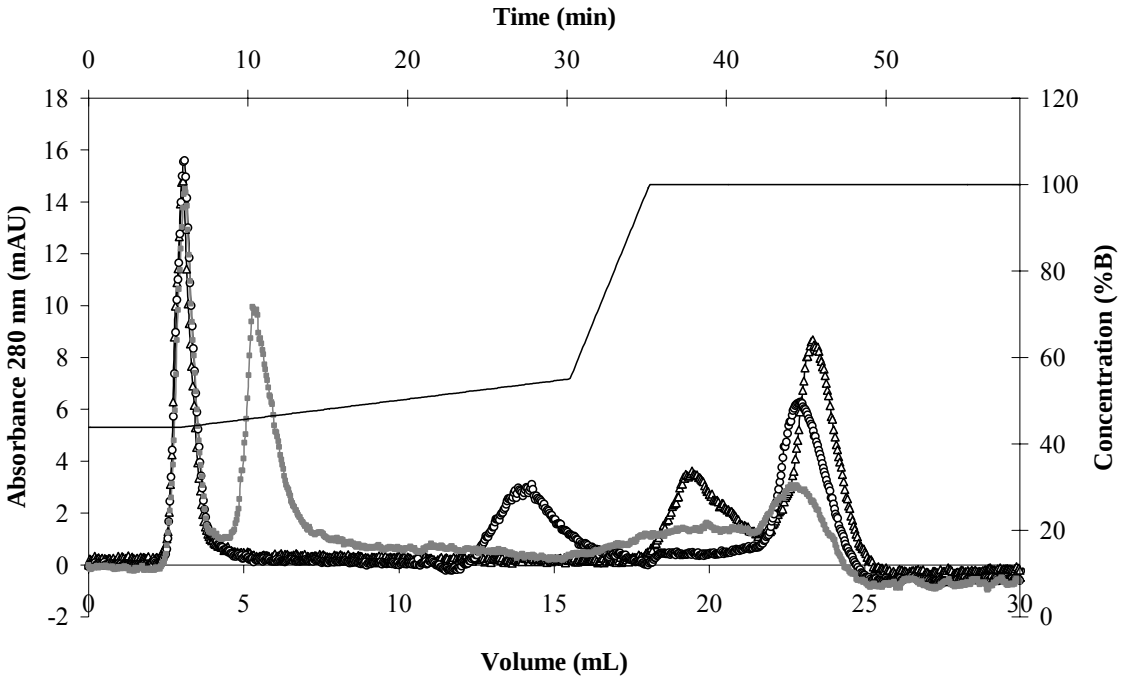
0.000
0.300
0.400
0.600
Unmodified RNAse A
Mono-PEG RNAse A
Di-PEG RNAse A

Figure 4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2
0
-2
Concentration (%B)
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0
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5
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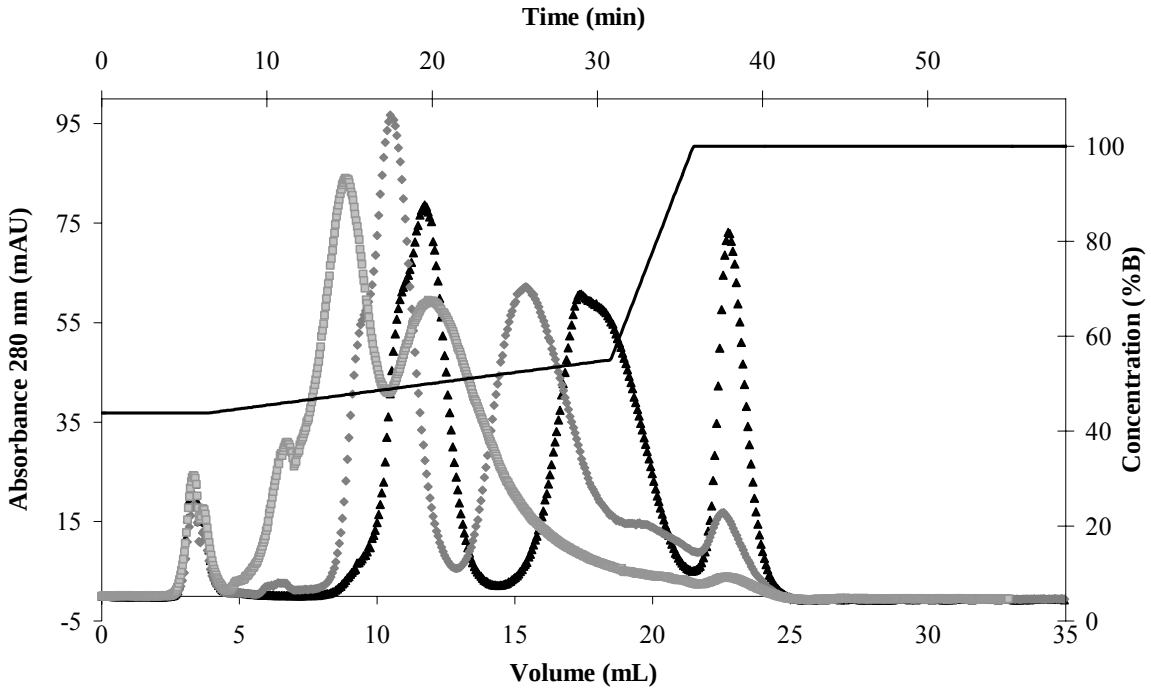
15
20
25
30
Volume (mL)

Figure 5 A)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Concentration (%B)
0
5
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15
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25
30
35
Volume (mL)

Figure 5 B)

Separation of PEGylated RNase A proteins from unmodified protein using a mild hydrophobic interaction chromatography with polyethylene glycol on sepharose

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Abstract

PEGylation, used to mitigate some problems that affect the effectiveness of therapeutic proteins, often results in a heterogeneous population of conjugated species and unmodified protein that presents a protein separations challenge. This study presents the use of a mild hydrophobic support (polyethylene glycol MW 5 kDa immobilized on sepharose) as an alternative to separate PEGylated proteins from their unmodified species. The effect of parameters such as pH, salt type and salt concentration upon the chromatographic behavior of native, mono-PEGylated and di-PEGylated ribonuclease A on this media was characterized. The separation of the native protein from the PEGylated species was achieved using a gradient elution between 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 or 2 M potassium phosphate, pH 7.0 and 25 mm potassium phosphate, pH 7.0. The pH of the mobile phases and the addition of PEG600 to the mobile phase B had no significant influence on chromatographic behavior of the species. This media provides a simple and practical chromatographic method for the separation of unmodified proteins from their PEG conjugates.

Key words: PEGylated protein separations, Mild Hydrophobic Interaction, Ribonuclease A.

Introduction

The effectiveness of proteins as therapeutic agents is occasionally reduced by their susceptibility to enzymatic degradation, short circulation time, low solubility and other factors. The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins – a reaction process known as "PEGylation"- mitigates these problems and improves protein therapeutic properties. However, this can present a challenging separation problem due to the fact that PEGylation reactions often result in a population of conjugate species in addition to the residual unmodified protein. The resulting conjugate species can differ both in terms of the number of attached PEG chains and their locations (Zalipsky, 1995).

Hydrophobic interaction chromatography (HIC) is a purification technique used to separate proteins on the basis of their surface hydrophobicity. Very little work has been done on utilizing this technique for the separation of PEGylated proteins (Fee and Van Alstine, 2006). HIC consists of injecting a protein sample in a column packed with a hydrophobic media under conditions of high salt concentration to drive hydrophobic interactions between the proteins and the media, analogous to a salting-out process. Bound proteins are eluted by lowering the salt concentration. In the case of a strongly bound protein, it is eluted by the use of a chaotropic agent or an organic modifier such as ethylene glycol (Mahn and Asenjo, 2005). The most common hydrophobic ligands used in HIC are linear chain alkanes or simple aromatic groups. However, these hydrophobic ligands may promote strong hydrophobic interactions that sometimes result in irreversible adsorption of the proteins or denaturation during elution with harsh mobile phases. Ligands with milder hydrophobic characters, such as polymers used in aqueous two-phase systems, could be more useful since they provide

moderate binding strength and bound species can be eluted by simply decreasing the salt concentration of the eluent (Queiroz *et al.*, 2001).

The combination of aqueous two-phase extraction (ATPE) and HIC procedures has been used to develop new mild hydrophobic ligands less denaturing to proteins than those utilized in traditional HIC. ATPE is a technique extensively described by Albertsson (1986) that exploits mild hydrophobic interactions. It is considered a simple technique, scalable and biocompatible for the recovery of biotechnological products, due to its high water content and low superficial tension. The systems are composed of either two polymers or a polymer and a salt. ATPE is a liquid-liquid partition method where the separation is based on the partition coefficient difference of the solutes. In order to get good separations, it is essential to optimize the partition coefficient of each component (concentration in upper phase divided by concentration in lower phase, *K*) through the appropriate selection of the system composition (Rito-Palomares, 2004).

It seems logical that the principles of ATPE partitioning could be used in a chromatographic format if one of these polymers were immobilized on the chromatographic support, thus resulting in a new type of HIC media. Columns with this kind of media have been used in the separation of enzymes (Mathis *et al.*, 1989; Diogo *et al.*, 1999) and blood cells (Matsumoto and Shibusawa, 1980), showing excellent results and promising to be a good alternative to separate standard and complex mixtures of proteins (Dias-Cabral *et al.*, 2005; Dias-Cabral *et al.*, 2003; Diogo *et al.*, 1999). Additionally, previous studies have shown that the partition coefficient (*K*) of PEGylated proteins in ATPE is related to the number of PEG molecules attached to the protein (Delgado *et al.*, 1997). PEGylated proteins would partition to a PEG

stationary phase if the mobile phase composition were incompatible with the PEG phase, such as in an ATPE. Columns with mild hydrophobic media could then be used to separate PEG conjugates. Even when traditional HIC has been used in the separation of these proteins (Seely and Richey, 2001; Clark *et al.*, 1999), the use of columns with PEG immobilized on a support has not been exploited.

This study presents an alternative approach to separate PEGylated proteins from corresponding unmodified species. A mild hydrophobic support was developed by immobilizing methoxy polyethylene glycol amine with a nominal molecular weight of 5 kDa (Nektar Therapeutics, mPEG-NH_2) on activated CH sepharose 4B (Amersham Biosciences). Ribonuclease A (RNase A) was chosen as a model protein. It has potential therapeutic application as an aspermatogenic and antitumor agent and studies have shown an improvement of the therapeutic effect when RNase A is conjugated with PEG (Matousek J *et al.*, 2002).

The behavior of native RNase A and the mono- and di-PEGylated species previously separated using size exclusion chromatography (SEC) was characterized on the proposed ATPE-HIC separation system. pH, salt type, and salt concentration were the parameters selected to define conditions under which the separation of the native protein from the PEGylated species can be achieved.

Materials and Methods

Materials

Bovine pancreatic ribonuclease A (cat. No. R5000, Lot 093K0765) was purchased from Sigma Aldrich (St. Louis, MO). Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular weight of 20kDa and methoxy-poly(ethylene glycol)-amine with a nominal molecular weight of 5kDa came from Nektar Therapeutics (Huntsville, AB). Sodium cyanoborohydride was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Activated CH sepharose 4B (cat. No. 17-0490-01 Lot 307571) and the chromatography column, a Tricorn 5/100, came from Amersham Biosciences (Uppsala, Sweden). Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific, Pittsburgh, PA), and potassium chloride (Sigma-Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific. Other salts and solvents used in this research were of reagent grade. All reagents were used as received.

PEGylation Reaction

A solution of RNase A (2 mL) at 3 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 30 mg of the nominal 20 kDa methoxy poly(ethylene glycol) butyraldehyde. The reaction mixture was stirred rapidly for 17 – 19 h at 4 °C. After this time, the reaction products were separated by size exclusion chromatography.

Analysis and separation of PEGylated protein mixture by Size Exclusion Chromatography (SEC) and Mass Spectrometry

The reaction mixture (2.0 mL) was analyzed via size-exclusion chromatography (Akta Explorer, Amersham Pharmacia, Uppsala, Sweden) using a Sephacryl S-300 column (1.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and buffer exchanged with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2.

Each peak was analyzed by mass spectrometry (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry, MALDI-TOF/MS). Prior to analysis, the samples were desalted using centrifugal microconcentrators (Microcon YM-3, Millipore). MALDI-TOF/MS was performed with a PerSeptive Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared by mixing 1.0 μL of sample with 1.0 μL of matrix. The products were characterized as diPEG, monoPEG and unmodified RNase A.

Coupling the m-PEG amine on the sepharose

PEG immobilized on sepharose was used as a mild hydrophobic support. Activated CH Sepharose 4B (Amersham Biosciences, Uppsala, Sweden Lot: 307571) was used as matrix and methoxy-polyethylene glycol amine (Nektar Therapeutics, Lot PT-04G-16, mPEG-NH_2) was used as a hydrophobic ligand. The mPEG-NH_2 was coupled to the matrix according to supplier instructions (activated CH sepharose 4B instructions, Amersham Biosciences). Briefly, the mPEG-NH_2 (0.31 g) was dissolved in the coupling buffer (0.1M $NaHCO_3$, pH 8 containing 0.5 M NaCl). Separately, 1.3 g of sepharose was washed with ~250 mL of 1 mM HCl and mixed with the ligand solution over 24 h at 4°C. After this time, the mix was let to settle and a sample of the supernatant was taken for future analysis. The excess ligand was washed away with ~250 mL of coupling buffer. In order to block the remaining active groups, the medium was transferred to 0.1 M Tris-HCl buffer, pH 8. After 1 hour, the sepharose was washed with 3 cycles of alternating pH (0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris-HCl buffer, pH 8 containing 0.5 M NaCl).

The amount of mPEG-NH_2 coupled to the matrix was estimated by a solution depletion method with a colorimetric assay for PEG (Nag *et al.*, 1996). The amount of mPEG-NH_2 (~30 μmol) on the matrix was calculated as a difference between the initial amount added to the coupling buffer and the remaining amount of mPEG-NH_2 in the solution after the coupling reaction.

Separation of PEGylated species from unmodified RNase A

Chromatographic experiments were carried out with an Akta Explorer 100 integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ) at room temperature. Protein samples collected from SEC experiments were applied to a Tricorn 5/100 column (Amersham Biosciences, 4.6 x 150 mm) packed with Sepharose-mPEG-NH_2 media (~3.5 mL) and 65 theoretical plates, as determined by injections of 1% v/v acetone pulses, at 25°C. Gradient elutions were carried out at a flow rate of 1 mL/min with a solvent A (3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0 or 2 M potassium phosphate, pH 7.0 or 3 M ammonium sulfate in 25 mM acetate buffer, pH 5.0) and a solvent B (25 mM potassium phosphate, pH 7.0 or 25 mM acetates buffer, pH 5). The following gradient profiles were selected: 0% B for 60 mL, 0 – 100 % B for 10 mL, 100% B for 25 mL, used in Figure 1; and 0% B for 6.5 mL, 0 – 100% B over 29.5 mL, 100% B for 15 mL; used in the other experiments. The UV response was measured at 215 nm, 254 nm and 280 nm.

Results and Discussion

In order to study the retention behavior of the proteins using the sepharose-PEG support, each protein was applied to the column with an initial concentration of 2 M ammonium sulfate (Figure 1). The PEGylated proteins are strongly retained on the support under these conditions and they can not be eluted until a very low salt concentration is achieved (100% B). However, the unmodified protein elutes as a wide peak at a volume at ~10 mL before the gradient is initiated. These results show that the PEGylated protein interacts with the PEG on the support strongly whereas the unmodified protein has only weak interactions with the

media. It is notable the difference in absorbance between the mobile phases used, reason why the baseline at the end of the gradient has higher absorbance that at the beginning.

This behavior is expected. This technique combines the partitioning of the proteins in ATPE and the chromatographic behavior in HIC. PEG modification of surfaces is typically used to prevent non-specific protein adsorption (Ostuni *et al.*, 2001) unmodified proteins should have weak interactions at best with the PEG HIC media. Since the initial mobile phase conditions used are those that would result in the formation of an ATPE if the PEG phase were free in solution, PEGylated species should preferentially interact with the PEG HIC media.

Previous studies have shown that the partition coefficient (*K*) of PEG-conjugates to the top PEG phase in a PEG-salt ATPE increases with the number of PEG molecules attached to the protein (Delgado *et al.*, 1997; Delgado et al, 1994).

This behavior opens the possibility for the separation of the unmodified RNase A from its PEG conjugates using a gradient elution. The chromatographic separation of the unmodified protein from the PEGylated forms using a gradient elution scheme is shown in Figure 2. The unmodified protein is more strongly retained in this higher salt concentration initial mobile phase, eluting at ~26 mL in the gradient while both mono-PEG and di-PEG species elute later at ~33.5 mL at the same percentage of mobile phase B (at). It is clear again that the retention behaviors of the unmodified and PEGylated forms are different. The non selectivity between mono and diPEGylated proteins could be caused by non-covalent interaction between the grafted PEG molecules and the protein surface groups, masking significantly the protein's

surface because the PEG has higher molecular mass than the protein. However, this observation needs more exploration in order to get resolution between PEGylated species.

Retention in hydrophobic interaction chromatography process is highly dependent on ionic strength and salt type. The separation strategy requires a careful selection of these conditions during both the adsorption and desorption steps (Mathis *et al.*, 1989). ATPE is also dependent on the type of system selected (in this case salt type) (Rito-Palomares, 2004). In order to characterize the hydrophobic column for the separation, the effect of salt was evaluated. Figure 3 shows the results of RNase A and its PEG-conjugates using 2 M potassium phosphate buffer as a mobile phase. No significant difference was detected in the behavior, even when the salt concentration used was lower than the used with ammonium sulfate. The differential retention between the unmodified and the PEGylated forms was slightly decreased, since unmodified protein elutes at ~27.3 mL and the PEG-conjugates at ~32.3 mL.

The pH of mobile phase can also be an important factor that affects the protein retention in HIC. Usually, an increase in the pH value (up to 9–10) decreases the hydrophobic interaction between proteins and the hydrophobic ligands, due to the increased hydrophilicity promoted by the change in the charge of the protein. On the other hand, a decrease in pH results in an apparent increase in hydrophobic interactions. Since each protein could have different behavior in distinct pH values and the effect of pH on protein retention in HIC is not well defined, this parameter should be used in the optimization of protein separation by HIC (Queiroz *et al.*, 2001). Partitioning of ATPE is also affected by the pH of the system and it is expected that a protein having a negative net charge migrates towards the PEG rich phase, that is positive charged. RNase A has a pI (isoelectric point) 9.3 (Raines, 1998) and it is expected

that the conjugate forms have the same or very similar pI, according to the PEGylation conditions (Kintsler *et al.*, 1996). Under both pH conditions studied, 5.0 and 7.0, the proteins will have a positive net charge.

The effect of the pH was tested using 3 M ammonium sulfate in acetate buffer at pH 5.0. The results at low pH (Figure 4) show similar chromatographic retention behavior to that at pH 7.0 (Figured 2); the unmodified protein elutes at ~25.5 mL and the PEGylated proteins at ~ 33 mL. Under the tested conditions, the pH does not have influence on the separation of the proteins on this media.

The influence of PEG concentration in the mobile phase was tested, adding 15 mM polyethylene glycol with a nominal molecular weight of 600 to the mobile phase B. Here, the idea was that free PEG in solution might compete with PEGylated protein for interaction sites on the media and encourage elution. In order to avoid pressure drop problems in the column, due to the viscosity of the solution, a lower flow rate was used (0.7 mL/min). The gradient was changed in order to match the one used in the experiments reported before. The addition of PEG to the mobile phase B had no effect on the volume retention of the proteins, showing that the interaction with PEG on the support is higher than the interaction with the PEG in the mobile phase.

Using a gradient elution between a high salt concentration solution (3 M ammonium sulfate or 2 M potassium phosphate) and a low salt concentration solution (25 mM potassium phosphate), the unmodified RNase A was separated from their PEG-conjugated forms. Established technique can then be used to further separate the different conjugate species.

However, it would also be interesting to evaluate the effect of additional parameters such as molecular weight of the polymer, polymer type and matrix support on the partition behavior of the PEG-conjugates species, in order to selectively separate each of the species.

Conclusions

It was demonstrated that the separation system developed here can exploit the approach of combining ATPE and HIC to separate unmodified proteins from their PEGylated conjugates. Selectivity was found between the unmodified protein and its PEGylated species using a gradient elution between 3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0 and 25 mM potassium phosphate buffer pH 7.0. Variations like the use of 2 M potassium phosphate at pH 7.0 instead of the ammonium sulfate solution, a decrease in pH (from 7.0 to 5.0) or the addition of PEG600 to the mobile phase B had no effect on the separation.

Acknowledgement

The authors wish to acknowledge the financial support of the National Science Foundation (Grant No. BES-0214183) and Biotechnology research chair from Tecnológico de Monterrey (Grant CAT005).

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Figure 1 Retention behavior of RNase A, MonoPEG and DiPEG-RNase A on the PEG HIC media. Mobile phase A, 2 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: water. The PEGylated proteins were separated by SEC and exchanged into a 25 mM potassium phosphate buffer, pH 7.0; 100 μL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 2 Behavior of RNase A and PEG-RNase A species with a gradient elution scheme. Mobile phase A: 3 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: 25 mM potassium phosphate, pH 7.0. The PEG-conjugate species were separated by SEC and exchanged into a 25 mM potassium phosphate buffer, pH 7.0; 100 μL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 3 Chromatographic separation of RNase A and PEG-RNase A species using potassium phosphate as a mobile phase. Mobile phase A: 2 M potassium phosphate pH 7.0, mobile phase B: 25 mM potassium phosphate pH 7.0. The PEGylated proteins were separated by SEC and put them in a 25 mM potassium phosphate buffer pH 7.0. 100 μL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 4 Effect of pH on the separation of RNase A and PEG-RNase A species. Mobile phase A: 3 M Ammonium sulfate 25 mM acetates buffer pH 5, mobile phase B: 25 mM acetates buffer pH 5.0. The PEGylated proteins were separated by SEC and put them in a 25 mM potassium phosphate buffer pH 7.0. 100 μL of sample were injected at room temperature. The flow rate was 1 mL/min.

Figure 5 Effect of PEG600 in the mobile phase on the behavior of RNase A and its PEG-conjugates. Mobile phase A: 3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0; mobile phase B: 15mM PEG600 in 25 mM potassium phosphate, pH 7.0. The PEG-RNase A proteins were separated by SEC and put them in a 25 mM potassium phosphate buffer pH 7.0; 100 μL of sample were applied to the chromatographic system at room temperature. The flow was decreased to 0.7 mL/min in order to avoid pressure drop problems.

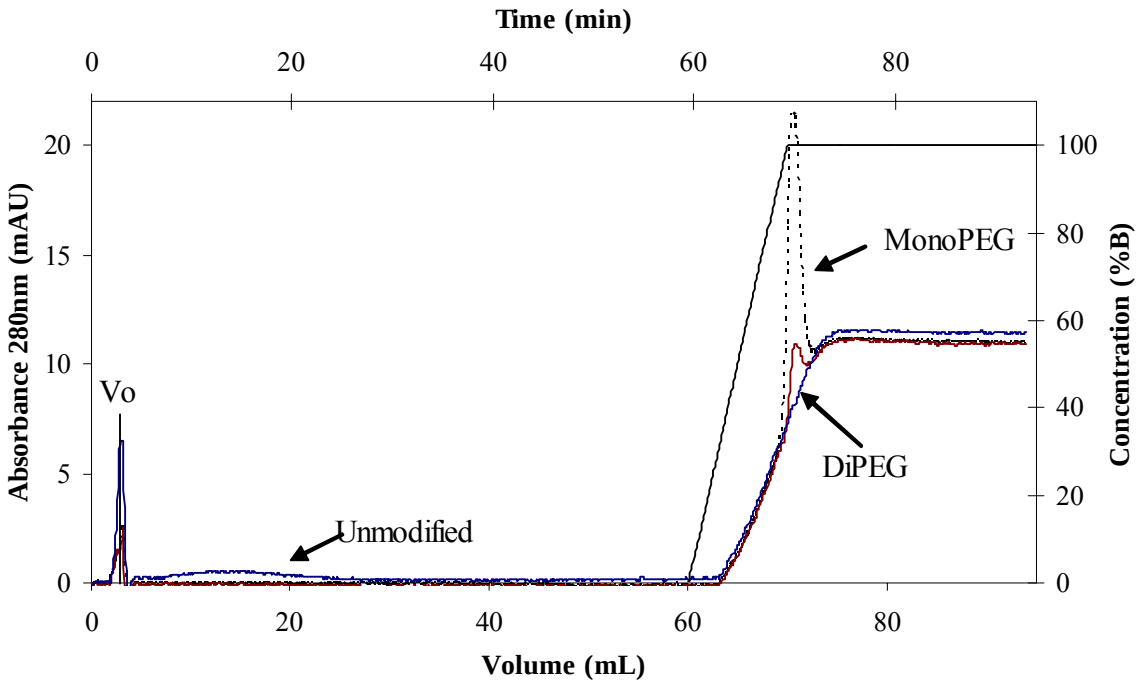
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Figure 1

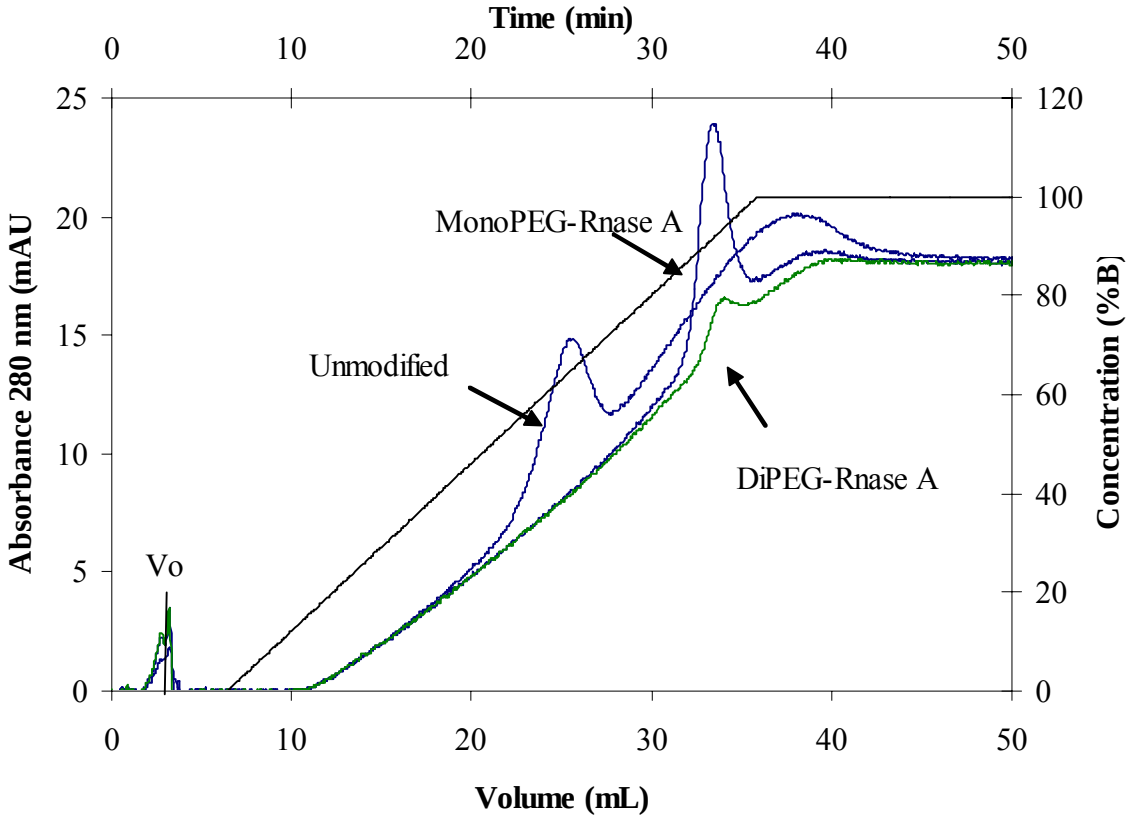
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Figure 2

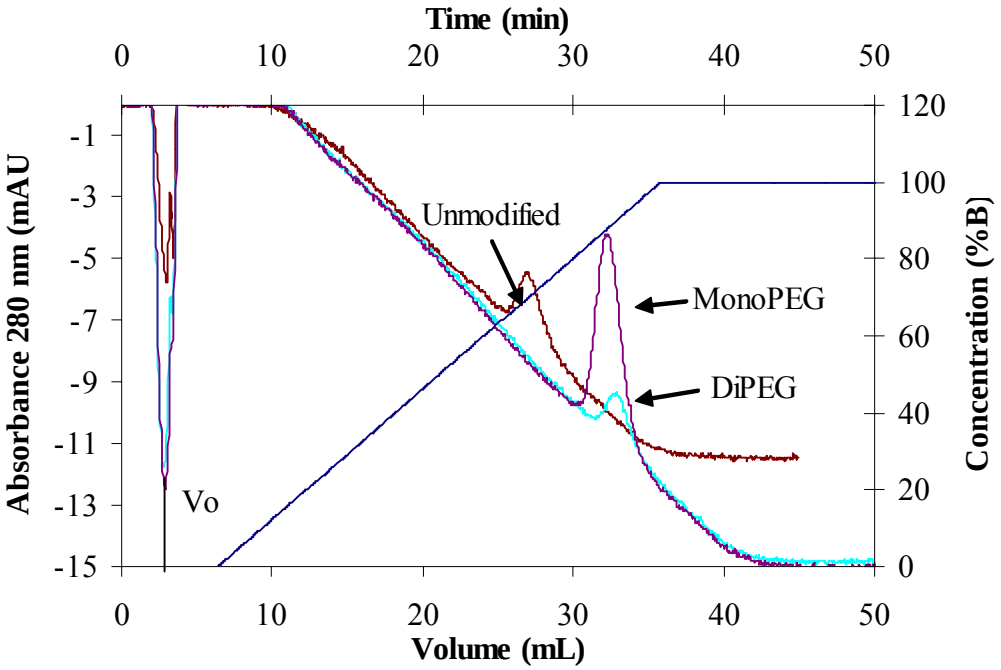
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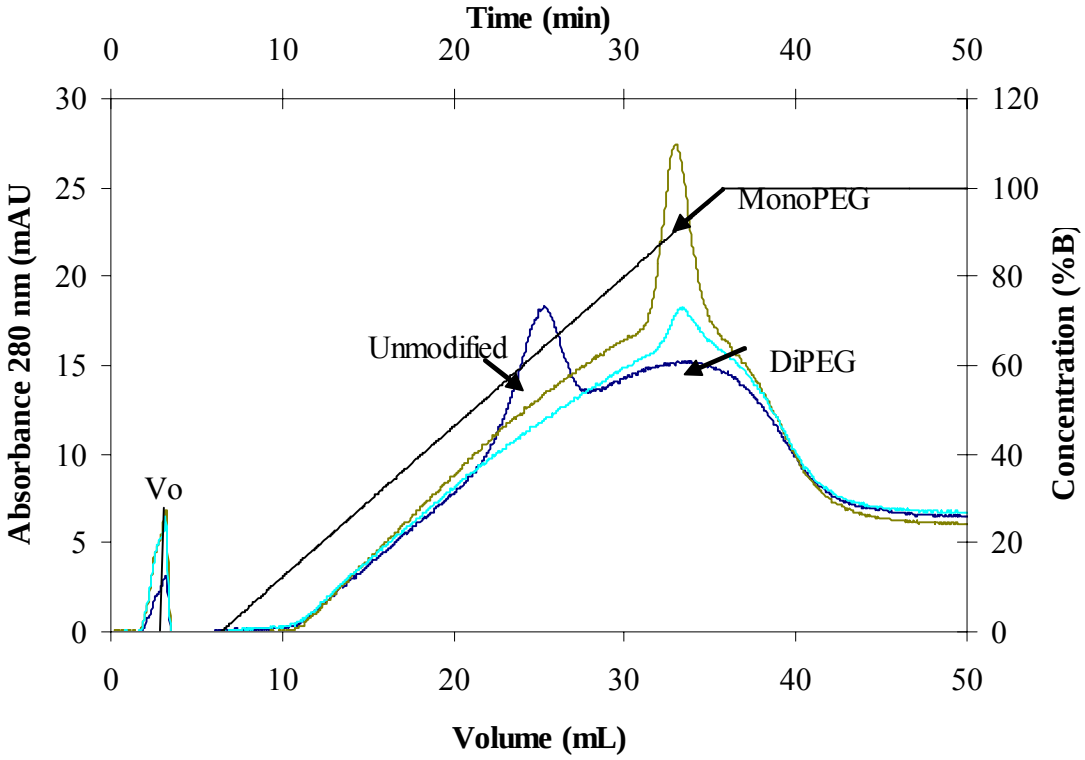
Figure 3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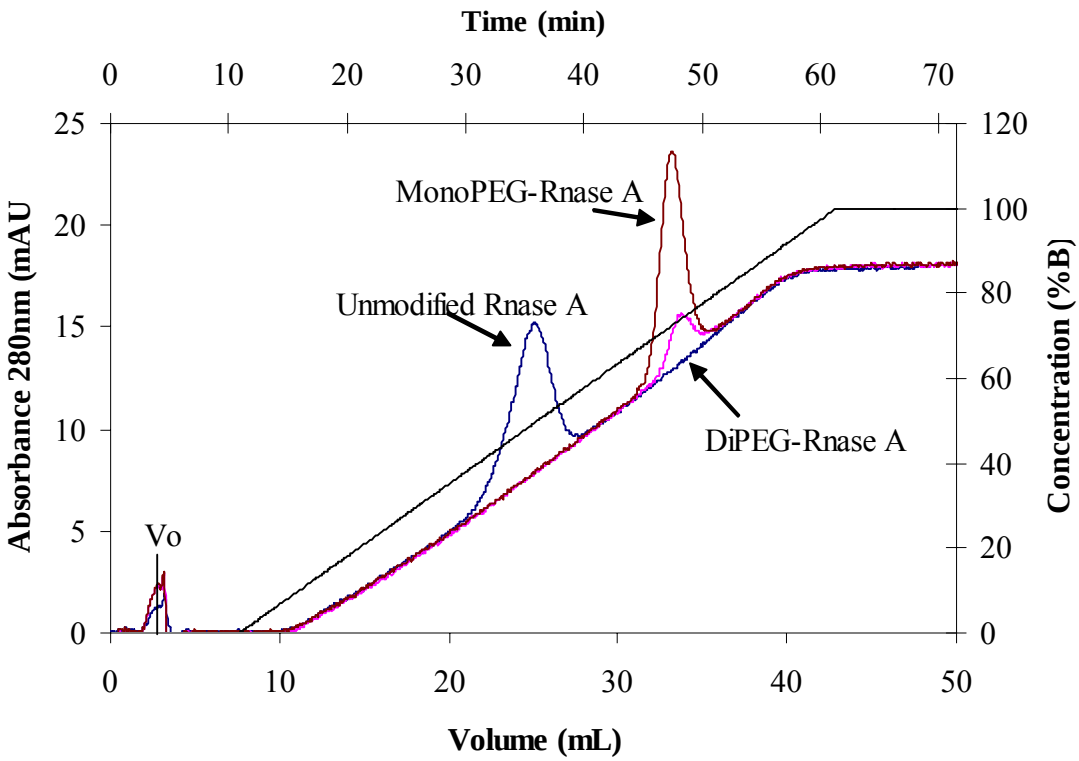
Volume (mL)

Figure 4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Concentration (%B)
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MonoPEG-Rnase A
Unmodified Rnase A
DiPEG-Rnase A
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Volume (mL)

Figure 5

Chapter 6.- Conclusions and suggestions

6.1 Conclusions

Currently, the most common techniques used for the separation of PEGylated proteins have been size exclusion chromatography (SEC) and ion exchange chromatography (IEX). The lack of reports describing the separation of PEGylation variants by exploiting their hydrophobicity is evident. The research presented here addressed the chromatographic behavior of PEG conjugates in reversed phase chromatography (RPC) and hydrophobic interaction chromatography (HIC) modes as a first step for the development of practical strategies for the purification of these species. The behavior of PEG conjugates in RPC and HIC was characterized under different process conditions, using as experimental models two proteins: RNase A and apo-αLac.

During this research, it was demonstrated that PEG-conjugates can be separated with better resolution and in less time using RPC at neutral pH rather than using SEC. RPC allowed the separation of the three species (mono-, di- and tri-PEG-RNase A) whereas only two conjugate species were resolved using SEC. In the case of RNase A species, mobile phase conditions could be selected such that the unmodified protein was not retained. This allowed the possibility of adjusting the chromatographic conditions so that the separation was dominated by the attached polymer and not by the properties of the protein. The findings reported here suggest that PEGylation confers some structural protection to the protein: the PEG chain(s) may shield(s) the protein from denaturing interactions with the hydrophobic support. On the

other hand, in the case of apo-αLac, it appeared that PEG modification was not sufficient to protect the structure from perturbation in RPC mobile phases. Thus, RPC may be suitable for the separation of PEG conjugates in cases where the intrinsic stability of the target protein is good.

It was found that the pH of the mobile phase does not play a significant role on the chromatographic behavior of RNase A PEGylated proteins. In this case, the PEG molecules dominate the elution and the unmodified protein is not retained. However, in the case of apo-αLac, the behavior of both PEGylated and unmodified proteins is affected in a similar manner. The unmodified protein was retained and its chromatographic behavior was affected by the pH of the mobile phase. These findings were useful to optimize separation conditions to obtain better resolution between conjugates species and allow the use of less unfolding environments. It was also established that temperature can be manipulated to increase the retention volume of the PEGylated proteins. Consequently, temperature could be used to improve the resolution between PEG-conjugates, especially if the protein is thermostable.

An alternative separation system exploiting the approach of combining aqueous two-phase extraction (ATPE) and hydrophobic interaction chromatography (HIC) was developed. It was demonstrated that a chromatographic medium in which PEG was immobilized on a Sepharose matrix can be used to separate the unmodified protein from its PEG-conjugates. The results obtainedopen the possibility of exploiting the attached PEG chains as hydrophobic purification tags.

During this research preparative equipment was used, making it easier to transfer an optimized purification process to larger scale columns. However, before scaling up the separation process reported in this study, additional information should be obtained in order to improve the resolution between monoPEG and diPEG αLac species. Since the results showed that this protein is not stable in the organic modifier used here, the option to use another solvent could be explored. To scale up RPC, it is important to keep in mind that this technique is based on strong hydrophobic interactions between the biological molecule and the ligands on the chromatographic support to obtain separation. In addition, elution requires organic solvents, which may affect the biological activity of the target protein, obtaining low recovery rates. Thus, use of this technique should be biased towards proteins which are stable under the described conditions.

6.2 Suggestions

Additional investigation to this work is suggested as described below. The work in this dissertation has shown that mobile phase conditions could be selected such that the unmodified protein is not retained. This allowed the possibility of adjusting the chromatographic conditions so that the separation was dominated by the attached polymer and not by the properties of the protein. For example, the pH of the mobile phase does not play a significant role on the chromatographic behavior of RNase A PEGylated proteins. This is an interesting observation and should be pursued in more detail. The research reported in this thesis was carried out using PEG with molecular mass bigger than the molecular mass of the model proteins. It would be interesting to analyze the chromatographic behavior of PEG-conjugates where the molecular weight of the protein is bigger than that of the attached PEG

and determine if it is possible to adjust the chromatographic conditions in order to make the PEG molecules to dominate the separation. PEG with 5 kDa molecular weight would be interesting to analyze, since some PEGylated pharmaceutical proteins approved for use in humans have been modified with this size PEG. Additional information about the stability conferred by the PEG molecules to the protein on the hydrophobic support could also be investigated by analyzing the structure of the adsorbed PEGylated protein. Techniques such as hydrogen exchange mass spectrometry and/or Raman spectroscopy could be very helpful in this matter.

Previous studies have reported conformational changes of PEG molecules at different temperatures. The results of this work have shown the evident effect that temperature has on the chromatographic behavior of PEGylated proteins. This observation is very interesting and should be analyzed in more detail. Information about the temperature effects on PEGylated proteins could be obtained using light scattering technique.

The mild hydrophobic support PEG-sepharose was used to separate the unmodified protein from its conjugate species, by changing salt concentration. It would be interesting to evaluate the effect of additional parameters such as molecular weight of the polymer, polymer type, immobilized polymer ligand density and matrix support type on the partition behavior of the PEG-conjugates species, in order to selectively separate each of the species. Information about the partition behavior of the proteins in aqueous two phase systems could be very helpful in finding the optimum parameters. Additionally, this information would explore in detail the relation between these techniques. Previous studies have reported that the partition coefficient of PEGylated proteins increases with the number of PEG molecules attached to the

protein. However, during this investigation no difference in the retention volume of the PEGylated proteins was observed, so it would be valuable to confirm this relationship.

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