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Novel bioengineering strategies for the recovery and purification of PEGylated lysozyme conjugates: *in situ* ATPS and affinity chromatography.

A dissertation presented by

Luis Alberto Mejía Manzano

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I, **Luis Alberto Mejía Manzano**, declare that this dissertation titled, “Novel bioengineering strategies for the recovery and purification of PEGylated lysozyme conjugates: *in situ* ATPS and affinity chromatography” and the work presented in it are my own. I confirm that:

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Luis Alberto Mejía Manzano

Monterrey Nuevo León, July 13th, 2017

Dedication

Dedicated first to God,
for all the blessings and because you have never left me.

Also, I dedicate this work to my family (father, mother and sister)
and friends

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To my family, my parents and sister for all their support. Although I have been away in many occasions, I love you! To my godparents, Ma. Refugio and Gilberto for being around.

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Novel bioengineering strategies for the recovery and purification of PEGylated lysozyme conjugates: *in situ* ATPS and affinity chromatography

By

Luis Alberto Mejía Manzano

Abstract

PEGylation is the modification of therapeutic proteins with polyethylene glycol (PEG) with the goal of improving their bioavailability and effectivity in the organism. During the PEGylation process, proteins with different degrees of PEGylation and positional isomers are generated. Numerous chromatographic and non-chromatographic techniques have been used for the purification of the adequate or most active conjugate. However, the obtained yields are still low, representing an interesting engineering challenge to address. Therefore, novel techniques to achieve this must be devised. In the present work, two strategies were explored: *in situ* aqueous two-phase systems (ATPS) and affinity chromatography.

Aqueous two-phase systems (ATPS) are a promising alternative for recovering modified proteins but this technique has not been tested with complete PEGylation reactions. In this work, lysozyme PEGylation reactions were used as part of the phase-forming chemicals to form *in situ* ATPS. This was best achieved by adding a 4M ammonium sulphate in a 20 mM Tris-HCl pH 7.0 solution. The phases were separated and analyzed by monolithic chromatography and SDS-PAGE. Results indicate that PEGylated lysozymes (mono- and di-) are mainly fractionated to the top phase (56% and 100% respectively) while native lysozyme was found in the bottom phase (97.7%).

On the other hand, PEG-modified and native lysozyme adsorption to Heparin Sepharose was described by Langmuir isotherms. The affinity of the conjugates decreased with the PEGylation degree with no significant binding of the reactive 20 kDa mPEG to the resin. A method in Heparin Affinity Chromatography (HAC) eluting with NaCl gradient was developed and optimized through Response Surface Methodology for the purification of mono-PEGylated lysozyme with a better yield, purity and productivity than other reported chromatographic modes. A formulated rate model could model and simulate the separation of mono-PEGylated and native lysozymes in HAC. Diverse mass transfer data were obtained from this simulation.

Finally, as an antecedent to the generation of immunosorbents to purify PEGylated proteins, the immobilization conditions of antibodies on NHS Sepharose 4 Fast Flow were

optimized with a factorial design. The antibody isotype showed effects on the coupling efficiency, being improved when isotype G was used.

In conclusion, *in situ* ATPS and heparin affinity chromatography combined with optimization tools such as design of experiments, modelling and simulation represent new and never reported before techniques in the recovery and purification of PEGylated lysozyme with several advantages with respect to currently used methods.

Key words: *PEGylation; PEGylated proteins; mono-PEGylated lysozyme; in situ ATPS; Heparin Affinity Chromatography (HAC); simulation; covalent immobilization; optimization.*

Estrategias innovadoras de bioingeniería para la recuperación y purificación de conjugados de lisozima PEGilada: SDFA *in situ* y cromatografía de afinidad.

Por

Luis Alberto Mejía Manzano

Resumen

La PEGilación es la modificación de proteínas terapéuticas con polietilénglicol (PEG) con el objetivo de mejorar su biodisponibilidad y efectividad en el organismo. Durante esta modificación, proteínas con diferentes grados de PEGilación e isómeros posicionales son generadas. Numerosas técnicas cromatográficas y no cromatográficas han sido usadas para la purificación del conjugado con mayor actividad. Sin embargo, los rendimientos obtenidos en procesos industriales son todavía bajos, representando un interesante reto desde el punto de vista ingenieril. Por lo tanto, nuevas técnicas para lograr este objetivo deben ser ideadas. En el presente trabajo, dos estrategias fueron exploradas con esta finalidad: sistemas de dos fases acuosas (SDFA) *in situ* y cromatografía de afinidad.

Los sistemas de dos fases acuosas (SDFA) son una alternativa prometedora para la recuperación de proteínas modificadas pero esta técnica no ha sido evaluada con reacciones de PEGilación completas tal cual se obtienen de este procedimiento. En este trabajo, las reacciones de PEGilación de lisozima fueron usadas para generar un SDFA *in situ*. La mejor opción para formar sistemas bifásicos fue agregar a la reacción una solución de sulfato de amonio 4M en Tris-HCl 20 mM pH 7.0. Las fases del sistema fueron separadas y analizadas por cromatografía monolítica y SDS-PAGE. Los resultados indican que las lisozimas PEGiladas (mono- y di-) presentan afinidad principalmente por la fase superior (56 % y 100% respectivamente) mientras la lisozima nativa fue encontrada en la fase inferior (97.7 %).

Por otro lado, la adsorción de lisozima modificada con PEG y la lisozima nativa a la resina de sefarosa modificada con heparina fue descrita por isotermas de Langmuir. La afinidad de los conjugados PEGilados disminuyó con el grado de PEGilación mientras el PEG reactivo (mPEG) de 20 kDa no se enlazó significativamente a la resina. Se desarrolló un método de Cromatografía de Afinidad de Heparina (CAH) eluyendo con un gradiente de NaCl, y se optimizó a través de Metodología de Superficie de Respuesta (MSR) para la purificación de lisozima mono-PEGilada

con rendimiento, pureza y productividad mejores a los obtenidos en otros modos cromatográficos reportados. Un modelo general de velocidad permitió modelar y simular la separación de lisozima mono-PEGilada y nativa en CAH. Diversos datos de transferencia de masa fueron obtenidos como resultado de esta simulación.

Finalmente, como antecedente en la generación de inmunosorbentes en la purificación de proteínas PEGiladas, las condiciones para la inmovilización de anticuerpos sobre el soporte NHS Sepharose 4 Fast Flow fueron optimizadas a través de un diseño factorial. El isotipo de anticuerpo mostró efectos sobre la eficiencia de acoplamiento, siendo mejor en aquellos casos donde el isotipo G fue usado.

En conclusión, los SDFA *in situ* y la CAH en combinación con herramientas de optimización como diseño de experimentos, modelamiento y simulación representan técnicas de recuperación y purificación de lisozima PEGilada novedosas y nunca antes reportadas, además de poseer diversas ventajas respecto a los métodos usados actualmente.

Paabras clave: PEGilación; proteinas PEGiladas; lisozima mono-PEGilada; *SDFA in situ; Cromatografía de Afinidad de Heparina (CAH); simulación; inmovilización covalente; optimización..*

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Chapter 1. Introduction

The global market of biopharmaceuticals was estimated at US$ 199.7 billion in 2013 and it is predicted to reach US$ 500 billion by 2020, with a compound annual growth rate of 13.5% between 2010 and 2020.[1] This is an indicator of the growing expansion of this industry mainly because of the constant search for new active biomolecules and production increase for the consumer market. This growth involves finding efficient methods for their production and separation. In fact, separation is one of the stages with the greatest impact on the final cost of the product, since in some cases it can represent up to 80% of the product cost.[2] This impact is also applicable for additional processes that aim to improve the delivery and bioavailability of these biomolecules in the organism to perform their therapeutic action. One of these processes is PEGylation.

1.1 PEGylation: generalities

PEGylation is defined as the covalent attachment of polyethylene glycol (PEG) molecules to a biomolecule,[3-5] usually a protein with therapeutic properties. Among the various benefits of PEGylation are: increased protein solubility, decreased renal filtration, immunogenicity, proteolysis, and increased bioavailability, thermal and mechanical stability.[8] However PEGylation processes also involve the formation of conjugates with different degrees of PEGylation,[9] which is important since usually only one of these species has the adequate properties and biological activity for its pharmaceutical administration. Generally, the mono-PEGylated conjugate is the desired isomer with the adequate properties and biological activity to the desired therapeutic function. The market of PEGylated drugs is calculated over US$ 8 billion per year.[5] There are more than 10 approved PEG-modified drugs (some of which displayed in **Table 1.1**) in the market while some 30 more are in clinical trials.

1.1.1 PEGylation chemistry

PEG chains with different lengths are used to carry out PEGylation. Polymer molecular weights lower than 50 kDa are preferred in proteins[3] although this depends greatly on the size of the biomolecule to be modified. PEG is functionalized through one of its hydroxyl groups[10] with

different chemical functionalities selected depending on the available chemical moieties (i.e. amino, hydroxyl, imidazole histidine, thiol, disulfide, carboxylic, hydrophobic or electrostatic residues) in the desired molecule that will be PEGylated.[11]

Table 1.1 *FDA-Approved and commercial PEG-protein drugs*

Commercial name	Active protein	Therapeutic use	Reference
Adagen®	Adenosine deaminase	Severe combined immunodeficiency disease	6
Oncaspar®	L-asparaginase	Leukemia	7
PEGasys®	Interferon α-2a	Hepatitis C	5
Krystexxa® (pegloticase)	Urate oxidase	Chronic gout	6
Neulasta®	Granulocyte colony-stimulating factor	Neutropenia	7
Somavert® (pegvisomant)	Growth hormone receptor antagonist	Acromegaly	6
Mircera®	Continuos erythropoiesis receptor activator	Renal anemia in patients with chronic kidney disease	6
Cimzia®	Anti-TNFα Fab`	Crohn´s disease and rheumatoid arthritis	6
PEGintrón®	Interferón α-2b	Hepatitis C	5
Macugen® (PEGaptanib)	Anti-vascular endothelial growth factor aptamer	Related macular degeneration	5

Modification of the N-terminal amine group of the protein is the most frequently PEGylation strategy used,[11] because this PEGylation reduces the number of PEGylation sites considerably[12] and consequently the number of isomers (also known as “PEGamers”). Also, in this amine group PEGylation, different agents have been used, the most used is methoxy-PEG(mPEG)-propionaldehyde because of its preference to modify the terminal α-amino group with respect to the ε-amino groups of lysine when using low pH values.[4] In these reactions, the α-amino group makes a nucleophilic attack on the carbon of the aldehyde group to form a secondary amine as it is shown in **Figure 1.1**.[4]

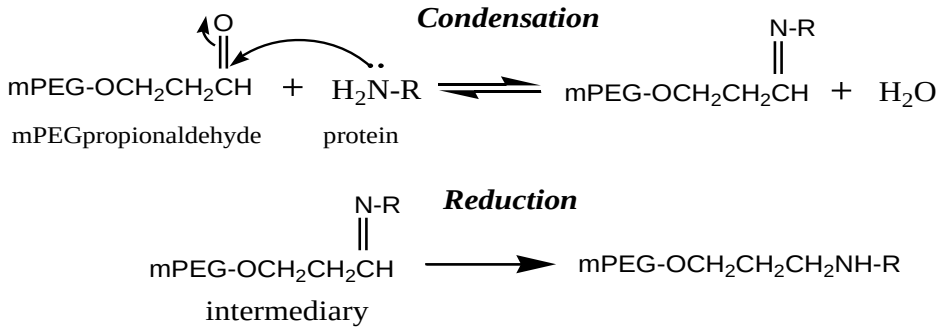

Figure 1.1 *PEGylation of the N-terminal amine group with mPEG propionaldehyde*

1.2 Current techniques in the recovery and purification of PEGylated proteins

The methods of separation of PEGylated proteins can be classified into two groups: chromatographic and non-chromatographic techniques.[12] The chromatographic techniques have been the most used due their high resolution while the non-chromatographic (e.g. aqueous two-phase systems (ATPS), electrophoresis or membrane separations)[13] are techniques recently implemented and which are used partially in the recovery or separation of these molecules. Generally, more than one operation or combinations of them are required for full purification.

Within the non-chromatographic techniques, ultrafiltration has been applied in the recovery of PEGylated ovalbumin, bovine serum albumin, α-lactalbumin[14] and ribonuclease A (RNase A), but low recoveries[15] and the high cost of the membranes are two of the great disadvantages of this procedure. On their part, ATPS demonstrated to be a promising technique in the fractionation of PEGylated proteins in PEG-phosphate systems[16, 17] but until now the tests have been carried out with individual purified standards instead of complete PEGylation reactions. Also, polyacrylamide gel electrophoresis (PAGE) has been used as a separation technique for PEGylated proteins although mainly for the characterization or analysis of the modified molecules. One negative characteristic of this operation is that staining procedures avoid the subsequent recovery of the proteins and an additional step for removing these dyes would be required.[12]

Regarding conjugate purification in chromatography, Size Exclusion Chromatography (SEC) is one of the most used methodologies for its ability to separate low molecular weight impurities and non-reacted protein. The principle of separation in SEC is the differences of hydrodynamic radii and size of the molecules. Nevertheless, SEC presents disadvantages such

as the inability to distinguish between positional isomers, poor resolution, high buffer consumption, dilution and long processing times.[18] At the industrial level, Ion Exchange Chromatography (IEX) is the most commonly applied technique because it takes advantage of the protein charge modification with different degrees of reaction by the effect of PEG shielding.[10, 19] The limitations of IEX are: low dynamic binding capacity of PEGylated proteins with long PEG chains,[20] the short lifetime of the chromatographic support with effect on the separation costs and the low load of the column that compromises the resolution. A rarely used option is Reverse Phase Chromatography (RPC) due to the denaturation of proteins caused by temperature effects[18] and the use of organic solvents in the mobile phase that results in a severe decrement in the protein biological activity. Finally, Hydrophobic Interaction Chromatography (HIC) has been recently exploited, approaching the hydrophobicity changes of the protein as a function of the molecular weight of PEG and the degree of PEGylation. Nonetheless, the lack of predictive understanding of molecule retention in HIC makes these processes not as straightforward to design as those based on IEX.[10] In addition, in HIC free PEG is also linked to the stationary phase.[12]

1.3 Aqueous Two-Phase Systems in protein recovery and its prospective role in the fractionation of PEGylated proteins

Aqueous Two-Phases Systems (ATPS) are a liquid-liquid extraction and primary recovery technique, which has been applied to the separation and total or partial purification of a wide range of nanoparticles, biomolecules, biological structures and cells.[21-23] As their name describes, ATPS are primarily constituted by water, reaching concentrations of about 85 or 90% of this component.[24] Diverse benefits are attributed to ATPS such as: low cost, potential for large-scale separation, process integration and biocompatibility.[25, 26] According with the constituents involved in phase formation, ATPS can be classified in polymer-polymer, polymer-salt, alcohol-salt, ionic liquid-based or micellar systems.[27] The constituents commonly used in the formation can be of diverse nature since common salts up to large molecules, like antibodies, enzymes, proteins, nucleic acids or cell fragments are used to form ATPS.[28]

Among the factors affecting the partitioning of proteins in ATPS are those related to the system components, their relative concentrations and those related to the protein properties. In the first case, within the factors dependent on the system are: constituent type, molecular weight and concentration of the components used, temperature, pH, density and viscosity of the phases,

settling time and interfacial tension.[29] On the other hand, the protein characteristics that determine the partitioning are the hydrophobicity, charge, protein concentration and bioaffinity.[26]

Some concepts to better understand the underlying ATPS theory are the following (**Figure 1.2**):

- Phase diagram: it is the potential working area for a characteristic two-phase system under specific conditions: pH, temperature, general phase-forming component concentrations, the concentration of phase components in the top and bottom phases, and the ratio of phase volumes.[30] On these diagrams the binodal curve, tie-line length, critical compositions and the operation point are presented.
- Binodal curve: curve that symbolizes the concentration boundary separating the monophasic from the biphasic region.[27]
- Tie-line length (TLL): line connecting two nodes on the binodal curve, representing the thermodynamic equilibrium between two phases. [30]
- Nodes: these are the final concentrations of phase components in the top and bottom phases as correlated by the tie-line. [30]
- Critical point (Cp): point at which the compositions and volumes of both phases are almost equal. [30]
- Volume ratio (Vr): it is the quotient of the top and the bottom phase volumes. [27]
- Partition coefficient (K_p): parameter which relates the quotient of the concentration of a particular solute in the top and bottom phase. [27]

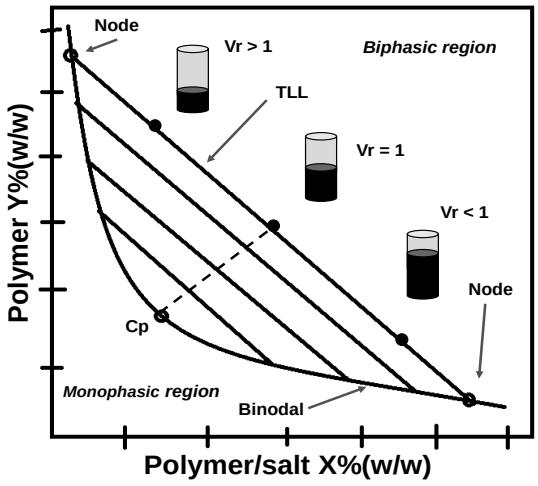

Figure 1.2 *Schematic representation of a phase diagram in ATPS*

The partitioning of PEGylated proteins in ATPS has been investigated previously with PEG-dextran and PEG-phosphate systems. In the first case, PEG 6000 g mol^{-1} and dextran T-500 were used to build and study the separation of a 5 kDa PEGylated bovine serum albumin (PEG-BSA) and PEGylated granulocyte-macrophage colony stimulating factor (PEG-GM-CSF) mixture. [16] Conjugates were mainly found in the top-PEG rich phase and a linear relationship between their partition coefficients (log K_p) and the degree of modification was observed.[16] This relationship was tried to be additionally validated by using PEG 8000 g mol^{-1} and Ig G, but it was determined that more experiments were required to analyze the influence of the size of the polymer, phase composition and type of protein.[31] The use of 3400 g mol^{-1} PEG-phosphate systems in countercurrent distribution (CCD) to fractionate lysozyme modified with 5000 g mol^{-1} p-nitrophenyl carbonate in 55 transfers suggested a more applied use of ATPS as a potential step in the recovery of PEGylated products. Although the separation was not completely achieved for unmodified, mono- and di-PEGylated conjugates.[32] Afterwards, González-Valdez and collaborators evaluated the influence of the molecular weight of the PEG, tie-line length (TLL) and volume ratio (Vr) in the partition of 20 kDa mono-PEGylated RNase A and α-lactalbumin, showing a tendency of these conjugates to partition to the top phase with good recoveries (98% and 77%, respectively).[17] In a second study with a protein concentration 20 times lower, the predilection of mono-PEGylated RNasa A was for the interphase (77%) followed for the top phase (14.7%).[33] Such results pointed out the potential of ATPS in the recovery of PEGylated proteins. However, all those antecedents present shortcomings or gaps for their practical application which are described next. First, older studies do not report product recovery yields and the followed strategies are time consuming in some cases, because the phases were formed and separated before and the PEG phase used later to partition the proteins (as the case of CCD), even the transfers may demand a lot of time if this operation is performed manually[32] instead of using a high speed countercurrent chromatography equipment. In more recent studies, the tests used purified conjugate samples and not the complete PEGylation reaction, which has a defined ratio and concentration of each protein specie (reagents and products). Therefore, the observed behavior with discrete samples could differ from that in the reaction mixtures. Additionally, the subsequent recovery of the conjugate of interest was not discussed or proposed in the previous works. On the other hand, from the economical aspect, the addition of PEG to form the phases is mandatory, representing an additional cost. Meanwhile the reactive mPEG propionaldehyde used in excess during PEGylation is discarded at the end, occurring in the same way for the spare protein that was not PEGylated. In summary, the existing ATPS strategies for PEG-protein

conjugates are not framed or oriented as a separation operation to be implemented in a production-separation process.

In this framework of expectations and considerations about the application of ATPS in the separation of PEG-modified proteins, ATPS formation directly using PEGylation reactions is proposed as a solution to locate it within a production-separation train. Then, the construction of an ATPS in the same vessel of the PEGylation reaction media is called "*in situ* ATPS". "*In situ"* procedures imply the performing of one operation in the site where others occur or have occurred, so these *in situ* processes include steps such as separations, product extraction or conversion.[34] Although the use of "*in situ"* removal techniques has been preferably used in biocatalytic processes[35] when the product is unstable or it causes product inhibition,[34, 36] its utilization out of these purposes is justified and considered as part of process integration. This means the substitution of two or more unit operations for a single one[37] improving the yield and cost-effectiveness of the process.[34]

1.4 Affinity chromatography, generalities and opportunities in the purification of PEGylated proteins

In literature, different definitions for Affinity chromatography (AC) are found among which are the following: "dynamic process in which a target biomolecule associates to or dissociates from an immobilized ligand",[38] "liquid chromatographic technique that uses a specific binding agent for purification or analysis"[39] and "selective method for the purification of a molecule or group of molecules from complete mixtures based on highly specific biological interaction between two molecules".[40] In general, these concepts have in common that AC is a kind of chromatography in which a specific interaction among two biomolecules occurs. This mode of AC is considered the most powerful fractionation technique in the large-scale purification of biotechnological products.[41] The advantages of AC are its high selectivity, sample concentration, high level of purification (greater than 1,000-fold), scalability,[42] conservation of biological activity using gentle operations and time saving as a consequence of its high selectivity and specificity. Due to the variety of ligands in nature, there are several sub-types or methods of AC according to the ligand and target involved in the purification (**Figure 1.3**).[39] **Figure 1.3** presents the ligands, targets and specific names of the sub-types of AC. For example, in immunoaffinity chromatography the antibody is generally the ligand and the corresponding antigen is the target. However, the inverse separation (antigen as ligand and antibody as target) may also may be

considered as immunoaffinity chromatography. This last, is defined as "an efficient antibody separation method which exploits the binding efficiency of a ligand to an antibody"[42] or "process in which the binding affinity of an antigen to a parent antibody is utilized as a basis of separation".[43] Other important AC sub-types which may be useful in the separation of proteins are enzyme, lectin, dye-ligand and biomimetic AC.

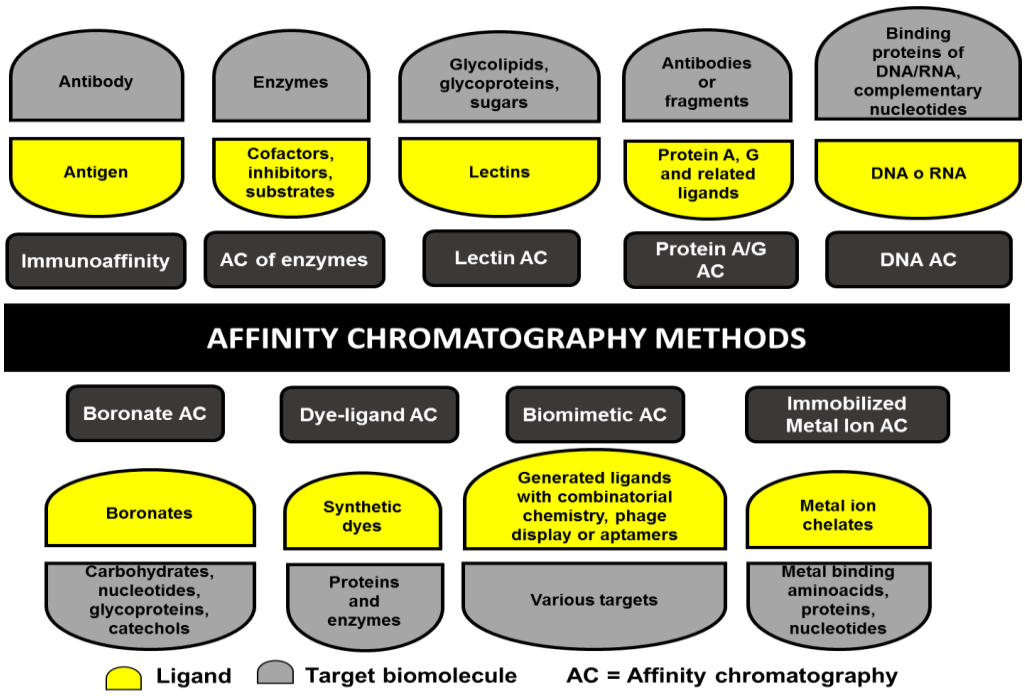

Figure 1.3 *Subtypes of affinity chromatography.*

The separation in AC is integrated by the following stages or steps[44] which are also presented in **Figure 1.4**:

- Equilibrium: stationary phase is conditioned with mobile phase to perform the separation.
- Adsorption: the sample is applied and the target molecule is bound to the immobilized ligand.
- Washing: this stage aims to remove impurities in the bulk fluid and in the stagnant fluid inside particle macropores or those non-specifically bound impurities to the stationary phase.
- Elution: the target is desorbed from the ligand through an eluting agent such as pH, chaotropic agents, polarity reducing agents or a competitive compound.
- Regeneration: this stage cleans the column and prepares it for a new separation cycle.

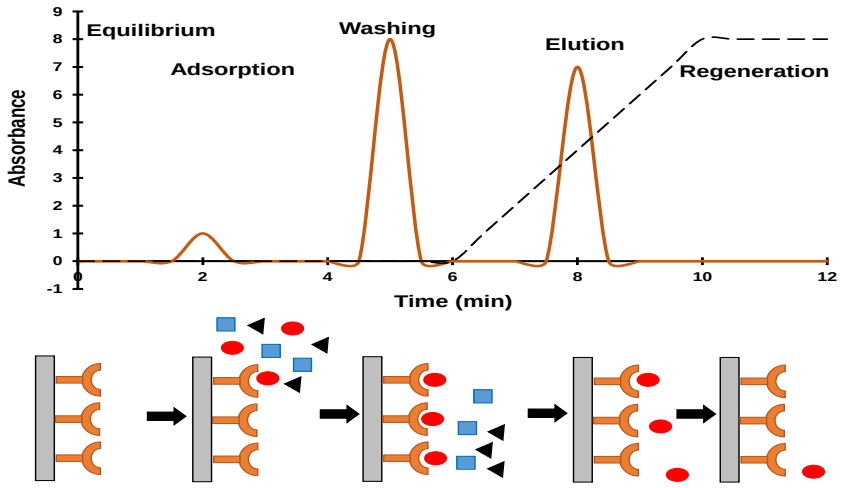

Figure 1.4 *Operational stages in affinity chromatography.*

One particular type of AC is Heparin Affinity Chromatography (HAC), which can be classified as DNA AC since heparin has the ability to bind to DNA binding proteins. This heterogeneous group of proteins has as common characteristic: its ability to bind DNA. Several initiation and elongation factors, RNA/DNA polymerases, restriction enzymes, transcriptional activators and repressors are included in the group.[45] Heparin is a linear, unbranched heteropolysaccharyde or glycosaminoglycan composed of disaccharide repeating units (hexuronic acid and glucosamine residues) negatively charged.[46–48] HAC has been used to fractionate growth factors, inhibitors, enzymes, lipoproteins, nucleic acids, hormone receptors or proteases, most of them presented in complex biological matrices such as blood, serum or tissues.[45, 48-50]

Literature involving PEGylation and affinity chromatography is scarce. The PEGylation effect on ligands in affinity chromatographic supports has been the most studied topic in this aspect. Two publications have addressed this issue, the first case was the PEGylation of concanavalin A with 2, 5 and 20 kDa mPEG succinimidyl propionate and its use in lectin AC, an improvement in the stability of 2 kDa PEGylated concanavalin was detected, keeping a 90% of binding capacity respect to the unmodified support.[51] In the same way, a second research showed a reduction in non-specific binding and increased recovery up to 15% of Ig G using PEGylated protein A with 5 and 20.7 kDa mPEG propionaldehyde.[52] Particularly, the application of AC in the purification of PEGylated proteins has been practically unexplored. Thus, for this reason and the positive

advantages of AC from other modes (i.e. selectivity, specificity, mild elution conditions) makes it an interesting study subject for the purification of PEGylated proteins. Moreover, as it has been previously stated, the recovery and purification of PEGylated proteins with low yields and purities using current techniques and their disadvantages constitute a contemporary problem.

The implementation of AC in the purification of PEGylated proteins can be achieved through three main approaches: the testing of existing affinity resins, the generation of novel affinity supports and the optimization of the performance of the mentioned supports through several tools such as design of experiments (DoE), modelling and simulation. These terms will be explained in the next sections. The evaluation of HAC to purify PEGylated proteins developing an optimized chromatographic method is located within the first and third approaches since it is intended to give a new application to a heparin adsorbent and it is optimized at the same time.

Another proposal is the generation of immunosorbents for PEG-proteins in immunoaffinity chromatography. These immunosorbents can be generated by the covalent attachment of antiPEGylated protein antibodies on N-hydroxysuccinimide (NHS) activated Sepharose 4 Fast Flow, a resin containing active esters to immobilize ligands through primary amino groups to form a stable amide linkage.[53] However, a challenge in the development of these immunosorbents is the low amounts and concentration of antiPEGylated antibody that can be procured. Moreover, the factors to perform the coupling reaction have not been examined widely. So, prior to the development of antiPEGylated protein immunosorbents, the influence of reaction factors in coupling of antibodies at low concentrations was studied. Then, it is essential to contextualize the importance of the optimization tools such as design of experiments and modelling and simulation.

1.5 Optimization tools: design of experiments, modelling and simulation in chromatography

As it is known, the market demands for fast, safe and efficient processes in the production of any biotechnological product. Moreover novel paradigms and trends in the biopharmaceutical industry are being adopted as the quality by design (QbD), which emphasizes product and process understanding and process control for guarantee the quality of the final product[54, 55]. These requirements can only be pursued by performing multiple experiments to evaluate a high number of parameters. The sample preparation and analytic techniques consume resources, and materials and time are limited.[44] In several cases, even human resources can be insufficient.

Therefore, process optimization is crucial and plays a preponderant role. Some tools that can be used in the optimization of bioseparations are design of experiments, modelling and simulation.

Design of experiments (DoE) is defined as a statistic technique for planning, conducting, analyzing and interpreting data from the experiments[56] or as an approach that involves systematic and efficient examination of multiple variables simultaneously to create an empirical model that correlates the process responses to the various factors.[57] The use of DoE is advantageous due to cost and time savings which are reached performing a small number of experiments.[58] The objectives of DoE can involve a screening of the factors significantly affecting the response on the process or an optimization finding the optimal set points.[57] When the impact of variables is tested on the process, model equations are obtained to predict results and define the robustness of the process through the variability analysis.[59] In bioseparations, there is scarce information about the application of DoE. Some of the works dealing with DoE in protein bioseparations are: Blue Sepharose for purification of recombinant human erythropoietin,[58] binding and elution of Granulocyte Colony Stimulating Factor (G-CSF) expressed from *E. coli* in mix mode chromatography,[60] definition of the process design space for phenyl hydrophobic interaction chromatography of Fc fusion protein expressed in a CHO cell line,[61] and antibody purification using mix mode chromatography.[62] In this context, the application of experimental designs[63] is immature in the bioseparations area and in the downstream of PEGylated proteins.

As it has been said**,** another tool in the optimization of separation processes is modelling and simulation. In several chromatographic separations, especially in those developed earlier, a deep knowledge and understanding of the basic phenomena involved in the operation are required for the evaluation of multiple parameters in a short time and with reagent saving as well as for future scale-up. In this scenario, mathematical modelling and simulation offer an alternative to those problems. Furthermore, chromatographic modelling and simulation can be performed from different points of view or approaches: statistic, thermodynamic, momentum, mass transfer or mixed. Mathematical models provide approximations or attempts of the phenomena and generally are supported by experimental data to restrict the model use to experimental conditions.[59] The number and type of variables or parameters included is related to the model complexity, and the more complex is a model, the more precise is the description of the studied phenomena. but in several cases a simpler model can be sufficient to describe a process, therefore it is necessary to set the aim of the study and to establish the appropriate limits.

Two are the main theories used to model protein chromatograms: the plate model (PM) and the general rate model (GRM). The PM was created in the 1940s and it assumes no mass transfer resistances and no axial dispersion considering a chromatographic column like a constant succession of compartments or theoretical plates.[64] In these plates, protein concentration in the stationary phase is considered in equilibrium with the mobile phase,[65] nevertheless PM theory is restricted to linear adsorption, diluted protein solutions and it does not explain the spreading in chromatographic peaks.[64] On its part, the GRM theory is another conception that has allowed the description of non-linear chromatography in terms not only of the protein-support interaction but also on the protein mass exchange between the stationary and mobile phases and it has been versatile enough to represent the separation in several studies.[65] So, as part of optimization in the application of AC to PEGylated protein purification, the simulation of HAC at different operation conditions using the general rate model theory was addressed as fourth issue in this thesis.

1.6 Lysozyme as experimental model

For this work, lysozyme from chicken egg white (**Figure 1.5**) was used as an experimental model to study PEGylation and the new implemented techniques here presented for its purification. Lysozyme has been a model protein in several studies about PEGylation and purification[66-68] that has allowed the extrapolation of the related techniques to other high-value therapeutic proteins with similar size like ribonuclease A (RNase A), granulocyte macrophage colony stimulating factor, fibroblast growth factor, interleukine-6 among others.[69] Additionally, lysozyme is relatively abundant and cheaper than other proteins (therapeutic or recombinant) since its production has increased in the last years.[70] Lysozyme (EC 3.2.1.17) is a glycosyl hydrolase monomer[71] of 14.3-14.6 kDa and isoelectric point of 10.7[72] with catalytic activity on (1-4)-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan and N-acetyl-D-glucosamine residues in dextrins.[71] So, lysozyme displays activity against peptidoglycan in the cell wall of Gram-positive organisms[66] and well as in some Gram-negative bacteria.[73] This protein has been used as preservative in milk based products, cheese and meat. It is a pharmacological agent too. Diverse studies register its role as biological marker and antibiotic synergist.[72] It was the first enzyme for which an atomic resolution x-ray structure was published and it has had importance in biochemical, molecular evolution and immunochemical studies.[74]

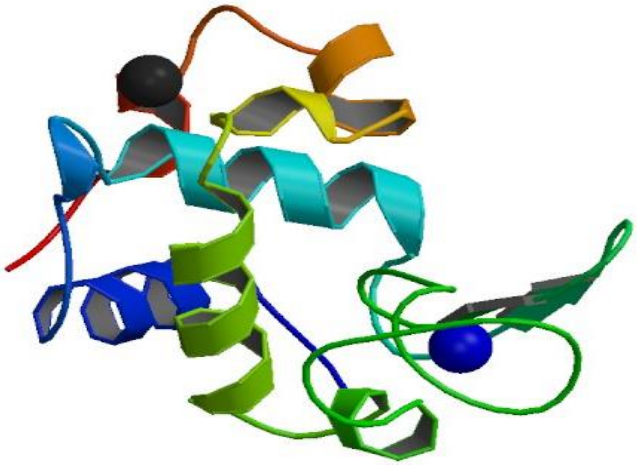

Figure 1.5 *Structure of lysozyme from chicken egg.*[71]

1.7 Hypotheses

The purification of PEGylated proteins is an important area because it is related with the production of therapeutic drugs for the treatment of various pathologies. Moreover, the current and future estimated markets of these products show their huge role in the economy of biopharmaceuticals. In this way, the development or improvement of strategies for the purification of PEGylated proteins such as *in situ ATPS* and affinity chromatography is an important research topic not only from the biopharmaceutical viewpoint but also from the arising opportunities of "PEGylaided" bioprocessing which may increase in a nearby future.[75] Under this foundation and the contextual framework established previously, this dissertation deals with the following four hypotheses:

I. It is possible to form aqueous two-phase systems using the leftover mPEG at the end of the PEGylation reaction and a polymer or salt solution to form an "*in situ* ATPS" and to recover PEGylated lysozyme.
II. Optimized Heparin Affinity Chromatography can purify mono-PEGylated lysozyme with improved yields, purity and productivity.
III. The separation of mono-PEGylated lysozyme in heparin chromatography from a PEGylation reaction can be simulated and explained at different flow and gradient length conditions, applying the general rate model.
IV. The coupling efficiency in the covalent immobilization of antibodies to a NHS sepharose 4 Fast Flow resin is affected by temperature, buffer and reaction times and by the antibody

isotype, however optimal conditions to perform this procedure can be found using a full factorial and a nested designs.

1.8 Objectives

Based on the previous hypothesis the next objectives were stated:

1. To propose a practical and profitable strategy to recover PEGylation products and reactants through the formation of ATPS exploiting PEGylation reaction media while providing new knowledge in the partitioning of the native and PEGylated proteins in the selected ATPS.
2. To understand and characterize the binding of native, PEGylated lysozyme and 20 kDa mPEG propionaldehyde to heparin-modified Sepharose 6 Fast Flow resins and to suggest an optimized chromatographic method for the efficient purification of mono-PEGylated lysozyme with the desired purity level based on Response Surface Methodology model.
3. To provide a mathematical model as a tool in the prediction and simulation of mono-PEGylated lysozyme separation in heparin chromatography at different operational conditions like flow and gradient length.
4. To offer a guide in the covalent generation of immunosorbents for PEGylated proteins through the evaluation of the effect of coupling buffer, temperature, reaction time and antibody isotype in the immobilization of antibodies.

1.9 Dissertation structure

In order to reach the stated research objectives the present thesis is divided in six chapters. The content for each chapter is described below.

Chapter 1 "Introduction" defines the term PEGylation and its importance, how it is performed and the separation methods that have been used for these conjugates until now. It includes the problem statement, fundamental concepts addressed in the solution overview, proposes the hypotheses and objectives and describes the thesis structure.

Chapter 2 presents an accepted manuscript in the *Journal of Chemical Technology and Biotechnology* under the name of "Recovery of the PEGylated and native lysozyme using an *in*

situ aqueous two-phase system directly from the PEGylation reaction". Such work integrates a recovery and concentration technique of PEGylated proteins using the same PEGylation reaction media to form two phases, called *in situ* ATPS.

Chapter 3 comprises a published paper titled "Optimized purification of mono-PEGylated lysozyme by Heparin Affinity Chromatography using Response Surface Methodology". This research focused in the development of a heparin chromatographic method to purify mono-PEGylated lysozyme and its joint optimization through a Response Surface Methodology using a Box-Behnken design for yield, purity and productivity, considering as factors the protein load, the flow and the gradient length. In this work, the plate theory is considered to explain the column performance and consequently the efficiency of the peak separation.

Chapter 4 summarizes the application of the general rate model theory to the modelling and simulation of the separation of PEGylated lysozyme reactions with heparin chromatography. A deep knowledge about the physical mass transfer processes in this purification are elucidated. The derived manuscript "Simulation of mono-PEGylated lysozyme separation in heparin affinity chromatography using a general rate model" has been accepted for publication in the *Journal of Chemical Technology and Biotechnology*.

Chapter 5 presents a published paper in the journal of *Separation Science and Technology* entitled: "Covalent immobilization of antibodies for the preparation of immunoaffinity chromatographic supports". This study shows the effect of reaction time, temperature, buffer and antibody isotype kind on the coupling efficiency of antibodies when these are covalently immobilized at low concentrations on a N-hydroxysuccinimide (NHS) activated sepharose resin.

Chapter 6 "Conclusions, contributions and perspectives" presents the general conclusions of the whole thesis, highlights the contributions and the potential research lines derived from this research.

It should be mentioned that the result chapters (chapters 2, 3, 4 and 5) contain research papers that have been published or been accepted for publication. The contents of these manuscripts are presented in the final accepted versions. However, some format editing was performed in them for the purposes of this thesis.

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Chapter 2. Recovery of PEGylated and native lysozyme using an in situ aqueous two-phase system directly from the PEGylation reaction

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2.1 Abstract

BACKGROUND: Purification of PEGylated proteins from reactions is still a challenge due to the formation of isomers. Despite the fact that aqueous two-phase systems (ATPS) have shown to be an attractive operation for their purification, the fractionation of complete PEGylation reaction mixtures has been poorly attended. In this work, the recovery of PEGylated lysozyme was studied in a novel integrative approach called *in situ* ATPS. The excess of PEG in the lysozyme PEGylation reaction (LPR) was used as part of the phase forming chemicals with different polymer (UCON, ficoll, dextran) and salt (sodium phosphates, potassium sulphates, sodium sulphate, ammonium sulphate, sodium carbonate) solutions. After selecting the system, cation exchange monolithic chromatography and SDS-PAGE were used to analyze proteins from each phase.

RESULTS: The best option for the *in situ* ATPS formation was the addition of a 4 M ammonium sulphate in 20 mM Tris-HCl (pH 7.0) solution to the PEGylation reaction. PEGylated conjugates of lysozyme exhibited a preferential partition to the top (PEG-rich) phase while native protein was partitioned to the bottom (salt-rich) phase. Di-PEGylated lysozyme partitioned entirely to the top phase (recovery of 100 %), while the mono-PEGylated protein presented a recovery yield of 56% in the in top phase. On its part, 97.7% of the native lysozyme was concentrated in the bottom phase.

CONCLUSION: The proof-of-concept presented in this work allows the recovery of PEGylated proteins directly from a PEGylation reaction. It is the first time that PEGylated proteins have been fractionated using a PEG-ammonium sulphate system taking advantage of the unreacted PEG left in a PEGylation reaction. Based on reaction engineering, i*n situ* ATPS are a potential mechanism for PEGylation control, a good alternative for “packed-bed” or on-column PEGylation processes and a pioneer strategy in the development of phase transfer catalysis in PEGylation.

Key words: *in situ ATPS; PEGylated lysozyme; PEGylation; Protein Recovery; Monolithic Chromatography*

2.2 Introduction

The chemical modification of proteins via PEGylation, is a common strategy to improve bioavailability of therapeutic proteins in the organism due to multiple advantages (*e.g.* reduced antigenicity, proteolysis and kidney filtration, increased solubility and mechanical and thermal stability) given by the covalent attachment of one or more polyethylene glycol (PEG) molecules to their structure.[1,2] Among the different PEGylation techniques available, N-terminal amine PEGylation is one of the site-specific PEGylation strategies in which methoxy PEG-propionaldehyde (mPEG) is used for the reaction at approximately pH 5.[3] However, despite of the relative acidic pH used to control the reaction, proteins with different PEGylation degrees are produced. Therefore, since in most cases only one of the conjugates possesses the required physicochemical properties, the separation of the desired PEGylated conjugate at high yields (generally the mono-PEGylated product) remains an important engineering challenge[4] since even when the PEGylation reaction has been fully optimized the conversion yield and selectivity are low.[4,5]

In this context, several chromatographic and non-chromatographic techniques[6] have been studied for the recovery of PEGylated conjugates. However achieving their total separation using only one technique with high yields is difficult. In this sense, two promising techniques readily available for the purification of these chemically modified proteins are aqueous two-phase systems (ATPS)[7,8] and chromatographic monoliths.[9-11] On their part, ATPS is a liquid-liquid extraction technique characterized by a high biocompatibility, reduced toxicity of phase-forming chemicals, ease of process integration, scale-up potential, continuous processing, rapid mass transfer and low denaturation of the biomolecules separated.[12-13] On the other hand, monoliths are continuous and homogenous supports packed within disks or columns used for the fast chromatographic separation of molecules,[14] these provide several processing benefits like the opportunity of using higher flow rates to shorten purification times, better resolutions and reduction of peak broadening in comparison to packed columns.[9,15]

In previous works concerning the recovery of PEGylated proteins exploiting ATPS,[7,8] purified samples of PEGylated proteins have been used at different concentrations mainly in a discrete manner (i.e. using samples of each one of the produced conjugates by separate). However, the direct use of complete PEGylation reactions has rarely been reported. In the better cases, “synthetic” mixtures of PEGylated proteins simulating the typical obtained yields of each

of the conjugates are prepared from purified stocks of these molecules. Furthermore, it is clear that in the use of ATPS, the removal of unreacted and phase-forming polymers as the concentration of the proteins is mandatory and remains an issue that has not been widely addressed. An interesting alternative involves the direct recovery of the proteins from the PEGylation reaction by generating an ATPS within the reaction and integrating the production and the primary recovery operations in a single stage. Consequently, an increment in product recovery and the reduction in the amount of phase-forming chemicals could be obtained while minimizing the operational costs. In this framework, process integration is understood as the appropriate selection and logical combination of purification techniques with a minimum number of stages.[16]

In the present work, an integrated process approach for the recovery of conjugated and native lysozyme molecules was proposed exploiting the generation of *in situ* ATPS directly from the PEGylation reaction and monolithic chromatography. Lysozyme was selected as model protein since besides presenting antibacterial and pharmacological activity, its PEGylation reaction has been widely reported.[17, 18] The term "*in situ* ATPS" refers to the generation of a biphasic system in the same site where a previous operation (e.g. fermentation, cell rupture, extraction, bioseparation, reaction or bioconversion) has been performed.[19-20] Specifically in this case, PEGylation reaction media was used to form an ATPS taking advantage of the excess of mPEG used in the PEGylation reaction. To assess the effectiveness of the operation, the effects of several polymers and salt solutions as potential phase-forming chemicals were compared studying the partition of the different PEG-lysozyme conjugates in the formed systems. In doing so, the top and bottom phases of the generated systems were processed by separate in an ion exchange (IEX) monolith disk.

2.3 Materials and methods

2.3.1 Materials

Lysozyme from chicken egg white (cat. no. L6876), ficoll 70,000 (cat. No. F2878), dextran 10,000 g mol^{-1} (cat. no. D9260-100G), dextran 75,000 g mol^{-1} (cat no. D8821-100G), and dextran 500,000 g mol^{-1} (cat no. 31392) were all purchased from Sigma-Aldrich (MO, USA). Methoxy-PEG-propionaldehyde (cat no. A3001-10) with nominal molecular weight of 20 kDa was obtained from Jen Kem Technologies (TX, USA). Sodium cyanoborohydride (cat. no. 1001911397) and

ficoll 400,000 g mol^{-1} (cat no. 46327-100G-F) were purchased from Fluka (MO, USA). Tris base (cat no. 4099-02) and ammonium sulphate (cat no. 0792-01) of ultra-pure grade were purchased from J.T. Baker (PA, USA). All other used chemicals were at least of analytical grade.

2.3.2 Lysozyme PEGylation reaction (LPR)

Lysozyme PEGylation reactions were prepared according the procedure originally reported by Daly *et al*.[21] Briefly, a 5.5 mL solution of lysozyme at a 3.0 mg mL^{-1} concentration in 100 mM sodium phosphate buffer pH 5.1 with 20 mM sodium cyanoborohydride was added to a vial containing 82.5 mg of 20 kDa mPEG. The reaction mixture was stirred and incubated at 4°C for 17 h. The reaction was stopped freezing the vials at -20 °C. For analytical purposes and the generation of protein standards, the mono and di-PEGylated lysozyme species were purified using Size Exclusion Chromatography (SEC), concentrated by ultrafiltration, lyophilized and stored at -4 °C as it has been reported previously.[22]

2.3.3 In situ ATPS formation and selection with different polymers and salt solutions

Dextran (MW 10,000, 75,000 and 500,000 g mol^{-1}) solutions at 30% (w/w); ficoll® 70,000 and 400,000 g mol^{-1} solutions at 30% (w/w); and UCON solutions at 30% and 50% (w/w) were prepared along with saline solutions of 30% (w/w) sodium phosphates, 40% (w/w) potassium phosphates, 20% (w/w) sodium sulphate, 20% (w/w) sodium carbonate, 4 M ammonium sulphate in water and 4 M ammonium sulphate in 20 mM Tris-HCl (pH 7.0). With these, ATPS formation, was assessed with a qualitative test was in which 100 µL of each polymer or salt solution were added individually to 2.5 mL of LPR in 15 mL centrifuge tubes up to a final volume of 3 mL. Each combination was then centrifuged at 10,000 rpm, at room temperature for 6 min. After each addition, the presence of two defined phases was verified, one system was selected considering the minimum formed phase volume and their stability over time. The partition of the different PEGylated lysozyme conjugates and the native species in the selected ATPS was measured using the natural logarithm of the partition coefficient (K_P), the top phase recovery yield (Y_{Top}) and the purification factor in the top phase (PF_{Top}) were all estimated as previously reported.[23, 24]

2.3.4 IEX chromatography using a monolithic disk

To perform the appropriate partition, yield and purification measurments, the phases of the *in situ* ATPS formed with 4 M ammonium sulphate in 20 mM Tris-HCl (pH 7.0) were analyzed and purified by cation exchange chromatography using a CIM™ Multus SO_3 monolith (inner diameter: 6.7 mm, length: 4.2 mm, CV: 1 mL) (BIA Separations; Ajdovščina, Slovenia). The monolith was connected to an Äkta Avant System 150 (GE Healthcare, Uppsala, Sweden). The mobile phases used were 20 mM Tris-HCl, pH 7.0 (buffer A) and 20 mM Tris-HCl pH 7.0 + 1 M NaCl (buffer B). The flow rate in the system was kept at 5 mL min^{-1} and absorbance was monitored at 280 nm. The top and bottom phases were carefully separated and diluted in ratios of 1:6 and 1:3, respectively in buffer A. The linear gradient for top phase separation was 25 column volumes (CVs) from 0 to 100 % buffer B. For the bottom (salt-rich) phase a gradient of 10 CVs was used in the same proportions. The recovered chromatographic fractions were concentrated using Amicon® Ultra 3 kDa centrifugal filter devices (Merck Millipore, MA, USA). After a concentrated volume of approximately 500 µL was reached, the samples were washed 4 times with 3 mL of deionized water for desalinization purposes. Calibration curves at 280 nm of native, mono and di-PEGylated lysozyme using purified standards as it has been mentioned before, were done in each chromatographic method for the identification and quantification of the different observed peaks in each of the samples.

2.3.5 SDS-PAGE analysis and protein identification

Sodium dodecyl sulphate-polyacrilamyde gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method[25] using a 12.5% (w/v) resolving gel and a 5% (w/v) stacking gel. The desalted fractions (0.014 mL) of each phase were mixed with 6X loading buffer and heated for 10 min at 99 °C. The gels were silver-stained for protein detection and afterwards stained using a barium–iodine method to visualize mPEG as it has been previously reported.[26]

2.3.6 Statistical analysis

Experiments of *in situ* ATPS formation were repeated at least three times. About twenty fractions per peak were pooled to perform SDS-PAGE analysis. Data is expressed as the average of the different results with the standard error of the corresponding number of replicates for each experiment.

2.4 Results and discussions

2.4.1 Selection of the in situ ATPS

The general strategy used for the recovery of PEGylated lysozyme species with *in situ* ATPS is described in **Figure 2.1**. As it has been stated, tests with different salt and polymer solutions were performed to select the best system to form these *in situ* ATPS. In this context, the polymer and salt solutions that were able to form the ATPS with the PEGylation reactions are shown in **Table 2.1**. High molecular weight dextrans of 75,000 and 500,000 g mol^{-1} were able to generate biphasic systems with the largest top phase volumes (approximately 400 µL). However, these were not selected as an alternative for the recovery of PEGylated lysozyme to avoid dilution effects in the target products. For this reason, the systems generated with ammonium sulphate were selected even when the obtained top phase volumes were small. In fact, there is a current trend regarding the handling of miniaturized phase volumes since their use concentrates the biomolecules while using a lower amount of reagents.[27]

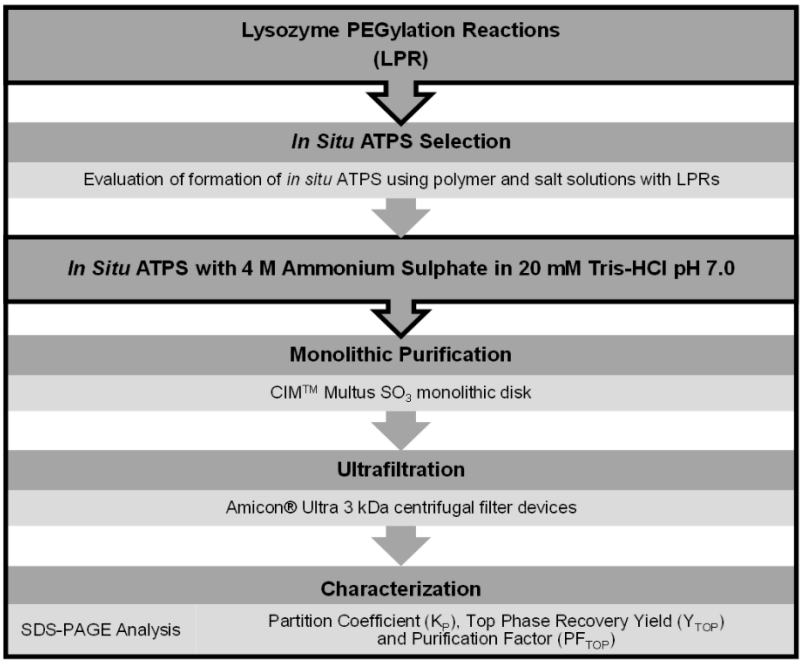

Figure 2.1 *General strategy for obtaining the in situ ATPS**.** Firstly, the adequate solution for in situ ATPS formation was evaluated, selected systems was repeated and phases were separated and purified in a monolith, the fractions were ultrafiltrated and characterized by SDS-PAGE.*

Table 2.1 *Tested solutions in the in situ ATPS formation with lysozyme PEGylation reaction*

Solution	pH	ATPS formation	Required volume to form phases (mL)	Total system volume (mL)	Formed top phase volume (mL)	V_R	Stability of the system up to 5 min (min)
***Polymer* solutions**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
30%(w/w) Dextran MW 9,000-11,000	-	NO	-	-	-	-	-
30%(w/w) Dextran MW 64,000-76,000	-	YES	3.6	9.1	0.4	0.046	5
30%(w/w) Dextran MW 450,000-650,000	-	YES	2.4	7.9	0.4	0.053	5
30%(w/w) Ficoll® MW 70,000	-	NO	-	-	-	-	-
30%(w/w) Ficoll® MW 400,000	-	NO	-	-	-	-	-
30%(w/w) UCON 50-HB-5100	-	NO	-	-	-	-	-
50%(w/w) UCON 50-HB-5100	-	NO	-	-	-	-	-
Salt solutions							
30%(w/w) sodium phosphates	8.6	YES	1.5	7	0.1	0.015	1
40%(w/w) potassium phosphates	7.4	YES	1.5	7	0.1	0.015	1
20%(w/w) sodium sulphate	8.6	YES	2.8	8.3	0.1	0.013	3
20%(w/w) sodium carbonate	11.4	YES	2.0	7.5	0.1	0.014	2.5
4 M ammonium sulphate	5.0	YES	1.6	7.1	0.15	0.022	2
4 M ammonium sulphate in 20 mM Tris-HCl pH 7.0	7.0	YES	1.8	7.3	0.2	0.028	4

It should be noted that all of the salt solutions tested were able to form *in situ* ATPS with the PEGylation reactions. In fact, the capacity to form biphasic systems with mPEG solutions of chemical species such as potassium phosphate, sodium sulphate and sodium carbonate has already been reported,[28] and according to the literature, the phase formation in polymer-salt systems is affected by the total amount and type of salt added.[29] In the different tests performed in this work using salt solutions, the top phase volume was smaller (less than 200 µL) when 20%

(w/w) sodium carbonate, 30% (w/w) sodium phosphates and 4 M ammonium sulphate were used. Furthermore, the stability of these systems over time was small since the systems were completely dissolved after a determined time. On the other hand, the system generated with 1.8 mL of 4 M ammonium sulphate in 20 mM Tris-HCl (pH 7.0) achieved an easy to handle top phase volume between the evaluated salt solutions (0.2 mL) and it was reproduced using 5.5 mL of LPR by triplicate. This last system was also selected since phase separation was preserved for a longer time when compared to the other systems indicating a larger stability and because the extractive capacity of 1.5-8 kDa PEG-ammonium sulphate systems for protein recovery has been previously reported.[30-32]

The theoretical concentration of free mPEG at the end of the PEGylation reaction (considering a water density of 1.0 g cm^{-3}) is about 1.14 ± 0.02% (w/v). After the addition of the 4 M ammonium sulphate in 20 mM Tris-HCl at pH 7.0 solution, the free mPEG concentration in the system is believed to change to 0.86 ± 0.02% (w/w). In the same way, the ammonium sulphate concentration in the total system was about of 11% (w/w). Both mPEG and ammonium sulphate concentrations are located outside of the biphasic zone in the phase diagram for the PEG 20,000 g mol^{-1} - ammonium sulphate systems reported by Zaslavsky.[33] However, since mPEG and PEG have different terminal groups (i.e. mPEG presents a metoxi (CH_3O-) and an aldehyde (HCO-) group in each end while PEG has only alcohol (HO-) groups in its molecular ends) the net charge of each molecule is expected to change causing the phase diagram to slightly shift as noted by the formation of the system using mPEG. The volume ratio (V_R; ratio between the top and bottom phase volumes) measured for the system was 0.028 and in this line, the small top phase volume favored the system handling while concentrating the proteins at the same time. It should be mentioned that grafted mPEG in the protein conjugates may have some contribution to the phase formation, however there are no reports regarding this and more experiments must be performed to confirm this hypothesis.

2.4.2 Partition analysis using IEX monolithic chromatography and SDS-PAGE

Analysis of PEGylated lysozyme species using ion exchange (IEX)[10,15] and hydrophobic interaction monolithic supports[9] has been previously reported with successful results. Some of these separations have been performed with randomly PEGylated species where small mPEG succinimidyl carbonates were used as activated PEG agents.[10,15] From these examples, mPEG propionaldehyde was only used in one case, where the separation was done with a CIM C4 A

monolithic disk.[11] Previous works reinforce the application of monolithic chromatography as a fast analytical technique that can be used for the phases generated from *in situ* ATPS. **Figure 2.2A** shows the chromatographic profile for the separation of proteins in the top phase. The first peak with an elution volume of 14.3 mL (lane 3) corresponds to di-PEGylated lysozyme (as compared to its corresponding standard in lane 4), which showed a band for protein staining (**Figure. 2.2B**) and a band for PEG staining in SDS-PAGE at 75 kDa (**Figure 2.2C**). The second peak (16.1 mL, lane 6) was attributed to the mono-PEGylated lysozyme (as compared to its corresponding standard in lane 7) at 50 kDa in both I_2-$BaCl_2$ and silver staining. Peak 3 (23.7 mL, lane 9) corresponds to native lysozyme, protein band with MW between 10-14 kDa (as compared to its corresponding standard in lane 10), this band can be clearly seen in SDS-PAGE for the PEG-rich phase (**Figure 2.2 B**).

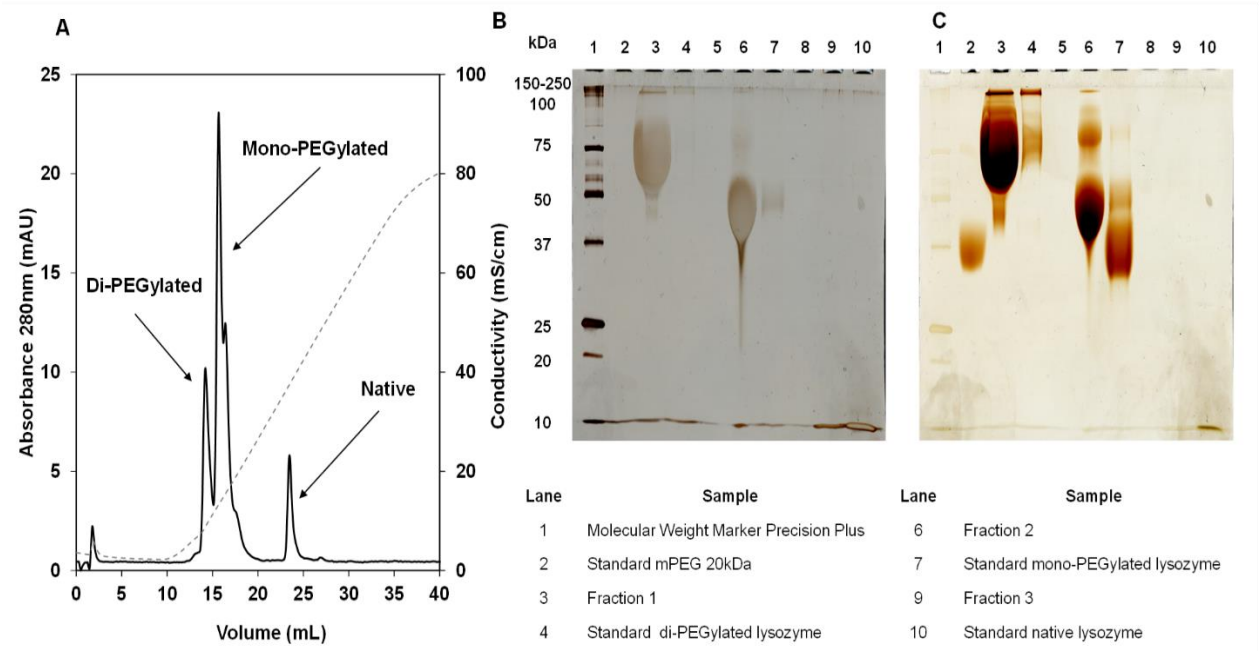

Figure 2.2 *Separation in monolith and characterization of fractions of top phase (PEG-rich phase) (**A**) Chromatographic profile of top phase in CIM™ Multus SO_3 monolith. Buffer A: Tris-HCl 20mM pH 7.0. Buffer B: Tris-HCl 20mM pH 7.0 containing 1M NaCl. Loop: 100 µL. Flow rate: 5 mL min^{-1}, lineal gradient from A to B: 25 CVs. (**B**) Silver staining for protein detection of SDS-PAGE analysis of the chromatographic fractions. (**C**) I_2-$BaCl_2$ staining for mPEG detection of SDS-PAGE analysis of the chromatographic fractions.*

Protein separation in the bottom phase is presented in **Figure 2.3A**. It shows a peak at 14.2 mL (line 4), which is the mono-PEGylated lysozyme (standard in lane 6) due to the match with the 50 kDa band protein and PEG detection in the SDS-PAGE gel (**Figure 2.3B** and **2.3C**). The peak at 17.6 mL (lane 7) corresponds to native lysozyme with a defined band at 10-15 kDa in silver staining (standard in lane 8). As it can be observed in the SDS-PAGE gels (**Figure 2.2** and **2.3**) the standard of the mono-PEGylated conjugate had traces of the 20 kDa mPEG. The band of 20 kDa mPEG was visualized at 37 kDa (lane 2).he low mobility of PEG and protein conjugates bands indicate a disparity in comparison to real molecular size, this behavior was also reported by González-González[34] for a PEGylated antibody and it is a consequence of the high hydrodynamic radius of PEG. The same effect has also been observed and explained for other PEGylated proteins such as PEGASYS.[35]

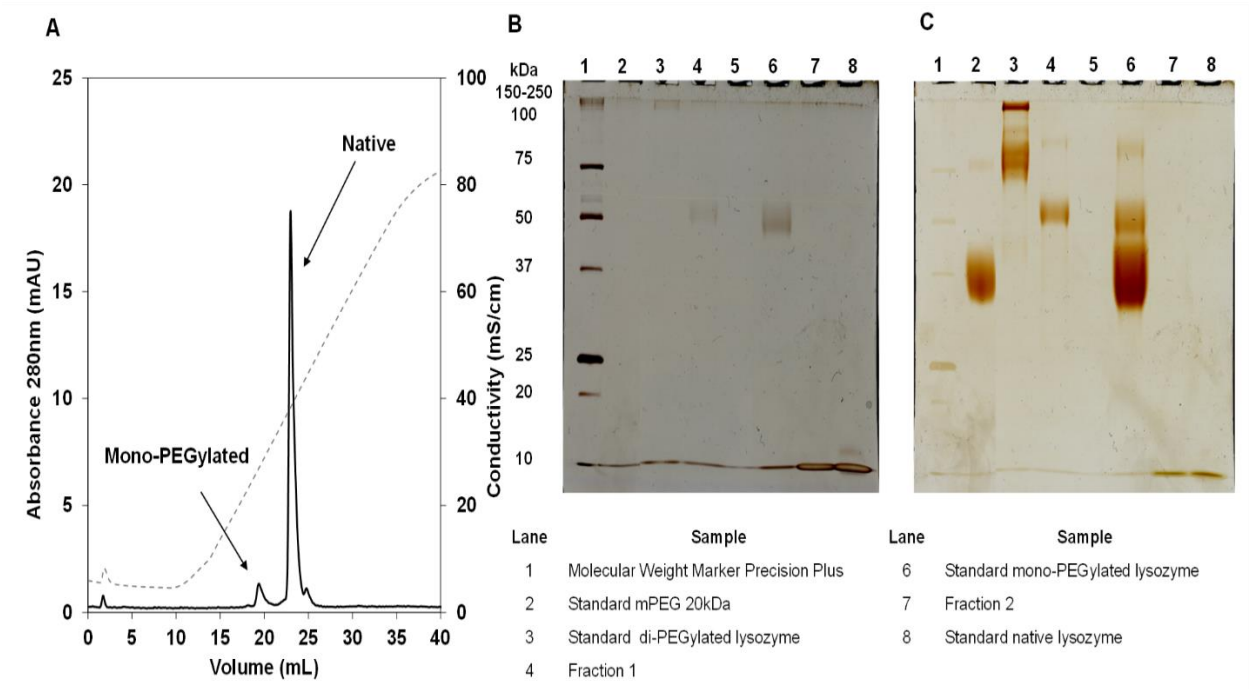

Figure 2.3 *Separation in monolith and characterization of fractions of bottom phase (salt-rich phase) (**A**) Chromatographic profile of bottom phase in CIM™ Multus SO_3 monolith. Buffer A: Tris-HCl 20mM pH 7.0. Buffer B: Tris-HCl 20mM pH 7.0 containing 1M NaCl. Loop: 100 µL. Flow rate: 5 mL min^{-1}, lineal gradient from A to B: 10 CVs. (**B**) Silver staining for protein detection of SDS-PAGE analysis of the chromatographic fractions. (**C**) I_2-$BaCl_2$ staining for mPEG detection of SDS-PAGE analysis of the chromatographic fractions.*

The elution order for PEGylated lysozyme species in chromatography for the top phase correlates to previous works [10, 36, 37] and this is explained based on the charge shielding effect of PEG on the protein.[38] Retention is described by steric hindrance between the ion exchange monolith and the charged groups on the protein surface.[39] In this tenor, the chromatographic analysis of both phases and mass balance allowed to estimate that the conversion yields of native protein transformed to mono-PEGylated and di-PEGylated conjugates were 56.65 ± 5.21% and 7.87 ± 0.57%, respectively. Mono and di-PEGylated lysozyme conjugates reached a final concentration of 4.06 ± 0.37 and 0.89 ± 0.06 mg mL^{-1} in the LPRs, while residual native lysozyme had a final concentration of 1.07 ± 0.09 mg mL^{-1}. The achieved conversion yield for the mono-PEGylated lysozyme conjugate is located in the same conversion range (50%) that has been reported for lysozyme with the use of 20 kDa mPEG propionaldehyde at optimized conditions,[5] but our results were higher than those with 10 and 30 kDa mPEG propionaldehyde modified lysozyme (30-45%).[40]

After the separation of the phases in the generated *in situ* ATPS and their chromatographic analysis, the partition coefficient, yields and purity were estimated. **Table 2.2** shows the natural logarithm of the partition coefficients (ln K_P), top phase recovery yields (Y_{Top}, %) and purification factors (PF_{Top}) of each of the studied species in the selected system. Positive ln K_P values indicate a partition preference of the products towards the top phase while negative values indicate a preference for the bottom phase.[41]

Table 2.2 *Natural logarithm of partition coefficients (ln K_p), recovery yields (Y_{Top}) and purification factors (PF_{Top}) of native lysozyme and its PEGylated conjugates*

Protein	Ln Kp	Y Top (%)	PF Top
Native lysozyme	-0.16 ± 0.06	2.23 ± 0.17	0.73 ± 0.07
Mono-PEGylated lysozyme	3.82 ± 0.09	55.71 ± 5.13	18.27 ± 2.03
Di-PEGylated lysozyme	>>>1*	100 ± 5.23	32.77 ± 2.65

* Di-PEGylated lysozyme was not detected in the bottom phase

The affinity of PEGylated conjugates towards the top phase (PEG-rich phase) has been explained by an increment in the hydrophobicity generated from the attached mPEG chains to the protein.[42] Initial observations about PEG association with proteins has been presented previously in Blomquist´s work.[43] Protein partition coefficients in polymer-salt ATPS increase directly with the PEGylation degree or the number of attached polymeric chains[44, 45].

Sookkumnerd & Hsu[46] proved this trend in countercurrent distribution (CCD) ATPS of 5000-p-nitrophenyl carbonate PEGylated lysozyme in PEG 3400 g mol^{-1} and potassium phosphate systems. In the same line, Delgado *et al.* [47] found a linear correlation between the PEGylation degree and the partition coefficient of the proteins in PEG-dextran ATPS. This corresponds to the obtained results where di-PEGylated lysozyme was recovered completely (~100%) in the top phase, while the mono-PEGylated lysozyme also presented ln K_P value of 3.82 ± 0.09 and a recovery yield (Y_{Top}) in the top phase of 55.71 ± 5.13% (the remaining ~44% was accumulated in the bottom phase). These results are also consistent with previous reports regarding the partition of PEGylated RNase A conjugates where top phase yields for di-PEGylate and mono-PEGylated RNase A where of 88% and 98%, respectively in PEG 8000-phosphate systems.[7]

Regarding the lower top phase yields obtained for mono-PEGylated lysozyme, it is believed that the difference between the volumes of the bottom (7.1 mL) and top phases (0.2 mL), may influence the partition of the conjugate towards the bottom phase once the top phase becomes saturated and unable to accommodate a higher amount of protein because of free volume and steric effects. This same effect was observed in PEG 8000-phosphate systems for mono-PEGylated RNase A at a tie-line length (TLL; line which shows points with the same final concentration of phase components in the top and bottom phases but with differing total compositions and volume ratios)[48] of 45% (w/w), with recovery yields of 41.05 ± 0.28% and 17.49 ± 0.55% in the top and bottom phases, respectively, the remaining percentage was assigned to the interphase.[8] In another study using the same PEG 8000-phosphate system but with a greater protein concentration (0.20g of 10 mg mL^{-1} solution), mono-PEGylated conjugates had preference for top phase with yields of 98% and 77% for RNase A and α-lactalbumin, respectively.[7] Nonetheless, as it has been stated previously, the evaluation of the separation of PEGylated proteins with ATPS has been mainly done with individual protein standards without considering the amounts and ratios of the protein conjugates or unreacted species existing in the PEGylation reaction. In this sense, this is one of the first occasions in which a complete PEGylation reaction mixture is fractionated in ATPS without further processing. On its part, native lysozyme presented a ln K_P value of -0.16 ± 0.06 indicating that unreacted protein can be recovered in the bottom (salt-rich) phase with a yield of 97.7%. This behavior has also been observed in ATPS lysozyme extraction and for proteins such as ribonuclease A (RNase A) and α-lactalbumin.[7] This behavior is mainly attributed to salting-out forces and also excluded volume effects.[49] At the end of the separation the purification factors in top phase were 18.27 ± 2.03 and 32.77 ± 2.65 for mono-

PEGylated and di-PEGylated lysozyme respectively. This also demonstrates the ability of this strategy to concentrate the different obtained conjugates.

In general, the recovery of proteins from complex mixtures in ATPS is difficult to achieve because there are only two phases for the distribution of molecules. However in most cases, a partial separation of the products of interest can be achieved with a good removal of contaminants or reactants that in some occasions presents similar yields as those found in chromatographic operations.[50] In addition to the obtained partition of the mono and di-PEGylated lysozyme species, the generated *in situ* ATPS allowed the concentration of said proteins in a small volume which might represent additional advantages in further processing operations.

From the perspective of PEGylation reactions and purification engineering, the application of *in situ* ATPS may present several advantages from a bioprocessing point of view. First, it can be devised as an unidirectional and integrative process[4] in the production of mono-PEGylated proteins at large reaction volumes, which can not be processed using packed bed or on column PEGylation processes due to the limiting saturation capacities or the large columns needed to do so.[4] Second, the fast remotion of PEGylated products would improve the reaction efficiency, being itself a regulation strategy to deal with undesirable conjugates while enabling the re-use of the leftover protein and reactants in the bottom phase to perform a second reaction which might also help in developing the concept called “phase transfer catalysis”.[51] Third, any alternative to improve desirable conversions in PEGylation reaction and approaching the recovery of reactants (mPEG or native protein) is interesting from an economical perspective specially in pharmaceutical environments.[4]

2.5 Conclusions

ATPS is highlighted as a promising technique for the recovery of several biomolecules including PEGylated proteins, however the direct application of this operation on reaction media has not been studied. An *in situ* ATPS was formed adding 4 M ammonium sulphate in 20 mM Tris-HCl (pH 7.0) to lysozyme PEGylation reactions. PEGylated conjugates of lysozyme had affinity for the top (PEG-rich) phase with ln K_P values higher than 1 while native lysozyme was mainly partitioned towards the bottom phase with recovery yields of 97-98%. 100% of di-PEGylated lysozyme and 56% of mono-PEGylated protein were recovered in the top phase. Approximately, 40% of total amount of native lysozyme remained unreacted. This proof-of-

concept demonstrated that an ATPS directly formed from the PEGylation reaction can separate PEGylation mixtures making their processing and purification easier and allowing a design of a shorter purification train. The findings reported here open the potential for the generic application of *in situ* ATPS for the recovery of high-value biological products, such as therapeutic proteins modified with different PEG sizes from large reaction volumes. It would be highly recommended to evaluate the economic advantages of integrating the reaction and primary recovery operations in a single stage as is the case for these *in situ* ATPS to further validate the attractiveness of these types of operations.

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The authors declare no financial or commercial conflict of interest.

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Chapter 3. Optimized purification of mono-PEGylated lysozyme by Heparin Affinity Chromatography using Response Surface Methodology

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3.1 Abstract

Background: The efficient, controlled and robust purification of conjugates from PEGylation has a growing demand in the biopharmaceuticals market. In general the yield and purity reached through the conventional chromatographic modes are not particularly high nor efficient. Affinity chromatography has so far been scarcely explored. The present work, introduces the purification of mono-PEGylated lysozyme from a PEGylation reaction by heparin affinity chromatography (HAC) for first time in a single step. Response Surface Methodology (RSM), particularly a Box-Behnken design (BBD) was employed to optimize the separation.

Results: Protein adsorption of PEGylated and native lysozyme on Heparin Sepharose 6 Fast Flow resin was described by Langmuir isotherms, showing a relatively low affinity for the PEGylated proteins. From the experimental design, optimal elution conditions in a linear gradient of sodium chloride (NaCl) for the three response variables (yield, purity and productivity) were: gradient length of 13 column volumes (CVs), flow at 0.8 mL min^{-1} and protein load of 1 mg mL^{-1}. Based on this optimization, a step gradient procedure was designed that achieved the purification of mono-PEGylated lysozyme with approximately 100% yield and purity in comparison with 92.7% and 99.7% with the linear gradient. Productivity was ca. 0.048 ± 0.001 mg mL^{-1} min^{-1} using 0.05 M NaCl for its elution.

Conclusions: Mono-PEGylated lysozyme was completely separated from a PEGylation mixture with high yield and purity using HAC for first time. Applying Response Surface Methodology (RSM), adequate conditions for more than one requirements were found as well as optimal conditions for a linear gradient of NaCl. Based on this optimization a step gradient procedure was designed that achieved the purification of mono-PEGylated lysozyme in one step with advantages in time, resolution, yield and purity compared to other chromatographic modes such as hydrophobic interaction chromatography (HIC) and cation exchange chromatography (CEX).

Key words: *Heparin Affinity Chromatography (HAC); mono-PEGylated lysozyme; PEGylation; optimization; Response Surface Methodology (RSM); Box-Behnken Design (BBD).*

3.2 Introduction

PEGylation is a drug delivery strategy which consists of covalently linked PEG (polyethylene glycol) to therapeutic proteins with the aim to improve significantly their biodistribution through positive characteristics among which are increased solubility, thermal and mechanical stability, reduced renal clearance, less immunogenicity and resistance to protease degradation.[1-3] There are several approved PEGylated proteins in the market used in the treatment of diseases[4] meanwhile, others are still in clinical trials.[5]

Nowadays, the purification of PEGylated proteins is a bottleneck in their production due to the fact that a mixture of bio-conjugates with different PEGylation grades are generated in this reaction,[6,7] even when a site-specific PEGylation method is used.[8] Chromatography due to its high resolution and purification obtained[9] continues to be the preferred technique for the separation of PEGylated proteins from the reaction mixture. Size Exclusion Chromatography (SEC), Ion Exchange Chromatography (IEX), Reverse Phase Chromatography (RPC) and Hydrophobic Interaction Chromatography (HIC) have been the modes used, however these present several drawbacks amongst which is the sample dilution, process times, low recovery and loss of biological activity[8, 10-12] of the PEG-conjugate. The total separation in these modes is reached with more than one chromatographic step. Thus, only affinity chromatography (AC) appears a suitable option.[13-15]This is a selective, high level-purification, fast, single step, reversible and mild technique[14, 16] used for separation of proteins, enzymes, antibodies, hormones, receptors, factors, vitamins, nucleic acids, cell components, viruses and phages[14] . Few publications refer to the combined use of PEGylation and AC. Some have modified the stationary phases with PEG and studied their impact in the chromatographic separation of antibodies and glucose oxidase[17, 18] or the use of AC in on-column PEGylation[19] to protect the enzyme´s active site. Nevertheless, purification of PEGylated proteins through AC has not been widely studied and characterized.

Heparin Affinity Chromatography (HAC) contains heparin as affinity ligand, a negatively charged (sulphated) glycosaminoglycan (alternating hexuronic acids with glucosamine residues) capable of binding to a wide range of biomolecules.[20, 21] Mainly, HAC has been used for the fractionation of proteins and coagulation factors in serum,[22] and also in the isolation of proteases, lipoproteins, polymerases, nucleic acids, growth factors in bovine bone marrow and proteins from diverse sources.[21, 23-24] Heparin is a high-cost ligand because it is obtained from animal sources,

however recent advances in chemoenzymatic synthesis of heparin and other related oligosaccharides are encouraging its large-scale production[25-27] and in the next years heparin use in diverse applications will be unlimited.

Aditionally, several process variables have to be taken in account during the operation design in a short time with limited availability of protein. In this point, design of experiment strategies as Response Surface Methodology (RSM) plays an important role and their use can provide advantages such as fast, control, scalability, robustness and quality in product.[28, 29] Despite the fact that RSM is now being used more routinely in the design of unit operations in biotechnological processes, [30] its employment in preparative chromatography optimization has been rare. [31, 32]

In this paper, the separation of PEG-conjugates of lysozyme produced by N-terminal site-specific PEGylation with 20kDa mPEG propionaldehyde in HAC is studied. Lysozyme is a protein model to study the PEGylation and the purification of conjugates in small therapeutic proteins, moreover lysozyme presents antibacterial activity[33] and it functions as preservative and antibiotic synergist.[34] Batch adsorption of PEGylated and native lysozyme were characterized through isotherms. Recovery of mono-PEGylated lysozyme was optimized in linear gradient elution via Box-Benkhen design (BBD) by Response Surface Methodology (RSM) to make the separation efficient. Using this result a highly efficient step gradient method was designed. So an optimized affinity chromatographic method is proposed to the purification of mono-PEGylated proteins.

3.3 Materials and methods

3.3.1 Materials

Lysozyme from chicken egg white (cat. no. 10837059001), barium chloride dehydrate (cat. no. B0750-100G) and iodine solution (cat. no. 319007-100mL) were purchased from Sigma-Aldrich (MO, USA). Methoxy-PEG-propionaldehyde (cat no. A3001-10) with a nominal molecular weight of 20 kDa came from Jen Kem Technologies (TX, USA). Sodium cyanoborhydride (cat. no. 1001911397) was purchased from Fluka (MO, USA). Tris buffer grade (cat no. TR-16514) was supplied by Winkler LTDA (Santiago, Chile). Sodium chloride (cat no. 106404) came from Merck Millipore (MA, USA). Also Coomassie Brilliant Blue G used in the Bradford reagent preparation was obtained from Sigma Aldrich. Heparin Sepharose 6 Fast Flow (cat no. 17099801)

was purchased from GE-Healthcare (Uppsala, Sweden). All solutions were made using Milli-Q-grade water (Merck Millipore, MA, USA).

3.3.2 Preparation of PEGylated lysozyme standards

The di- and mono-PEGylated lysozyme standards were obtained from the purification of lysozyme PEGylation reactions. PEGylation reactions were prepared as described by Daly et al and Cisneros-Ruiz,[35, 36] consisting of a solution of 5.5 mL of lysozyme at 3.0 mg mL^{-1} in 100 mM sodium phosphate buffer pH 5.1 with 20 mM sodium cyanoborhydride, and 82.5 mg of 20 kDa mPEG propionaldehyde stirred for 17 h at 4 °C. The reaction mixture was resolved by Size Exclusion Chromatography (SEC) on Äkta Explorer System (GE Healthcare, Uppsala, Sweden) with a Sephacryl S-300 Hi Prep column (2.6 cm ID, 60 cm long, GE Healthcare, Uppsala, Sweden) at 1 mL min^{-1} using 10 mM sodium phosphate buffer pH 7.2, containing 150 mM potassium chloride.[11] Fractions absorbing at 280 nm were collected and concentrated by ultrafiltration with a 10 kDa Diaflo membrane (Amicon Inc., MA, USA) in an Amicon chamber. Finally, proteins were lyophilized and stored at -20 °C.

3.3.3 Batch adsorption

The adsorption of native and PEGylated lysozyme on Heparin Sepharose adsorbent was carried out individually by batch experiments at room temperature at different concentrations. Total volume of gel slurry was equilibrated for 0.5 h with 5 volumes of 20 mM Tris-HCl pH 7.5 (buffer A) and dispensed into 1.5 mL microcentrifuge tubes (0.12 mL of gel slurry per tube). Then the resin was covered with 5 volumes of binding solution adding a mixture of buffering solution and protein solution to obtain final concentrations in the range of 0.5 mg mL^{-1} to 4 mg mL^{-1} of protein. Adsorption equilibrium was measured after incubation in a thermomixer comfort (Eppendorf, NY, USA) for 5 h at 1,000 rpm. After that, the solution was removed by centrifugation at 9,800 rpm for 5 min, the resin was washed with buffer A and protein desorption was done with 20 mM Tris-HCl pH 7.5 containing 2M NaCl. All experiments were carried out in triplicate. Adsorbed protein (q_e) was calculated by a mass balance from protein determination in solution using the Bradford assay[37] in a spectrophotometer. Prior to sample analysis, each protein was calibrated in both buffers. Equilibrium adsorption capacity, q_e (mg g^{-1}), was calculated by mass balance as shown in Eq. (3.1), considering Co and Ce, the initial and equilibrium protein

concentrations (mg mL^{-1}), V, the volume of aqueous solution (mL) and m, the heparin adsorbent mass (g).

$$\mathbf{q_e} = \frac{\left((\mathbf{Co}-\mathbf{Ce})*\mathbf{V}\right)}{\mathbf{m}} \quad (3.1)$$

Three adsorption models (Langmuir, Freundlich and Temkin) commonly described for protein adsorption were tested. The respective equations are the following:[38]

$$\mathbf{q_e} = \frac{\mathbf{q_o}\,\mathbf{bC_e}}{\mathbf{1}+\mathbf{bC_e}} \quad (3.2)$$

$$\mathbf{q_e} = \mathbf{K_F Ce}^{\frac{\mathbf{1}}{\mathbf{n}}} \quad (3.3)$$

$$\boldsymbol{q_e} = \frac{\boldsymbol{RT}}{\boldsymbol{B_t}}\mathbf{ln}(\boldsymbol{At} * \boldsymbol{Ce}) \quad (3.4)$$

Where q_o is the maximum monolayer adsorption capacity (g L_{gel}^{-1}) and b is the Langmuir isotherm constant in the Langmuir model (L g^{-1}). K_F is the Freundlich isotherm constant ((g L_{gel}^{-1})*(L g^{-1})$^{2.36}$) and n is the adsorption intensity. In the Temkin equation, R is the Universal Gas Constant (8.314 J K^{-1} mol^{-1}), T is temperature (K) and B_t is the Temkin isotherm constant and At is the Temkin isotherm binding equilibrium constant (L g^{-1}).

3.3.4 PEG test binding

In order to study the non-specific interactions between PEG and the heparin adsorbent, 0.12mL of gel slurry were put in contact with PEG solution at 3 mg mL^{-1} as described for protein batch adsorption. Binding, washing and elution solutions were analyzed for PEG quantification by the iodine/barium chloride method as reported by Gong *et al,*[39] and the amount of PEG adsorbed was calculated by mass balance as for protein.

3.3.5 Chromatographic method

Chromatographic experiments were performed in an Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) equipped with a 200 µL injection loop. Heparin Sepharose 6 Fast

Flow was flow packed in a 1 mL HR 5/5 column (100 mm x 5 mm ID). Packing performance was checked injecting 1% acetone pulses. All experiments were performed at room temperature. The liquid phases used in the chromatography were 20 mM Tris-HCl pH 7.5 (buffer A) and 20 mM Tris-HCl pH 7.5 containing 1 M sodium chloride (buffer B). Solutions were filtered on a membrane of 0.2 µm (Advantec, WI, USA) and subsequently degassed. The column outlet was monitored at 215 nm. Two elution modes were tested: an increasing linear salt gradient and a step salt gradient, which are described below. The yield and purity were calculated using the plate model according to Belter.[40] Productivity was estimated as before.[40, 41]

3.3.5.1 Linear gradient

Some test runs were done injecting protein mixtures of mono-PEGylated and native lysozyme in buffer A as a representative solution of the lysozyme PEGylation reaction. The separation was done with a linear salt gradient from A to 100% B. As mono-PEGylated and native lysozyme were not separated totally, optimization was suggested by design of experiments. For the experimental design the linear gradient elution mode was done using different conditions: gradient length (CVs), flow (mL min^{-1}) and protein load (mg mL^{-1}) according to the points generated in the experimental design. Once the separation conditions were optimized, the individual standards (di-PEGylated, mono-PEGylated and native lysozyme) were evaluated at those operational conditions to identify retention and elution behavior of the proteins.

3.3.5.2 Design of experiments (DoE) and result analysis in linear gradient

Response Surface Methodology (RSM) was applied to optimize the purification of mono-PEGylated lysozyme in HAC by linear gradient elution using as proof of concept a mixture of mono-PEGylated and native lysozyme at a ratio 4:1; this proportion was chosen based on previous knowledge about the concentrations at the end of the PEGylation reaction of these proteins. Di-PEGylated lysozyme was omitted in this mixture because it was not retained by the chromatographic column. The effect of gradient length from 5 to 25 column volumes (CVs), flow from 0.8 to 1.2 mL min^{-1}, and protein load from 0.25 to 1.75 mg mL^{-1} in three response variables: yield (%), purity (%) and productivity (mg mL^{-1} min^{-1}) were evaluated. A Box-Behnken design (BBD) with two central points and two replicates was generated on Minitab software and the experiments were done according to the specified run order. The data was analyzed individually for each response variable on the same software. Non-significant terms in the quadratic model

were eliminated until a reduced model was obtained. The model fit was checked by analyzing lack of fit and the R^2. Also, the analysis of variance (ANOVA) assumptions were verified: normality, constant variance and independence of the residuals. The three response variables were jointly maximized through the "optimizer" option on Minitab. Target value, minimum and importance values were defined according to the current requirements and end-use of mono-PEGylated lysozyme and these are indicated in **Table A1.** Optimal conditions were slightly adjusted for practical operational purposes.

The surface plots were created for the different responses studied. To confirm the optimum predicted average from the model the number of replicates was obtained with "power and sample size" from Minitab. After performing the appropriate replicates at optimal conditions comparison with predicted responses was done using a one sample t-test at 95 % confidence level. Finally, confidence intervals were computed for the optimum predicted average.

3.3.5.3 Step gradient

In order to improve the purification of mono-PEGylated lysozyme, a step gradient was developed with a lysozyme PEGylation reaction diluted 1:3 in buffer A at a flow of 0.8 mL min^{-1}. The initial concentrations were established according to the corresponding percentages of phase B where the mono-PEGylated and native lysozyme were eluted in the linear gradient; the method was modified to achieve the separation. The step gradient started with 5% phase B (5 CVs), 25% phase B (5 CVs) and 100% phase B (2 CVs).

3.4 Results and discussion

3.4.1 Adsorption isotherms

Adsorption data of proteins were tested with the linearized equations of three adsorption models: Langmuir, Freundlich and Temkin models which are the most frequently used models in the literature to describe protein adsorption on chromatographic resins,[42-44]including affinity chromatography;[14, 45] the regression coefficient (R^2) was the criteria for the selection of the model to fit. For the three proteins (di-, mono-PEGylated and native lysozyme) the best adjustment was observed with the Langmuir model (R^2 = 0.997, R^2 = 0.908, R^2 = 0.977, respectively) (**Figure S1**), while in the other models the values of regression were low (in Freundlich model these were:

0.635, 0.841 and 0.932 and in Temkin model these were 0.960, 0.902 and 0.643, respectively). The Langmuir behavior has been observed in the most of adsorption studies with PEGylated proteins on other chromatographic supports, mainly on ion exchange or hydrophobic adsorbents.[46, 47]

Of the three proteins studied in batch adsorption, mono-PEGylated lysozyme showed the highest adsorption capacity (**Table 3.1**) (10.8 g L^{-1} of gel) with the Langmuir model followed in decreasing order by native and di-PEGylated lysozyme with 4.9 and 2.8 g L^{-1} of gel, respectively. It was expected that the mono-PEGylated conjugate had less binding capacity compared to the native protein due to the change caused by PEG-modification which has also been observed with other PEGylated proteins[46, 48] on ion exchangers, nevertheless the discrepancy may be explained because the adsorption at static conditions not only might be driven by the ionic interaction with the protein but also with the PEG chain slightly as it has been happened on hydrophobic resins.[49]

Table 3.1 *Estimated parameters for Langmuir isotherms of native and PEGylated lysozyme.*

Langmuir parameter	Native lysozyme	Mono-PEGylated lysozyme	Di-PEGylated lysozyme
qo (g g^{-1})	4.884	10.787	2.797
b(L g^{-1})	41.377	2.971	5.204
Ka (M^{-1})	6.082 x 10^5	1.030 x 10^5	2.847 x 10^5

The low capacity observed for di-PEGylated lysozyme could be more related to a reduced accessibility to the pore space of the resin due to the increased size of the protein. Regarding the Langmuir association constant or affinity constant, native lysozyme had the highest affinity (6.08 x 10^5 M^{-1}) and it was lower for mono-PEGylated (1.03 x 10^5 M^{-1}) and di-PEGylated (2.85 x 10^5 M^{-1}) lysozyme; this value shows that PEGylation decreased the affinity of lysozyme for the resin. Examples where PEGylation also influences the affinity of the proteins are the reduction in the binding for glucose oxidase of PEGylated concanavalin A,[17] and the increase of the dissociation constant (kd) with anti-native RNase antibody when ribonuclease was modified with 4 and 9 mPEG 5,000 molecules.[50]

The 20kDa mPEG propionaldehyde exhibited a binding percentage between 1.0 and 3.0%, which was not considered significant, hence there appears to be no specific interactions between the PEG and the heparin adsorbent.

3.4.2 Heparin Affinity Chromatography (HAC) purification

Initial chromatographic tests in the 1 mL packed column with Heparin Sepharose 6FF showed a separation profile for PEGylated and native lysozyme using a linear gradient of NaCl at 1 M (**Figure 3.1**). Di-PEGylated lysozyme was not retained in the column at dynamic conditions and it was eluted in the washing step. This result is different from the detected binding in the batch isotherms but the effect of the flow could have affected the retention of the di-PEGylated conjugate. The mono-PEGylated and native lysozyme appeared early and relatively close in the elution at low salt concentrations as can be seen in **Figure. 3.1**. The identity of the peaks separated from the reaction components was verified with the injection of the individual standards. The amount of salt required to elute unmodified lysozyme (0.2 - 0.35 M NaCl) agrees with the reported 0.3 M NaCl for lysozyme purification from egg white at pH 7.4 on heparin-Ultrogel A4R.[51]The observed elution order of lysozyme isomers in the present study correlates with that for PEGylated proteins on cation exchange supports,[48, 52] however the profile presented here for HAC shows the complete resolution between mono- and di-PEGylated lysozyme conjugates which was not achieved in cation exchange chromatography (CEX) using Toyopearl Gigacap S-650 M and TSKgel SP-5PW resins.[48, 52] Based on this and the role of heparin as a weak cation exchanger with some proteins,[53] the affinity between lysozyme and heparin could be influenced mainly by an ionic effect, by decreasing of the charge in PEGylation caused by the PEG addition,[54] however no experimental demonstration is shown and affinity may be affected by different kind of interactions such as: hydrophobic interactions, hydrogen bonding or Van der Waals forces.[55] More studies to know the nature of these interactions between lysozyme and PEGylated proteins need to be done.

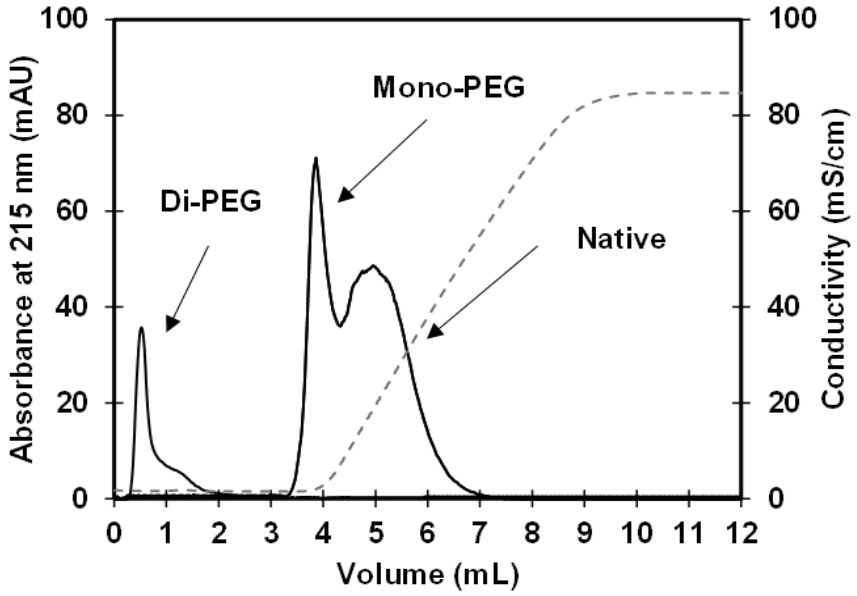

Figure 3.1 *Chromatographic profile in initial tests for the separation of a lysozyme PEGylation reaction (1:3) separation on HAC using a protein mixture of di-PEGylated lysozyme, mono-PEGylated and native lysozyme. Buffer A: Tris-HCl pH 7.5. Buffer B: Tris-HCl pH 7.5 containing 1M NaCl; linear gradient from A to B of 5 CVs, flow rate: 0.8 mL min^{-1}, loop: 200 µL. [Di-PEGylated lysozyme]: 2 mg mL^{-1}, [Mono-PEGylated lysozyme]: 4 mg mL^{-1}, [Native lysozyme]: 1 mg mL^{-1}.*

3.4.2.1 Optimization of linear gradient purification by Response Surface Methodology (RSM)

Since an incomplete separation of the mono-PEGylated and native lysozyme in the PEGylation reaction was observed, optimization of the separation of mono-PEGylated lysozyme in the linear elution gradient was pursued. In this optimization Response Surface Methodology (RSM) was applied through a Box-Behnken design (BBD). Although RSM has already been applied in chromatographic purifications of several therapeutic products and proteins such as recombinant erythropoietin on Blue-Sepharose,[56] so far the application of RSM for the optimization of the chromatographic purification of PEGylated proteins has not been explored. The experimental points were done with a mixture of the above-mentioned proteins in a 4:1 ratio. The factor protein load and its levels were chosen based on the maximum amount of protein available to test this variable; gradient length and flow levels were studied in a reasonable space.

The analysis of variance (ANOVA) results for the yield, purity and productivity is shown in **Table 3.2**. Additionally, **Figure 3.2** depicts the corresponding surface graphs. The lack of fit p-values in the three response variables: yield (0.537), purity (0.450) and productivity (0.254) were not significant, proving the fitting of the models. Also, the corresponding coefficients of determination (R^2) (yield (92.80%), purity (93.31%) and productivity (99.53%)) suggest the data and the variation in each response is explained by that percentage with the model. ANOVA assumptions (normality, constant variance and independence of the residuals) were verified and satisfied for all the models developed.

For yield, the protein load quadratic term was deleted from the model and the interaction flow-protein load was significant; protein loads from 0.25 mg mL^{-1} up to 1.0 mL min^{-1} combined with low flow (0.8 mL min^{-1}) (**Figure 3.2A**) maximize the yield. With regards to purity, the flow quadratic term was omitted from the model since it was not significant, but the interaction gradient length-flow was held, keeping protein load at 1 mg mL^{-1}. Low flows and gradients between 15 and 25 CVs improve the purity (**Figure 3.2B**), although in general the purities reached in the design are good. For productivity, all the interactions between the three factors were significant and the gradient length had an inverse impact on the productivity. **Figure 3.2C** shows the joint effect of gradient length and flow, the shorter the gradient (5 CVs) and the flow, the greater the productivity (0.050 mg of mono-PEGylated lysozyme (mg mL^{-1} min^{-1}). Regarding the gradient length-protein load interaction, if the gradient is around 5 CVs, regardless of protein load, productivity was between 0.040 and 0.045 (mg mL^{-1} min^{-1}). The interaction of flow-protein load at gradient length of 15 CVs (**Figure 3.2D**) indicates that if the flow goes from 0.8 to 0.9 mL min^{-1}, productivity will be between 0.035-0.040 (mg mL^{-1} min^{-1}), and it is the highest value if the flow is moved inside the design space.

Table 3.2 *ANOVA for the BBD in the optimization of mono-PEGylated lysozyme separation in linear elution gradient of HAC. P-value < 0.05 was considered significant*

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P-value
Yield (%) ANOVA					
Model	1215.40	6	202.567	54.72	0.000
GL	504.03	1	504.032	136.16	0.000
F	211.46	1	211.457	57.12	0.000
PL	204.12	1	204.117	55.14	0.000
GL^2	196.17	1	196.173	52.99	0.000
F^2	51.82	1	51.824	14.00	0.001
F*PL	89.76	1	89.755	24.25	0.000
Error	70.33	19	3.702		
Lack of fit	20.29	6	3.382	0.88	0.537
Pure error	50.04	13	3.850		
Total	1285.74	25			
Purity (%) ANOVA					
Model	5.69509	6	0.94918	59.13	0.000
GL	4.31269	1	4.31269	268.65	0.000
F	0.42639	1	0.42639	26.56	0.000
PL	0.08728	1	0.08728	5.44	0.031
GL^2	0.73327	1	0.733327	45.68	0.000
PL^2	0.16130	1	0.16130	10.05	0.005
GL*F	0.11243	1	0.11243	7.00	0.016
Error	0.30501	19	0.01605		
Lack of fit	0.09810	6	0.01635	1.03	0.450
Pure error	0.20691	13	0.01592		
Total	6.00010	25			
Productivity (mg mL^{-1} min^{-1}) ANOVA					
Model	0.003041	7	0.000434	759.97	0.000
GL	0.001977	1	0.001977	3458.81	0.000
F	0.000938	1	0.000938	1641.16	0.000
PL	0.000022	1	0.000022	38.26	0.000
GL^2	0.000032	1	0.000032	55.48	0.000
GL*F	0.000062	1	0.000062	108.64	0.000
GL*PL	0.000004	1	0.000004	7.1	0.016
F*PL	0.0000006	1	0.000006	10.37	0.005
Error	0.000010	18	0.000010		
Lack of fit	0.000004	5	0.000001	1.51	0.254
Pure error	0.000007	13	0.000001		
Total	0.003051	25			

In previous table GL = Gradient length, F = Flow, PL = Protein load.

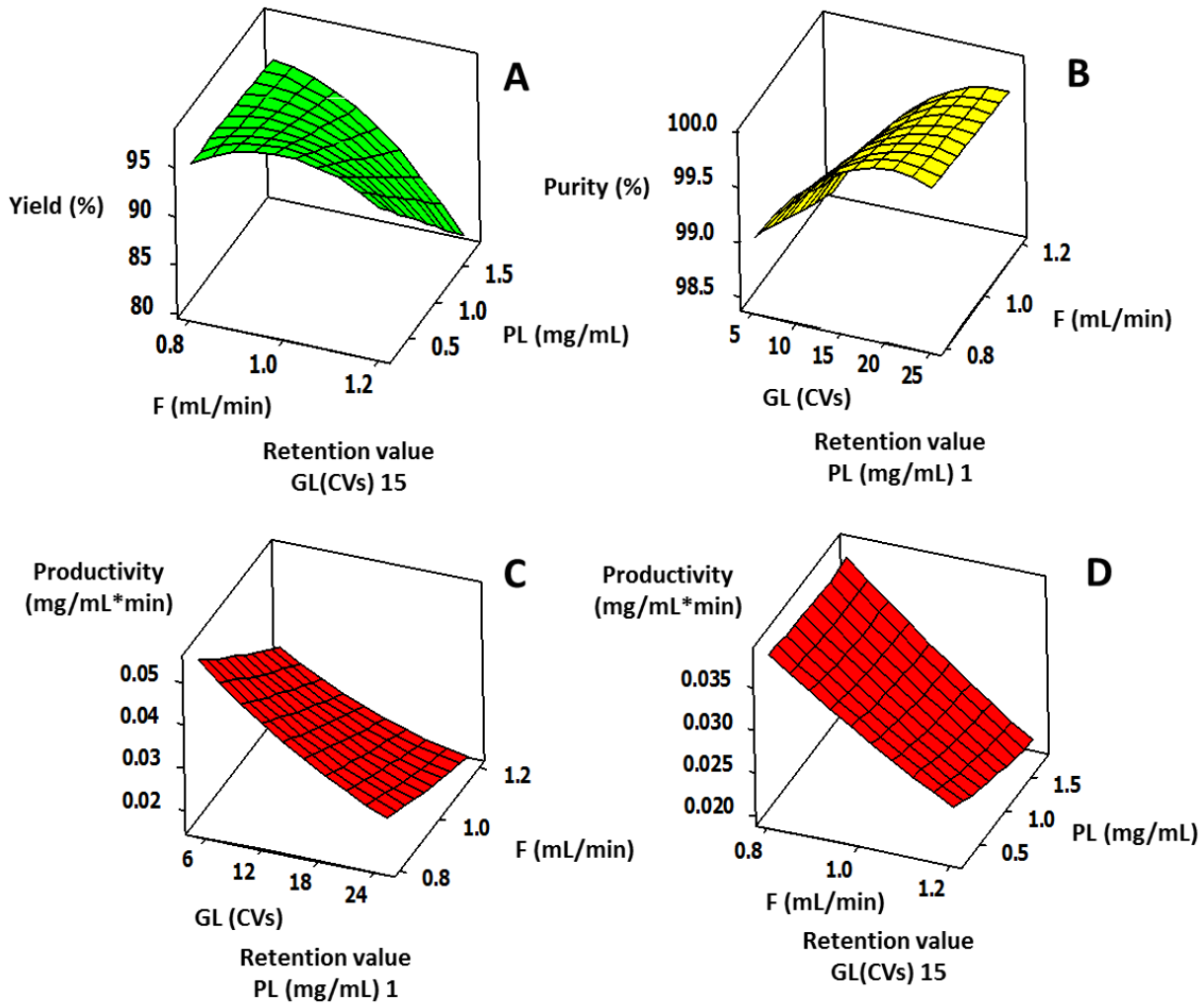

Figure 3.2 *Surface plots for yield, purity, resolution and productivity responses. (**A**) Yield: flow versus protein load (gradient length fixed at 15 CVs). (**B**) Purity: gradient length versus flow (protein load fixed at 1 mg mL^{-1}). (**C**) Resolution: flow versus protein load (gradient length fixed at 15 CVs). (**D**) Resolution: gradient length versus protein load (flow fixed at 1 mL min^{-1}). (**E**) Productivity: gradient length versus flow (protein load fixed at 1 mg mL^{-1}). (**F**) Productivity: flow versus protein load (gradient length fixed at 15 CVs).*

Expressions describing quadratic models are listed in equations (S3.1) to (S3.3) in Supplementary Material. Since the optimal conditions for the three response variables were slightly different and opposed regarding productivity and yield, a joint optimization was carried out using the optimizer application of Minitab. The yield, purity and productivity were maximized and

their respective top and objective values and importance; therefore, productivity was ranked with the highest importance followed by purities greater than 99 %. The optimal conditions given by the optimizer were gradient length of 13.5 CVs, flow of 0.8 mL min^{-1} and protein load of 0.95 mL min^{-1} with a desirability or global satisfaction of the committed solution of about 0.89. Final conditions were fixed as 13 CVs for gradient length, 0.8 mL min^{-1} for flow and 1 mg mL^{-1} for protein load. The desirability for the modified conditions was 0.87, not far from the previous estimation. These conditions make it possible to obtain mono-PEGylated lysozyme at a yield of 92.71%, purity of 99.69% and productivity of 0.0407 mg mL^{-1} min^{-1}. Finally, the model validation for each response variable was done running chromatographs at optimal conditions. In all the responses, the experimental values were not significantly different from the predicted values in the quadratic model (**Table 3.3**), confirming the precision of the model with a confidence level equal to or higher than 95% ($\alpha < 0.05$). All experimental response variables were in the range of the estimated confidence intervals (also included in **Table 3.3**). The chromatography results obtained by this optimization are shown in **Figure 3.3**

Table 3.3 *Statistic results in one-sample test for quadratic model validation in the mono-PEGylated lysozyme HAC at optimal conditions using linear gradient (1 M NaCl)*

Response variable	Predicted value by model	Experimental value	Confidence level (%)	*P*-value	Estimated Confidence Interval
Yield (%)	92.71	93.76	95	0.182	91.52-95.99
Purity (%)	99.69	99.55	96	0.044	99.41-99.70
Productivity (mg $^{-1}$mL min^{-1})	0.0407	0.0406	95	0.634	0.0397-0.0415

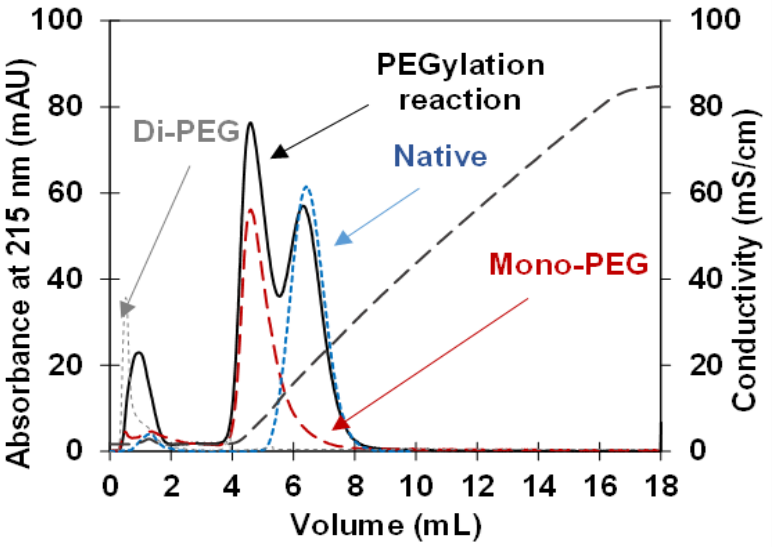

Figure 3.3 *Chromatographic profile of purified di-PEGylated, mono-PEGylated, native lysozyme and a lysozyme PEGylation reaction (1:3) separation on optimized linear gradient conditions in HAC. Buffer A: Tris-HCl pH 7.5. Buffer B: Tris-HCl pH 7.5 containing 1 M NaCl; linear gradient from A to B of 13 CVs, flow rate: 0.8 mL min^{-1}, protein load: 1 mg mL^{-1}, loop: 200 µL. [Di-PEGylated lysozyme]: 2 mg mL^{-1}, [Mono-PEGylated lysozyme]: 4 mg mL^{-1}, [Native lysozyme]: 1 mg mL^{-1}. Each protein standard and the reaction mixture were analyzed separately. The chromatograms were superimposed.*

3.4.2.2 Step gradient purification

Based on the optimal conditions of the linear gradient and salt concentrations at which mono-PEGylated and native lysozyme eluted, 0.18 M and 0.32 M NaCl, respectively, a step gradient method was designed to separate the lysozyme PEGylation reaction products. Several tests were assayed changing the percentage of phase B and the duration of the step for the first two steps; the third was fixed at 100% of B. Almost the complete separation of proteins was achieved with a first step at 0.05 M NaCl with 5 CVs, and a second step at 0.25 M NaCl with 5 CVs (**Figure 3.4**). These results point out that the small differences in the salt concentration make it possible to elute mono-PEGylated lysozyme from the native one (**Figure 3.4**); the low NaCl concentration is favorable to recover the protein in solution and even the subsequent desalting operation may be omitted. One sign of total purification in the step gradient procedure is the reached resolution (2.35 ± 0.001), which is greater than 1.5, a resolution factor greater than or equal to 1.5 is considered a complete separation of peaks[41] So, the yield and purity estimated by the plate model theory were 100% approximately; these values are 1.078 and 1.003 times greater

than the yield and purity reached during the optimal conditions in the linear gradient, respectively. Productivity (0.048 ± 0.001 mg mL^{-1} min^{-1}) improved slightly with respect to the linear gradient prediction (0.041 mg mL^{-1} min^{-1}). Clearly, the step gradient procedure improves the separation of the lysozyme PEGylation reaction.

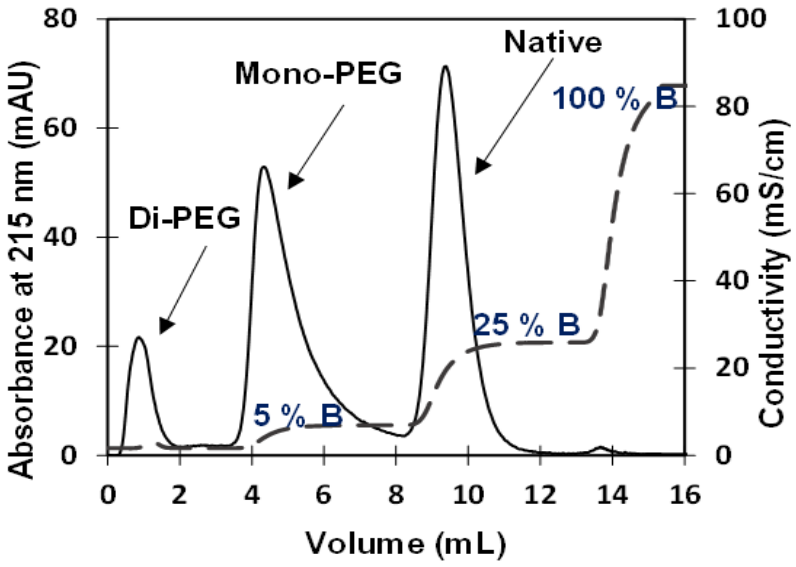

Figure 3.4 *Step gradient elution profile of lysozyme PEGylation reaction (1:3) on HAC. Buffer A: Tris-HCl pH 7.5. Buffer B: Tris-HCl pH 7.5 containing 1 M NaCl; step gradient from A to B. First step: 5% of B (5 CVs), second step: 25% of B (5 CVs), third step:100% of B (2 CVs), flow rate: 0.8 mL* min^{-1}*, loop: 200 µL.*

Since no detailed information on yield and purity have been published for other chromatographic separations of mono-PEGylated lysozyme it was not possible to compare our results with previously reported ones. Our yields and purities (either those obtained by the optimized linear gradient or step gradient) are better relative to those reached for mono-PEGylated ribonuclease A purification in hydrophobic interaction chromatography (HIC) using butyl sepharose (85% yield and 97% purity)[57] and sepharose 6B-PEG5000 (96% yield and 85 % purity).[58] In these separations the plate model was also applied to estimate yield and purity, and ribonuclease A is a protein with similar size to lysozyme (13.6 kDa vs 14.3 kDa), so it is a good guide to compare. The calculated productivity in the above-mentioned HIC purifications was 0.0039 mg mL^{-1} min^{-1} and 0.0031 mg mL^{-1} min^{-1}, respectively; these values are lower than those presented in this work.

Comparatively, HAC presented better resolution between conjugates than the observed when cation exchange resins separated 5, 10 and 30 kDa PEGylated lysozyme reactions[48, 52]. Also, the separation with Toyopearl Gigacap S-650M was longer (400 min)[48] than the required time to perform a chromatographic run in HAC at optimized step gradient method (16.8 min). In summary, HAC for PEGylated proteins purification is a promising technique.

3.5 Conclusions

The adsorption of di-PEGylated, mono-PEGylated and native lysozyme to heparin Sepharose 6 Fast Flow adsorbent is described by the monolayer Langmuir model. The PEGylated conjugates had less affinity for the heparin adsorbent than the native protein while 20 kDa mPEG propionaldehyde did not display unspecific binding with the resin.

A robust, efficient and novel chromatographic method for the purification of mono-PEGylated lysozyme from a PEGylation reaction mixture was developed with Heparin Affinity Chromatography (HAC). The linear salt gradient elution using 20 mM Tris-HCl with 1 M NaCl was optimized via a Box-Behnken design, for which the adequate conditions used in the separation were a gradient length of 13 CVs, flow at 0.8 mL min^{-1} and protein load of 1mg mL^{-1}. In this elution mode the predicted values by the model for the yield, purity and productivity were validated experimentally with an error level lower than 5% ($\alpha < 0.05$). The linear salt gradient found helped in designing a step gradient procedure to obtain a higher yield and purity of around 100 % approximately and a productivity of 0.048 mg mL^{-1} min^{-1}. These yields, purities and productivities achieved for mono-PEGylated lysozyme by HAC are superior to those found in the purification of PEGylated proteins using other types of packed-bed chromatography, particularly HIC and advantageous in time saving and resolution respect to CEX.

The optimization strategy implemented in operation stage with Response Surface Methodology (RSM) offers the possibility to streamline other chromatographic purifications with PEGylated proteins as a first step to design proper, efficient and fast purification procedures.

3.6 Supplementary material

Table A1. *Criteria for joint optimization for mono-PEGylated lysozyme purification in linear gradient of HAC*

Response variable	Goal	Target	Lower	Importance
Yield (%)	Maximize	95.00	88.00	1.0
Purity (%)	Maximize	99.80	99.00	1.5
Productivity (mg mL^{-1} min^{-1})	Maximize	0.040	0.030	8.0

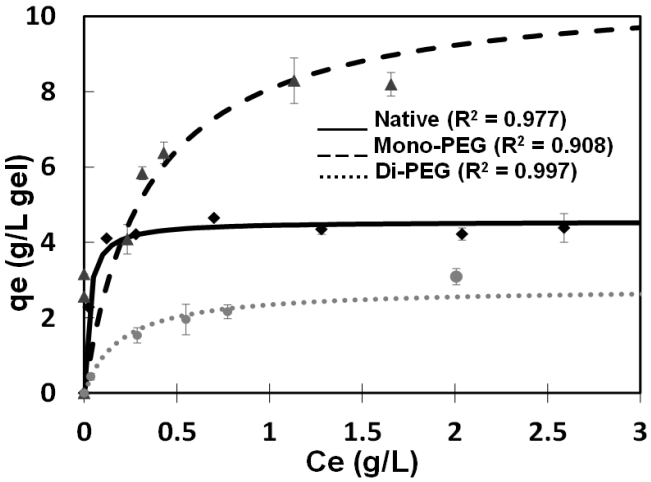

Figure S1. *Langmuir adsorption isotherms of native and PEGylated lysozyme on Heparin Sepharose 6 Fast Flow obtained after 5 h at room temperature.*

Expressions describing quadratic models:

$$Yield\ (\%) = -2.885 + 2.337GL + 156.257F + 17.568PL - 0.059GL^2 - 76.052F^2 - 22.330F * PL \quad \text{(S3.1)}$$

$$Purity\ (\%) = 99.329 + 0.101GL - 1.705F + 0.70PL - 0.004GL^2 - 0.302PL^2 + 0.059GL * F \quad \text{(S3.2)}$$

$$Productivity\ \left(\frac{mg}{mL*min}\right) = 0.108631 - 0.003281GL - 0.053439F + 0.302756PL - 0.000023GL^2 - 0.001393GL * F - 0.000095GL * PL - 0.005739F * PL \quad \text{(S3.3)}$$

Where GL is gradient length (CVs), F is flow (mL min^{-1}) and PL is protein load (mg mL^{-1}).

3.7 Acknowledgements

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Chapter 4. Simulation of mono-PEGylated lysozyme separation in heparin affinity chromatography using a general rate model

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4.1 Abstract

BACKGROUND: The bioavailability of therapeutic proteins is improved through PEGylation. This chemical modification involves the production of isomers with different number and site of attached PEG chains, which are difficult to separate efficiently. Their purification with chromatography requires the understanding of the operation and the evaluation of different operational conditions. We applied the General Rate Model (GRM) for modelling the linear salt gradient elution of mono-PEGylated and native lysozyme in Heparin Affinity Chromatography (HAC) considering mass balance equations for proteins in the bulk-fluid phase, in the particle phase and the kinetic adsorption.

RESULTS: The model was able to simulate the individual proteins and the separation of these in a PEGylation reaction using as proof-of-concept a mono-PEGylated and native lysozyme mixture under the change of operational parameters such as the gradient length (5, 13, 25 column volumes) and flow (0.8 and 1.2 mL min^{-1}) with a relative error in retention times of less than 6% and correlation coefficients greater than 0.78.

CONCLUSION: Simulation of the elution curves of PEGylated lysozyme in HAC was performed in this work and the diverse information generated by the model is explained through the physicochemical protein properties. This simulation represents a tool for optimization, prediction and future scale-up of PEGylated proteins purification, which would reduce the investment in time and resources to test several operation conditions.

Key words: *Simulation; mono-PEGylated lysozyme; Heparin Affinity Chromatography (HAC); General Rate Model (GRM); PEGylation.*

4.2 Introduction

PEGylated proteins are therapeutics with improved biodistribution, physical and chemical properties caused by the addition of polyethylene glycol (PEG) to the protein such as good solubility, resistance to proteolysis, retarded kidney elimination, and non-toxicity.[1, 2]

Lysozyme is a model enzyme which interacts with large substrates, and it has demonstrated bactericidal activity with numerous applications as a food preservative, antibiotic and pharmacological agent,[3, 4] it is due to this reason that the PEGylation of this protein and its use are being widely studied.[5, 6] The lysozyme PEGylation reaction contains reactive PEG, unmodified proteins and PEG-modified proteins or conjugates; of the latter only the mono-PEGylated conjugates have the appropriate characteristics and efficacy for their beneficial action. Despite the fact that diverse chromatographic modes in packed bed column have been tested in one-single step purification, such as Size Exclusion (SEC), Ion exchange (IEX), Reverse Phase (RPC) and Hydrophobic Interaction (HIC), the yield and purity are usually being low.[7] Recently, it has been demonstrated that heparin affinity chromatography (HAC) is able to separate the products of the lysozyme PEGylation reaction with high yield and purity.[8] However, purification of mono-PEGylated lysozyme and any other PEGylated proteins at pilot production level demand to have knowledge about the mechanism governing the separation so as to understand, optimize, control, predict and scale-up the chromatographic operation. Simulation and modelling of chromatographic processes is a tool to reach these aims,[9] in addition to selecting strategic directions in the design, thus saving time and resources.[10] Until now, simulation of chromatographic separation of polymer grafted proteins has not been studied and it can help to overcome or make efficient the current challenges and difficulties in the post-production of PEGylated proteins. Since the original physical and chemical properties of the PEG-modified proteins are changed by the PEGylation,[11] it is interesting to evaluate if the separation of PEGylated proteins can be predicted through the simulation. In this way, many trials in the chromatographic purification of PEGylated proteins may reduce costs in the optimization of expensive PEG-modified proteins.

The theoretical relationships in a process such as chromatography are described in a mathematical model, a set of expressions; then these equations are solved under specific conditions.[12] Among the most used models to represent and simulate the behavior of adsorptive chromatography with proteins is the General Rate Model (GRM). [10, 13] Particularly, in affinity

chromatography the examples of application of the GRM are few. These include the scale-up of the separation of a bovine serum albumin (BSA) and hen egg white lysozyme solution on Cibacron Blue F-3GA column,[14] the salt gradient elution of Bovine Serum Albumin (BSA) and rabbit hemoglobin (Hb) from Blue Sepharose CL-6B, and the pH gradient elution of a three-mouse antibody mixture (Ig G1, Ig G2a and Ig G2b) from protein A.[15]

The objective of this research was to model and simulate the elution curves of mono-PEGylated lysozyme and native lysozyme in HAC with a linear salt gradient elution applying the theory of the GRM. Also the simulation efficiency when changes in operational parameters (flow and gradient length) was evaluated.

4.3 Theory

4.3.1 General rate model

The general rate model (GRM) is a mathematical model used for studying chromatographic phenomena using rate expressions which represent the mass transfer of the components (protein and modulator or salt) in the system. The GRM considers in the mathematical formulation the adsorbent properties, process conditions and different mass transfer processes.[13, 16] The model is integrated by three sets of differential equations (Equation 1 to 3 in Supplementary Material), two of them describing the mass balance of the components in the bulk-fluid phase and in the particle phase inside the bed,[17] and the third representing the adsorption mechanism of the proteins to the adsorbent.

In the formulation of the GRM, the assumptions considered are: isothermal chromatography, spherical and uniform diameter of adsorbent particles, negligible radial dispersion in the column and no convective flow inside the macropores. There is an instantaneous equilibrium between macropore surfaces and the stagnant fluid inside the particles; diffusional and mass transfer parameters are constant and independent from the mixing effects of the components involved; the column was pre-equilibrated. Before the sample load the system does not contain protein; symmetric distribution of the compounds inside the adsorbent; and the column outlet protein dispersion flux is null.[13, 17]

Mass balance of the protein in the bulk-fluid phase takes into account diffusion, convection, accumulation and interfacial flux from bulk-fluid to particle (Eq. (S4.1) in Supplementary Material). For the salt component, interfacial mass transfer is not considered. The mass balance of the particle inside the pore involves accumulation in the micropore, accumulation in the macropore, and radial diffusion inside the porous particle (Eq. (S4.2) in Supplementary Material). Finally, the adsorption of the protein is modeled as a second-order kinetic binding reaction (Eq. (S4.3) in Supplementary Material) and initial and boundary conditions are given by equations (S4.4) to (S4.10) in Supplementary Material.

The affinity of the proteins for the ligand and the modulator concentration in the mobile phase were described with a linear relationship, which has been proposed by Melander *et al*[18] and validated by Sandoval *et al*[15] in affinity chromatography.

$$\mathbf{log_{10}b_i} = \boldsymbol{\alpha_i} - \boldsymbol{\beta_i C_{b,N+1}} \tag{4.1}$$

with b_i being a parameter in a Langmuir isotherm, in Eq. (4.2), that considers equal saturation capacities (C^{∞}) for all the components:

$$\boldsymbol{c_{pi}^{*}} = \frac{\boldsymbol{a_i c_{pi}}}{\mathbf{1}+\sum_{j=1}^{N} \boldsymbol{b_j C_{0j} c_{pj}}} \tag{4.2}$$

a_i and b_i are related to Damkhöler numbers of adsorption and desorption through Eq. (4.3)

$$\boldsymbol{b_i C_{oi}} = \frac{\boldsymbol{Da_i^a}}{\boldsymbol{Da_i^d}}, \quad \boldsymbol{a_i} = \boldsymbol{C^{\infty} b_i} = \boldsymbol{c_i^{\infty}} \frac{\boldsymbol{Da_i^a}}{\boldsymbol{Da_i^d}} \tag{4.3}$$

4.4 Materials and methods

4.4.1 Materials

The resin Heparin Sepharose 6 Fast Flow (Cat. No. 17099801) was purchased from GE Healthcare (Uppsala, Sweden). Lysozyme from chicken egg white (Cat. No. 10837059001) was acquired from Sigma Aldrich. Methoxy-PEG-propionaldehyde (Cat No. A3001-10) with a nominal molecular weight of 20 kDa was obtained from Jen Kem Technologies (TX, USA). Tris buffer grade (Cat. No. TR-16514) came from Winkler LTDA (Santiago, Chile). Sodium chloride (Cat. No.

106404) came from Merck Millipore (MA, USA). The mono-PEGylated lysozyme standard was prepared and purified as indicated by Mayolo-Deloisa *et al.*[19]

4.4.2 Chromatographic experiments

Resin was packed into a 5/5 HR column (5 cm length, 0.5 cm diameter, Pharmacia Biotech) and chromatographic experiments were performed in an Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) equipped with a 200 µL injection loop using a linear salt gradient of 1M of sodium chloride as it was pointed out by Mejía-Manzano *et al.*[8] As mobile phases A and B, 20 mM Tris-HCl pH 7.5 and 20 mM Tris-HCl pH 7.5 containing 1 M NaCl were used, respectively. The detection of native and mono-PEGylated lysozyme was done at 215 nm.

Individual standards of the proteins were injected separately to obtain the elution curves at a flow of 0.8 mL min^{-1} and 13 column volumes (CVs). To represent the lysozyme PEGylation reaction, mixtures containing mono-PEGylated and native lysozyme in mass ratio (4:1)[8] were prepared at a total protein load of 1 mg mL^{-1}. These mixtures were analyzed in the Äkta Purifier 10 System at different combinations of flow and gradient length as shown in **Table 4.1**.

Table 4.1 *Operation conditions used for simulating elution of mono-PEGylated and native lysozyme mixture in HAC*

Tested condition	Flow (mL/min)	Gradient length (CVs)
A	0.8	13
B	1.2	13
C	1.2	5
D	0.8	25
E	0.8	5
F	1.2	25

4.4.3 Software and numerical methods for simulation

The formulated rate model was translated to algorithms programmed in Matlab® R2014a software (The Mathworks, Natick, MA, USA) based on programming guides established by Sandoval *et al*[15] and Gu *et al.*[17] The bulk-fluid and the particle phase expressions were discretized

in space with finite elements (with 5 quadratic elements) and orthogonal collocation methods to obtain an Ordinary Differential Equations (ODEs) system.[15] This ODEs system was solved with the Matlab *ode15s* routine. All simulations were carried out on a laptop computer with Windows 8.1 operating system.

4.4.4 Parameter definition and estimation of kinetic parameters for individual protein standards

Parameters are classified in physical, operational, dimensionless mass transfer parameters and adsorption kinetic parameters. The first refers to the physical characteristics of the adsorbent, sample, phases and column (**Table 4.2**), and were found and established according to Orellana *et al*,[20] Hahn,[21] Geankoplis[22] and Hage and Cazes.[23] Since for this particular chromatography the reference in the values of tortuosity (τ_{tor}) and bed void volume fraction (ε_b) was absent, it was decided to perform preliminary simulations with individual standards and select their adequate value in the approximation of the kinetic parameters. The values tested for τ_{tor} were 2, 4 and 6 and for ε_b were 0.2, 0.3 and 0.4, taking as positive criteria the absence of an initial peak and how long took the simulation. Also interior orthogonal collocation points (Nr) with values of 2, 4 and 8 were evaluated.

Table 4.2 *Physical parameters used in chromatographic simulations*

Physical parameter	Value
Bed void volume fraction, ε_b	0.2[a]
Column capacity, C ∞(M)	Lys nat 0.660[b]; Lys mon 0.622[b]
Column length, L(cm)	5[c]
Column volume, V (mL)	1
Density of the mobile phase, ρ (g cm^{-3})	0.99823[d]
Inner diameter of the column, d (cm)	0.5[c]
Macroporous particle diameter, d_{porous} (nm)	300[e]
Molecular weight, MW (kDa)	Lysnat 14.7[f]; Lysmon 34.7[g]
Particle porosity, ε_p	Lysnat 0.75[h]
Particle radius, R_p (cm)	0.0090[i]
Tortuosity, τ_{tor}	2[j]
Viscosity of the mobile phase, μ (g cm^{-1} s^{-1})	0.010015[d]

[a][20, 21], [b][8], [c] Column dimensions (Pharmacia Biotech), [d][22], [e][23], [f] Sigma Aldrich, lysozyme (Cat. No. 10837059001), [g] Calculated, [h][21], [i][20], [j][14]

The dimensionless mass transfer parameters and related variables (Re, Pe_{Li}, Rh, D_{pi}, d_{mi}, D_{mi}, k_i, Bi_i) were determined through the same equations as in Sandoval *et al*[15] and Orellana *et al*[20] (Eq. (S4.11) to (S4.20) in Supplementary Material).

Adsorption kinetic parameters (α, β, Da^d) were estimated applying Eq. (4.3) for each protein from the experimental curves of the pure proteins at a flow of 0.8 mL min^{-1} and a gradient length of 13 CVs, starting off with approximated values and after obtaining the precise values through the algorithm "fminsearch" of Matlab with termination tolerance on $1x10^{-4}$.

Operational parameters such as gradient length and flow are those which can be modified during each run such as flow, protein concentration and gradient length.

4.4.5 Simulation of PEGylation mixture separation at different operational conditions

Once kinetic parameters were determined for mono-PEGylated and native lysozyme, protein mixtures at different conditions were simulated.

4.4.6 Statistical analysis

To evaluate the simulation effectiveness, two criteria were considered: the relative error between simulated and experimental retention times and the correlation of the simulated and experimental data. Relative error in retention times was calculated using the following equation:

$$\mathbf{Error}\ (\%) = \left|\mathbf{1} - \frac{\mathbf{t_{sim}}}{\mathbf{t_{exp}}}\right| \cdot \mathbf{100} \qquad (4.4)$$

Where t_{sim} is simulated retention time and t_{exp} is the average of experimental retention time. Correlation (Corr) was estimated by comparing point by point the simulated absorbance with the experimental absorbance.

4.5 Results and discussion

In the present work, PEGylated lysozyme separation in Heparin Chromatography from native and mono-PEGylated lysozyme were simulated applying the general rate model (GRM) theory and resolving the derived ODEs system through a numerical method on Matlab software.

4.5.1 Parameter definition and estimation for individual protein standards

As it has been described in methods, preliminary tests with different τ_{tor}, ε_b and Nr were done to select the best values for the simulation and kinetic parameter estimation. Therefore, adequate combination in this pre-selection was $\tau_{tor} = 2$, $\varepsilon_b = 0.2$ and Nr = 4.

The estimated kinetic parameters for individual standards of proteins (native and mono-PEGylated lysozyme) are shown in **Table 4.3**. From this table it can be seen that each kinetic parameter is different for both proteins. Constant α encompasses all the characteristic system parameters (electrostatic and hydrophobic interactions), β is only a descriptor parameter of the electrostatic interactions,[18] and Da^d is a dimensionless term which describes the relationship between the dissociation velocity of the ligand and protein and the mass transfer rate.[24] In this study, we observed that β is higher for mono-PEGylated than for native lysozyme, as it was expected that retention of the proteins in this heparin support is inverse to the magnitude of β parameter. Despite the fact that β is a function of the diverse properties such as protein charge (number, distribution and size), salt counter-ion and charge of the stationary phase,[25] it is obvious that the change in β is attributed to the decrease in number and size of charges in mono-PEGylated conjugates respect to native proteins, since that elution was performed at the same elution conditions and with the same heparin adsorbent. As described by some authors, [11,26] there is a charge-shielding effect due to the PEGylation, and in ion exchange chromatography PEGylated proteins are weakly retained. The observed difference in β parameter also can be explained by a change in the isolectric points (pI). Some studies,[5,27,28] have pointed out that mono-PEGylated lysozyme may be modified in other 5 lysine residues alternatively to the N-therminal residue (position 1), however the most abundant isomers of mono-PEGylated lysozyme are at position 1 and lysine 33, those had calculated pI between 11.07 and 11.12 in comparison to 11.28 of native lysozyme,[28] so a slightly decreasing in pI occurred, although an specific correlation is difficult to stablish in this moment, more analysis including calculated pI and other PEGylated proteins would need to be done. The Damköler number for desorption (Da^d) was greater for the

native than for the modified lysozyme. For the Damköhler desorption numbers (Da^d), the values were intermediate without indicating apparently some prevalence of desorption on diffusion rate. From the Da^d, the respective Damköhler adsorption numbers (Da^a) were estimated (9.18×10^{-18} for native and 3.02×10^{-45} for mono-PEGylated) and the adsorption rate is the rate-limiting the mass transfer step ($Da^a < 1$).[24] The respective Da^a for each protein shows that the adsorption of the PEG-conjugate shape occurs faster than in the native lysozyme. In general, to compare our estimated kinetic parameters using the Melander relationship with those published before is difficult since affinity supports and proteins are different.

Table 4.3 *Kinetic parameters estimated and used to simulate chromatographic profiles of individual and protein PEGylation mixtures in Heparin Affinity Chromatography*

Protein	α	β	Da^d	Error (%)	Corr*
Native lysozyme	3.849	16.956	1.765	1.62	0.995
Mono-PEGylated lysozyme	2.626	42.881	0.558	0.87	0.997

α, β and Da^d are dimensionless kinetic parameters.

* Correlation coefficient between simulated and experimental absorbance data.

The dimensionless number at flow 0.8 mL min^{-1}, such as Re, showed that the flow through the packed heparin bed is laminar (Re <<100). Pe were greater than 280, thus indicating that the mass transfer process in both proteins is controlled by convection rather than diffusion. The estimated Bi numbers for the studied proteins were greater than 100; therefore, the external film mass transfer is negligible in the pore diffusion,[10] predominating the intraparticle diffusion rate. In this last, although diffusion has few intervention on the separation, the calculated molecular diffusivity (Dm) showed that mono-PEGylated lysozyme (1.12×10^{-6} cm^2 s^{-1}) diffuses slower than the unmodified lysozyme (8.40×10^{-7} cm^2 s^{-1}). Estimated diffusivity for native lysozyme here was agree with the experimental coefficient measured by Brune and King[29] for this same protein in water, reinforcing the possible use of the mono-PEGylated lysozyme coefficient in future calculations. The diffusivity correlates inversely (if Stokes-Einstein equation is considered and the proteins are treated as rigid spheres[30]) with estimated protein viscosity radii (Rh) using the Fee and Van Alstine proposed model,[31] 20.087 Å for native and 50.31 Å for mono-PEGylated. As it can be concluded, the viscosity radii in the modified protein is approximately 2.5 times greater than that for unmodified lysozyme, and as consequence it diffuses faster. So, the separation may be slightly driven for these diffusivity differences in addition to other main processes such as convection and adsorption rate.

The experimental curves for native and mono-PEGylated lysozyme are shown in **Figure 4.1A** and **4.1B**. The peak of the native lysozyme is symmetric while the PEGylated one is asymmetric presenting peak tailing, which has been associated with the low desorption reaction rate when some fraction of the molecules bound to the ligand are dissociated slowly.[17] The peak tailing observed for the mono-PEGylated conjugates makes more difficult to simulate it; however, simulated and experimental absorbance data at 215 nm of both proteins had correlation coefficients (Corr) higher than 0.990. Regarding the retention times, standards showed a low relative error (below 2%). Therefore, individual standards were successfully simulated at 0.8 mL min^{-1} and at a linear elution gradient of 13 CVs.

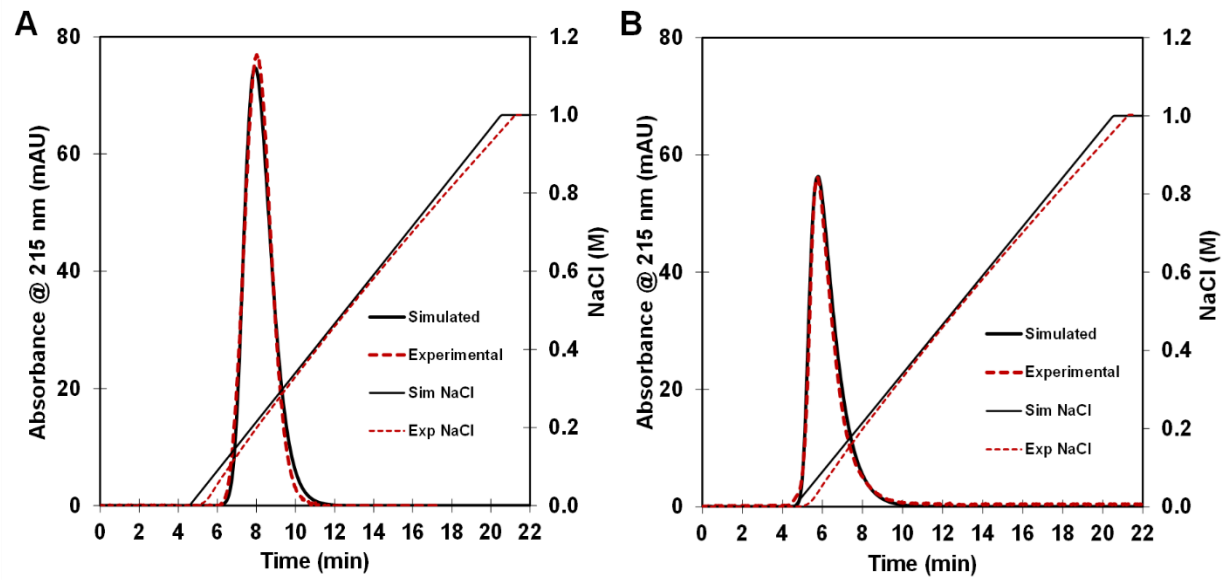

Figure 4.1 *Experimental (dotted line) and simulated (continuous line) profiles in Heparin Affinity Chromatography of individual standards and salt concentration: native (**A**) and mono-PEGylated lysozyme (**B**) at flow 0.8 mL min^{-1} and gradient length 13 CVs.*

4.5.2 Simulation of PEGylation mixture at different operational conditions of flow and gradient length

The comparison in retention times between the simulation and experimental profiles for mixtures at each operational condition from **Table 4.1** is indicated in **Table 4.4**, while their respective chromatographic profiles are shown in **Figure 4.2**.

Table 4.4 *Retention times and relative error of simulated and experimental peaks for individual and protein PEGylation mixture at different operational conditions using HAC*

Mixtures of proteins (conditions)	Native lysozyme			Mono-PEGylated lysozyme			Corr*
	t_{sim}	t_{exp}	Error (%)	t_{sim}	t_{exp}	Error (%)	
A	7.86	7.90	0.51	5.76	5.68	1.41	0.991
B	5.11	5.33	4.13	3.77	3.82	1.31	0.920
C	4.00	4.29	6.76	3.48	3.53	1.42	0.853
D	10.31	9.72	6.07	6.41	6.30	1.75	0.798
E	6.10	6.32	3.48	5.33	5.15	3.50	0.943
F	6.63	6.42	3.27	4.21	4.25	0.94	0.784

* Correlation coefficient between simulated and experimental absorbance data.

The protein mixture tested at condition A (flow at 0.8 mL min^{-1} and 13 CVs, **Figure 4.2A**) represents lysozyme PEGylation separation at the optimal conditions for the purification of mono-PEGylated lysozyme using a linear salt gradient found by our group in a previous study[8]. The relative error for the retention time of native lysozyme in mixture A (0.51%) was lower than that in the individual standard (1.62%), while for the mono-PEGylated lysozyme the error increased slightly (0.87 vs 1.41%). The correlation between the simulation and the experimental curve in this mixture was also good (0.991), only the peak of the simulated unmodified protein was slightly smaller than in the experimental mixture. Gradient of NaCl or modulator was also well simulated. The error in retention times of the native lysozyme at the other conditions increased relative to that observed in the individual standard. The same behavior was observed for the modified protein.

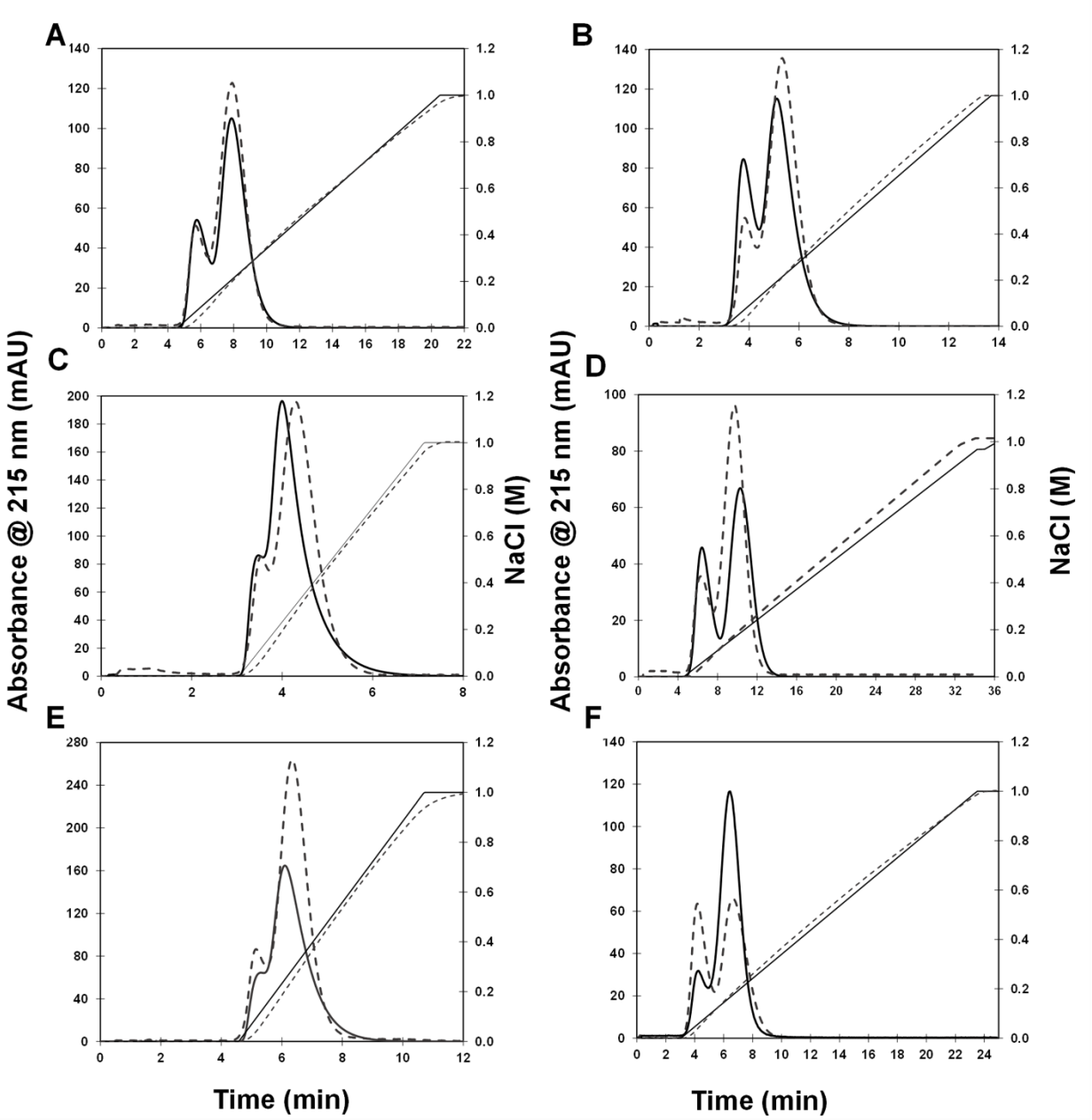

Figure 4.2 *Experimental (dotted line) and simulated (continuous line) profiles in Heparin Affinity Chromatography of protein PEGylation mixture (native and mono-PEGylated lysozyme): (**A**) 0.8 mL min^{-1} and 13 CVs, (**B**) 1.2 mL min^{-1} and 13 CVs, (**C**) 1.2 mL min^{-1} and 5 CVs, (**D**) 0.8 mL min^{-1} and 25 CVs, (**E**) 0.8 mL min^{-1} and 5 CVs, (**F**) 1.2 mL min^{-1} and 25 CVs.*

The biggest relative errors in the simulation of the mixtures were for the native lysozyme, up to 7%, while the PEG-protein errors did not exceed 4%. The error of retention times of native lysozyme in mixtures was the highest (above 6%, **Figure 4.2C-D**) when the mixture was simulated at extreme and opposite tested operational conditions: a high flow (1.2 mL min^{-1}) with a short

gradient length (5 CVs) as the mixture C or a low flow (0.8 mL min^{-1}) with a large gradient length (25 CVs) as the mixture D. The error for mono-PEGylated lysozyme was kept below 2% in almost all mixtures except for case E (**Figure 4.2E**), when a gradient length of 5 and a flow at 0.8 mL min^{-1} were used. In summary, mono-PEGylated lysozyme is better simulated than unmodified protein in mixtures. These results show a slightly bigger difference in the modelling at operational conditions (flow and gradient) with respect to the simulation in the separation of conalbumin, α-lactalbumin and BSA with the anionic Q-Sepharose Fast Flow, which errors ranked from 0 to 4.6%,[20] but our relative errors are lower than those obtained in the separation of BSA and hemoglobin with Blue Sepharose (values between 1.78 and 17.62%).[15]

The correlation indicates the overlapping of the curves and indirectly the amount of the predicted protein. In mixtures, the less accurate correlation for the simulations was for mixtures D and F (0.798 and 0.784, respectively). These had in common the same gradient length of 25 CVs but a different flow; also, the relative error for native lysozyme was somewhat higher. This may be due to small changes of the kinetic parameters at different conditions as seen by Orellana *et al,*[20] which would require their re-adjustment at each tested condition.

The average time in the simulation of the mixtures takes between 40 and 100 s, which is a very short time compared to performing a chromatogram (6 min) at the fastest conditions (flow at 1.2 mL min^{-1} and gradient length of 5 CVs), without considering the time involved in the preparation of samples or equipment. This suggests that simulation saves time and experimental costs in the determination of the adequate operational conditions.

It is important to point out that in the mentioned simulations, despite the fact that native lysozyme is a well-known protein, the mono-PEGylated conjugate has not been completely studied and characterized, and its properties are unknown with precision (molecular weight, viscosity radii, diffusivity), nevertheless, the results allowed to validate the used or calculated properties.

In our study, di-PEGylated lysozyme was not included in the PEGylation protein mixture because in our previous work[8] this protein was determined not be retained in the heparin support at dynamic conditions and it was proven that a mixture of mono-PEGylated lysozyme and native lysozyme in a 4:1 ratio represents the separation observed in a lysozyme PEGylation reaction.

Nowadays, model predictions based on pure proteins provide a good approximation to the real separation.[32]

The modelling of elution curves of PEGylated proteins or any other kind of polymer grafted-protein has not been researched; hence the results shown here establish a reference for future simulation of polymer-protein conjugates, particularly PEGylated proteins. In the same way, mass transfer data obtained in the simulation offers a guide for future scale-up procedures.[12]

As applications of the present work, we suggest the simulation of other PEG-protein conjugates: di-PEGylated, tri-PEGylated or poly-PEGylated isomers varying the size of the linked mPEG-chains in other random PEGylation mixtures. Actually, simulation of lysozyme PEGylation separation in other types of adsorption chromatographies such as ion exchange, hydrophobic or reverse phase and their comparison (from the technical and economical viewpoints) will provide information about the most robust technique in its purification. Also, the simulation of step gradient methods may increase its range of performance. As mentioned above, scale-up of Heparin Chromatography with lysozyme PEGylation reaction at pilot scale design process is a future recommended application of the simulation when conditions as the flow and gradient length are varied.

4.6 Conclusions

The purification of the adequate PEGylated protein conjugate from a reaction mixture with high yield and purity still continues being a challenge**.** Therefore, the purification of mono-PEGylated lysozyme in HAC is limited by both the understanding of the operation itself and the great number of conditions to test; thus, its simulation and modelling is a strategy to deal with these hurdles*.* In this work the separation of a lysozyme PEGylation mixture, representing the PEGylation reaction, was simulated under different operational conditions (flow and gradient length) using the GRM approach. Retention times for both proteins in mixtures were predicted with relative errors less than 6%, indicating that unmodified lysozyme was slightly more difficult to be simulated in extreme and opposite conditions of flow and gradient length. Correlation between simulated and experimental data was the lowest when a large gradient was used; however, the rate model was able to simulate the elution curves of the separation between mono-PEGylated and native lysozyme in HAC. The processes that controlled the separation were the adsorption/desorption rate, the convection and the pore diffusion.

In the future, scale-up to a pilot plant purification through HAC may be done taking as a basis the information collected by the simulation done; furthermore, the application of the GRM to chromatography of PEGylated proteins may be extended to other modes for optimizing each process individually**.**

4.7 Supplementary material

4.7.1 General rate model

Derived from continuity equations, Eq. (S4.1) represents the mass balance of the protein in the bulk-fluid phase, taking into account the terms of diffusion, convection, accumulation and interfacial flux from bulk-fluid to particle.[15] For the salt component or modulator, the last three terms are omitted because the interfacial mass transfer is not considered.

$$\frac{-1}{Pe_{Li}}\frac{\partial^2 c_{bi}}{\partial z^2}+\frac{\partial c_{bi}}{\partial z}+\frac{\partial c_{bi}}{\partial \tau}+\xi_i\left(c_{bi}-c_{pi,r=1}\right)=0 \tag{S4.1}$$

The mass balance of the particle inside the pore involves accumulation in the micropore, accumulation in the macropore, and radial diffusion inside the porous particle, and is given by Eq. (S4.2)

$$\left(1-\epsilon_p\right)\frac{\partial c^*_{bi}}{\partial \tau}+\epsilon_p\frac{\partial c_{bi}}{\partial \tau}-\eta_i\left[\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial c_{pi}}{\partial r}\right)\right]=0 \tag{S4.2}$$

The third equation expresses a second-order kinetic binding:

$$\frac{\partial c^*_{pi}}{\partial t}=Da^a_i c_{pi}\left(c^\infty_i-\sum_{j=1}^{N}\frac{C_{oj}}{C_{oi}}c^*_{pj}\right)-Da^d_i c^*_{pi}=0 \tag{S4.3}$$

The initial and boundary conditions are given by Eq. (S4.4):

$$\mathbf{c_{bi}}(\mathbf{0},\mathbf{z})=\mathbf{0} \tag{S4.4}$$

$$\frac{\partial c_{bi}(\tau,0)}{\partial z} = Pe_{Li}\left[c_{bi}(\tau,0) - \frac{C_{fi}(\tau)}{C_{oi}}\right] \quad \text{(S4.5)}$$

$$\frac{\partial c_{bi}(\tau,1)}{\partial z} = 0 \quad \text{(S4.6)}$$

for i = 1, …, N+1

$$c_{pi}(0,z,r) = 0 \quad \text{(S4.7)}$$

$$c^{*}_{pi}(0,z,r) = 0 \quad \text{(S4.8)}$$

$$\frac{\partial c_{pi}(\tau,0,z)}{\partial r} = 0 \quad \text{(S4.9)}$$

$$\frac{\partial c_{pi}(\tau,1,z)}{\partial r} = Bi_i\left[c_{bi}(\tau,z) - c_{pi}(\tau,z,1)\right] \quad \text{(S4.10)}$$

for i=1, …, N

4.7.2 Estimation of dimensionless mass transfer parameters and variables[15]

Re number was calculated as:

$$Re = \frac{2\,R_p v \rho}{\mu} \quad \text{(S4.11)}$$

For Peclet number (Pe_{Li}) with 0.001 < Re < 1000:

$$Pe_{Li} = \frac{L}{2R_P \epsilon_b}(0.2 + 0.011 Re^{0.48}) \quad \text{(S4.12)}$$

Solute molecular diameter (dm) was estimated multiplying for 2 the viscosity radius (Rh), which was calculated for native (Rh_{prot}) and PEGyated lysozyme ($Rh_{PEG\text{-}prot}$) using the proposed equations by Hagel[33] and Fee and Alstine,[31] respectively. For viscosity radius for mPEG (Rh_{PEG}) was used the expression indicated by Kuga.[34]

$$\mathbf{Rh_{prot}} = \mathbf{0.82}\ (\mathbf{MW})^{\frac{1}{3}} \tag{S4.13}$$

$$\mathbf{Rh_{PEG}} = \mathbf{0.1912}\ (\mathbf{MW})^{\mathbf{0.559}} \tag{S4.14}$$

$$\mathbf{Rh_{PEG-prot}} = \frac{\mathbf{1}}{\mathbf{6}}[\mathbf{108Rh_{prot}^3} + \mathbf{8Rh_{PEG}^3} + \mathbf{12}\left(\mathbf{81Rh_{prot}^6} + \mathbf{12Rh_{prot}^3 Rh_{PEG}^3}\right)^{\frac{1}{2}}]^{\frac{1}{3}} + \frac{\mathbf{2}}{\mathbf{3}}\frac{\mathbf{Rh_{PEG}^2}}{[\mathbf{108Rh_{prot}^3}+\mathbf{8Rh_{PEG}^3}+\mathbf{12}\left(\mathbf{81Rh_{prot}^6}+\mathbf{12Rh_{prot}^3 Rh_{PEG}^3}\right)^{\frac{1}{2}}]^{\frac{1}{3}}} + \frac{\mathbf{1}}{\mathbf{3}}\mathbf{Rh_{PEG}} \tag{S4.15}$$

$$\mathbf{dm} = \mathbf{2Rh} \tag{S4.16}$$

For molecules with MW > 1000, molecular diffusivity (Dm) is estimated with the Polson`s correlation:

$$\mathbf{Dm} = \mathbf{2.74\ x\ 10^{-5}}(\mathbf{MW})^{\frac{-1}{3}} \tag{S4.17}$$

Effective diffusivity (Dp_i) was calculated using equation (S4.18), which λ=dm/dp

$$\mathbf{Dp_i} = \frac{\mathbf{Dm}\left(\mathbf{(1-2.104\lambda+2.09\lambda^3-0.95\lambda^5)}\right)}{\tau_{\mathbf{tor}}} \tag{S4.18}$$

Film mass transfer coefficient (k_i) was determined with:

$$\mathbf{k_i} = \mathbf{0.687v^{\frac{1}{3}}}\left(\frac{\mathbf{1}}{\mathbf{Dm}}\in_{\mathbf{b}}\mathbf{R_p}\right)^{\frac{-2}{3}} \tag{S4.19}$$

And Biot number was calculated using:

$$\mathbf{Bi_i} = \frac{\mathbf{k_i R_p}}{\in_{\mathbf{p}}\mathbf{Dp_i}} \tag{S4.20}$$

4.8 Nomenclature

Bi_i	Biot number for mass transfer for component I, $k_iR_p/\varepsilon_pD_{pi}$
Co_i	Maximum concentration of protein i, equal to initial feed concentration of the component
C^∞	Maximum capacity of the column
$C_{A,N+1}$	Initial dimensionless concentration of the modulator in the mobile phase
c_{bi}	Dimensionless concentration of component i in the bulk-fluid phase
$C_{fi(\tau)}$	Feed concentration of component i
c_{pi}	Dimensionless concentration of component i adsorbed to the resin
d	Inner diameter of the column
Da_i^a	Damköler number for adsorption of component i, $Lk_{ai}Co_i/v$
Da_i^d	Damköler number for desorption of component i, L_{kdi}/v
D_{bi}	Axial dispersion coefficient of component i
d_{mi}	Solute molecular diameter
d_p	Solute molecular diameter
D_{mi}	Molecular diffusivity
D_{pi}	Effective diffusivity of component i
F	Flux of the mobile phase
L	Column length
k_{ai}	Adsorption rate constant for component i.
k_{di}	Desorption rate constant for component i.
k_i	Mass transfer coefficient of component i
N	Number of proteins in the sample. Modulator corresponds to component N+1
Nr	Radial dimension, interior orthogonal collocation points
Nz	Axial dimension, finite element discretization points
Pe_{Li}	Peclet number for mass transfer component i, vL/D_{bi}
r	Dimensionless radial coordinate
R_p	Radius of the adsorbent particle
Re	Reynolds number, $2R_pv\rho/\mu$
Rh	Viscosity radii
v	Interstitial velocity, $4F/\pi d^2\varepsilon_b$
z	Dimensionless axial coordinate
α_i, β_i	Experimental parameters for the exponential elution relationship
ε_b	Bed void volume fraction
ε_p	Adsorbent particle porosity
τ	Dimensionless time
τ_{tor}	Tortuosity
ηi	Dimensionless parameter for component i, $\varepsilon_{pi}D_{pi}L/R_p^2v$
μ	Viscosity of the mobile phase
ξ_i	Dimensionless parameter for component i, $3Bi_i\,\eta_i\,(1-\varepsilon_b)/\varepsilon_b$
ρ	Density of the mobile phase

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Chapter 5. Covalent immobilization of antibodies for the preparation of immunoaffinity chromatography supports

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5.1 Abstract

Immunosorbents in Inmunoaffinity Chromatography (IAC) are prepared immobilizing expensive antibodies without guidelines for ensuring the best coupling efficiencies, and avoid low binding capacities. Covalent immobilization of antibodies on N-hydroxysuccinimide (NHS) activated Sepharose 4 Fast Flow resin was optimized using human Ig G via full factorial design with incubation times (4, 9, 14, 19 and 24 h), temperatures (4 ºC and 20ºC) and coupling reaction buffers (sodium bicarbonate and triethanolamine). The best coupling efficiency (CE) (83.4 ± 8.7%) was reached with triethanolamine buffer, 14 h and 4 ºC. Comparison of antibody isotypes (Ig G or Ig M) by a nested factorial analysis suggested that antibodies in the Ig G isotype presents the best coupling efficiency.

Key words: *Coupling Efficiency (CE); Ig G; Ig M; Immunoaffinity Chromatography (IAC); Optimization.*

5.2 Introduction

Immunoaffinity chromatography (IAC) is a separation technique with high specificity and selectivity that takes advantage of the antigen-antibody recognition as the principle for the separation of different biotechnological products.[1,2] Diverse target biomolecules (i.e. viruses, hormones, peptides, enzymes and pharmaceutical substances) with different characteristics have been purified with this technique using antibodies as ligands.[3,4] These antibodies are immobilized on solid supports that consist of several low or high performance materials such as cellulose or agarose, modified silica, methacrylate, acrylamide and polyethersulfone among others.[5] An optimal support must be mechanically and chemically stable and should avoid non-specific protein binding[6] Antibodies and the support constitute the immunoaffinity matrix or immunosorbent.

In preparing IAC supports, there are three different antibody immobilization methods: random immobilization, site-specific immobilization and adsorption to secondary ligands.[7] The choice of the method for antibody incorporation should be based on ligand availability and cost, frequency of use, and the final objective of the immunoaffinity operation. In this sense, a typical method involves the covalent attachment of antibodies to the support because this technique

allows retaining and stabilizing these biomolecules during the chromatographic elution phase[8]. Besides, this strategy allows the reuse of the chromatographic resin[7] avoiding considerable losses of antibodies by leaching.

The covalent immobilization of antibodies uses amino, hydroxyl and carboxyl groups in the biomolecule for random immobilization while thiol groups and carbohydrate residues are exploited in site-selective immobilization[5]. Immobilization by primary amines represents the most used route since this group is an effective nucleophile which can react with carboxylic acids present in the resin through activated agents such as N-N´-carbonildiimidazol, aldehyde, isothiocyanate, cyanogen bromide, epoxide, tresyl/tosyl chloride or N-hydroxysuccinimide (NHS).[9] Furthermore, NHS esters are the most common activating groups for carboxylic acids, due to their capacity as leaving groups that form stable amide groups, when these react with amines.[10] However, this process is sometimes incomplete potentially due to steric hindrance or improper orientation which reduce antibody binding capacity.[7] For this reason, optimizing and assuring a more efficient antibody coupling to the support is important.

Until now, optimization of the covalent coupling of antibodies to produce IAC resins has not been studied thoroughly but should be an important reference for the immobilization of difficult to produce and/or expensive antibodies with potential use in the purification of high-value biomolecules, biomarkers or cells. In this context, statistical experimental designs have been applied in several biological, environmental, pharmaceutical, food and industrial processes to screen which factors influence an experiment while finding the optimal settings for the procedure,[11] reducing time and using resources efficiently. Finding the optimal coupling conditions in the preparation of IAC supports by using a model antibody such as human Ig G is important in establishing efficient reaction parameters for other expensive antibodies that might be used in the recovery of high-value biotechnological products via IAC. Furthermore, the coupling of antibodies of different kinds or with different physicochemical characteristics to chromatographic supports is also important to validate the obtained human Ig G optimized conditions. One of these antibodies, anti CD-133, has received particular attention due to its capacity of recognizing the stem cell marker CD-133.[12] In fact, diverse bioprocesses are being investigated right now for future purification of stem cells using the anti CD-133 antibody.[13] So preparation of an immunosorbent by coupling the anti-CD133 antibody can potentially contribute to the downstream processing of stem cells. Another important and potentially useful IAC ligand is the anti-PEG (polyethylene glycol) antibody, a novel murine antibody produced to evaluate PEGylation efficiency,

pharmacokinetics, pharmacodynamics and immunogenicity of PEGylated drugs.[14] This biomolecule may be used in the future for the purification of PEGylated proteins or PEG removal from different streams *via* IAC.

The aim of this work was to optimize the covalent coupling of isotype G antibodies on activated-Sepharose 4 Fast Flow resin using human Ig G as a model ligand and examining factors such as reaction time, temperature and buffer type. The optimized coupling conditions are compared with other isotype molecules (i.e. Ig M) to assess their applicability in the general preparation of IAC supports since comparison of the coupling of antibody isotypes into this kind of resin has not been previously reported. By knowing the adequate levels for each reaction factor, a high and optimal antibody coupling percentage can be reached. Although an increase in coupling efficiency does not necessarily correlates to an increase in the binding capacity,[15] a larger number of antibodies immobilized in a resin would extend the number of binding sites for the target molecule in the purification process.

5.3 Materials and methods

5.3.1 Materials

NHS-activated Sepharose 4 Fast Flow was purchased from GE-Healthcare (Uppsala, Sweden). Human Immunoglobulin Ig G (Cat. No. 340-21) was acquired from Lee Biosolutions (MO, USA). Anti PEG Ig M antibody (Cat. No. A01795) was purchased from Gen Script (NJ, USA) and anti CD-133 antibody was kindly donated by Dr. Richard C. Willson of the University of Houston. Bradford reagent, hydrochloric acid, triethanolamine and ethanolamine were purchased from Sigma-Aldrich (MO, USA). Sodium chloride, sodium acetate, glacial acetic acid, and sodium bicarbonate were all of analytical grade and acquired from J.T. Baker (PA, USA). Tris (hydroxymethyl aminomethane) and glycine were purchased from BioRad (CA, USA). Deionized water was produced using a Milli-Q-integral Water Purification System (EMD Millipore, Darmstadt Germany). All other salts and reagents used were from analytical grade or higher.

5.3.2 General experimental strategy

The followed strategy for the optimization of antibody coupling on NHS-activated Sepharose 4 Fast Flow resin is described in **Figure 5.1**. The first stage consisted of optimization

Ig G coupling through a full factorial design. After that, a nested factorial design study about the effect of antibody isotype on CE was performed.

5.3.3 Ig G coupling experimental design

A factorial design was generated in Minitab 15.1.20.0 (2007) software (Minitab Inc., PA, USA) using CE as a response output and as factors the reaction times (4, 9, 14, 19 and 24 h), temperature (4 or 20°C) and kind of coupling buffer (sodium carbonate ($NaHCO_3$) or triethanolamine) with two replicates. The results analysis was also performed using the Minitab software as specified below.

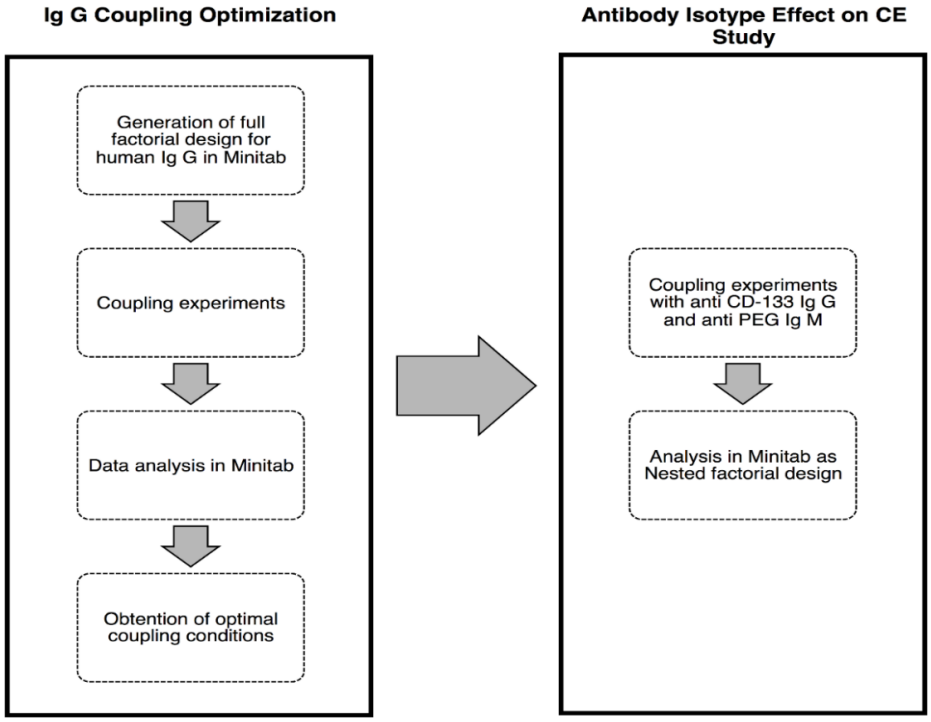

Figure 5.1 *General methodological scheme for the covalent optimization of antibody coupling used in this work.*

5.3.4 Preparation and coupling of antibodies to the activated resin

Preparation and coupling of human Ig G was carried out testing different variables along the recommendations provided by the manufacturer. Briefly, NHS-activated Sepharose 4 Fast

Flow (200 µL) was thoroughly washed with 2.5 V (gel volumes) of a cold 1 mM solution of HCl three times centrifuging at 16,000 rpm (27, 000 x g) for 5 min in each one of them. Afterwards, the resin was equilibrated twice with 2 V of the corresponding coupling buffer. 0.5 mg mL^{-1} stock solutions of antibodies were prepared in 0.2 M $NaHCO_3$ pH 8.3 + 0.5 M NaCl and 0.15 M triethanolamine pH 8.3 + 0.5 M NaCl buffers. Then, 1 mL of the antibody solutions was added to the chromatographic gel and incubated at the corresponding temperature and time conditions according to the factorial experimental design on an adjustable Lab rotator revolver (Labnet International, NJ, USA). Next, the samples were centrifuged at 16,000 rpm (27, 000 x g) for 5 minutes and the solution with residual antibody was removed. The unmodified activated groups in the gel were blocked with 1 V of 0.5 M ethanolamine pH 8.3 buffer + 0.5 M NaCl at 4 °C for 17 h, and afterwards alternating with 1.5 V of 0.1 M Tris-HCl pH 8.5 buffer and 1.5 V of 0.1 M acetate pH 4.5 buffer + 0.5 M NaCl, this cycle was repeated three times. All samples were collected and kept at 4°C until analysis was performed.

5.3.5 Sample analysis

After coupling, residual solutions were analyzed for total protein content using the Bradford colorimetric assay at 595 nm[16] in an EPOCH multiple well plate reader (Biotek, NY, USA). Calibration was done using as standards human Ig G (0.010-1.4mg mL^{-1}) and anti-PEG Ig M (0.010-0.060 mg mL^{-1}). Coupling efficiency (CE) was calculated as a percentage using the following equation:

$$\mathbf{CE}\ (\%) = \frac{(\mathbf{Mo}-\mathbf{Mr})}{\mathbf{Mo}} * \mathbf{100} \qquad (5.1)$$

Where Mo is the mass of antibody in the coupling solution before immobilization and Mr is the amount of antibody in the residual and wash solutions after reaction. Antibody mass is estimated as the product of concentration and volume. Finally, the optimum coupling efficiency (OCE) percentage at optimum immobilization conditions for human Ig G and its estimated confidence interval were calculated using the following equation:

$$\mathbf{OCE}\ (\%) = \mathbf{Y} + (\mathbf{Temperature_{4°C}} - \mathbf{Y}) + (\mathbf{t_{14h}} - \mathbf{Y}) + (\mathbf{Buffer_{Triethanolamine}} - \mathbf{Y}) + [(\mathbf{t_{14h}}\mathbf{Buffer_{Trietanolamine}} - \mathbf{Y}) + (\mathbf{Temperature_{4°C}}\mathbf{Buffer_{Triethanolamine}} - \mathbf{Y})] \qquad (5.2)$$

Where $\bar{Y}$ is the CE average for all the design points, $Temperature_{4°C,}$ t_{14h} and $Buffer_{Triethanolamine}$ are the CE means corresponding to the treatments at 4°C, 14h and triethanolamine buffer and t_{14h} $Temperature_{4°C}$, $t_{14h}Buffer_{Triethanolamine}$ and $Temperature_{4°C}$ $Buffer_{Triethanolamine}$ are the corresponding interactions, which were identified as the better conditions for coupling of human Ig G.

5.3.6 Study of antibody isotype effect on immobilization

To compare the effect of antibody isotype in coupling versus the obtained results for human Ig G, a test at optimal coupling conditions was done with anti CD-133 Ig G and anti PEG Ig M antibodies at concentrations of 0.5 mg mL^{-1} per duplicate.

5.3.7 Experimental design analysis for human Ig G coupling and for antibody isotype effects

Experimental design analysis of human Ig G CE was performed using the Minitab software through the option “balanced ANOVA” while the antibody isotype effect test (nested factorial design) was performed using “general linear model” option and means comparison through a Tukey test in the same software.

5.4 Results and discussion

5.4.1 Ig G coupling experimental design

Balanced ANOVA for Ig G CE (**Table 5.1A**) evaluating the effect of reaction times (5, 9, 14, 19 and 24 h), temperature (4 or 20 ºC) and buffer (sodium carbonate ($NaHCO_3$) and triethanolamine) indicated that reaction temperature and buffer as individual factors had a significant effect (p-value < 0.05) in the coupling efficiency as seen in **Figure 5.2A**. Double interactions between time and temperature, time and buffer and temperature and buffer were also significant. All these statistical analyses comply with the ANOVA assumptions of normality, homogeneity of variances and independence of residuals. The R-squared value in this analysis is 86.6% that indicates that this variability percentage in the response (CE) is explained by the statistical model or analysis.[17]

Table 5.1 *Antibody coupling optimization ANOVAS. Results for Ig G coupling (**A**) and for the effect of antibody isotype (**B**) on this same parameter are shown*

A. Balanced ANOVA for coupling efficiency (CE) in optimization of Ig G coupling

Source	**DF[a]**	**F[b]**	**P[c]**
Time	4	0.67	0.616
Temperature	1	23.71	0
Buffer	1	23.18	0
Time*Temperature	4	8.41	0
Time*Buffer	4	7.72	0
Temperature*Buffer	1	41.51	0
Error	24		
Total	39		
S[d] = 4.86015		R-Sq[e] = 86.64%	

B. ANOVA for coupling efficiency (CE) in effect of antibody isotype

Source	**DF[a]**	**F[b]**	**P[c]**
Isotype	1	32.42	0.011
Antibody (Isotype)	1	0.03	0.883
Error	3		
Total	5		
S[d] = 1.33618		R-Sq[e] = 91.54%	

[a] Degrees of freedom
[b] F-statistics
[c] *P*-value
[d] Standard deviation
[e] Squared regression coefficient

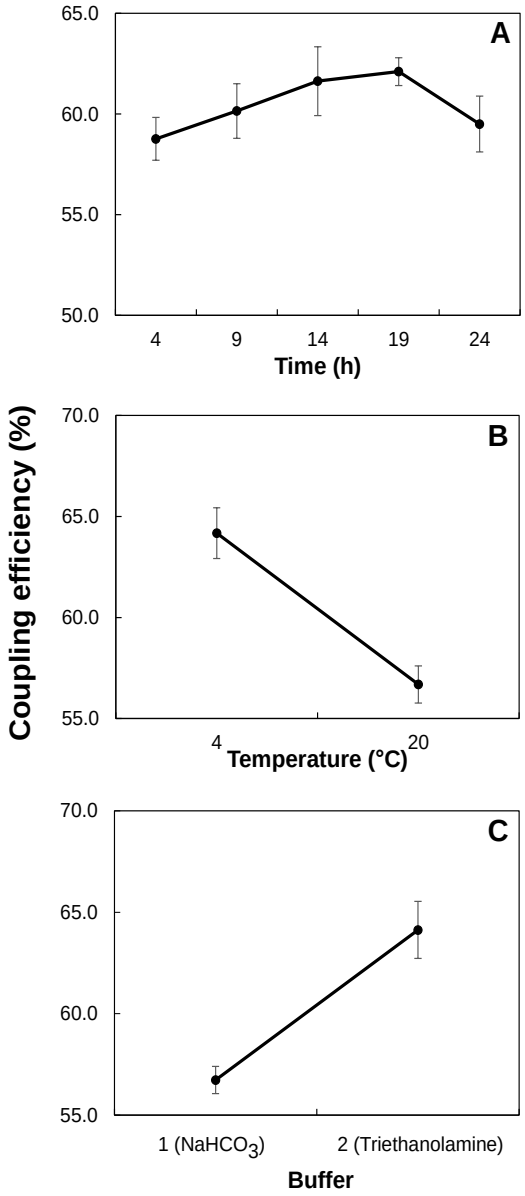

Figure 5.2 *Main effect plots for reaction time (**A**), temperature (**B**) and buffer (**C**) on coupling efficiency (CE) percentage (%) in the optimization of human Ig G immobilization on NHS activated Sepharose 4 Fast Flow resin.*

NHS esters are fast hydrolyzed.[18] Some studies report that the half-life for these activated groups is on the order of hours at physiological pH,[19] but reported coupling protocols do not specify reaction times to work with antibody immobilization. In this context, the time-temperature interaction (**Figure 5.3A**) indicates that after 4 h of incubation there is no change in CE whether it is carried out at 4 ºC or 20 ºC. However, if reaction times are longer (9, 14 and 24 h) and

temperature is set at 20 ºC the amount of immobilized antibody drastically decreases with respect to reactions carried out at 4 ºC for the same time lengths. These observations can be explained considering antibody denaturation caused by temperature and long immobilization times.[20] Other factors involved in antibody degradation are the selected reaction buffer solution, salt concentration and antibody concentration[21] and should all be explored further. In fact, Zheng and Janis[22] have found that the degradation by deamidation of the LA298 antibody occurs faster at 25 ºC. Therefore, antibody immobilization must be preferably performed at low temperatures such as 4 ºC.[20] The sole exception to this time-temperature correlation results was observed at 19 h where the CE was higher at 20 ºC (64.6 ± 4.9%) than at 4ºC (59.6 ± 6.2%). This may be due to additional antibody aggregation to the support induced at room temperature or even as a resulting difference of analysis variation.

For reaction time and buffer combinations, triethanolamine buffer shows better coupling results than with reactions using the $NaHCO_3$ buffer (see **Figure 5.3B**). This trend is kept at 4 ºC, but not at 20 ºC where Ig G coupling shows no significant change with temperature regardless of the buffer being used. Furthermore, antibody coupling is slightly lower at 4 ºC than at 20 ºC when using the $NaHCO_3$ buffer in the reaction (see **Figure 5.3C**). In this sense, despite the fact that both buffer solutions have the same pH value, the chemical components in them greatly influence antibody coupling. This is due to the differences in charges between triethanolamine and $NaHCO_3$ because this is a nucleophilic substitution reaction,[23] in which the predominant negative charged groups in the triethanolamine molecules may promote the selectivity of the nucleophilic attack of N-terminal primary amines in proteins to carbonyl groups whereas the scarcity of negative charges in the bicarbonate group can only do it at a lower rate. Also, among the two buffers the ionic strength is different, triethanolamine buffer has a calculated ionic strength of 0.65 M while the ionic strength for bicarbonate buffer is 1.1 M. This high ionic strength of the bicarbonate buffer may have adversely affected the solubility and coupling reaction of the antibody to the solid support. There are few reports about ionic strength affecting coupling reactions to chromatographic resins, for instance in the research of Matson & Little[24] the effect of concentration of NaCl (0.15, 0.5, 1.0, 2.0 and 3.0 M) on CE was evaluated. They found that CE is improved at high salt concentration which is divergent with the trend observed in our experiments.

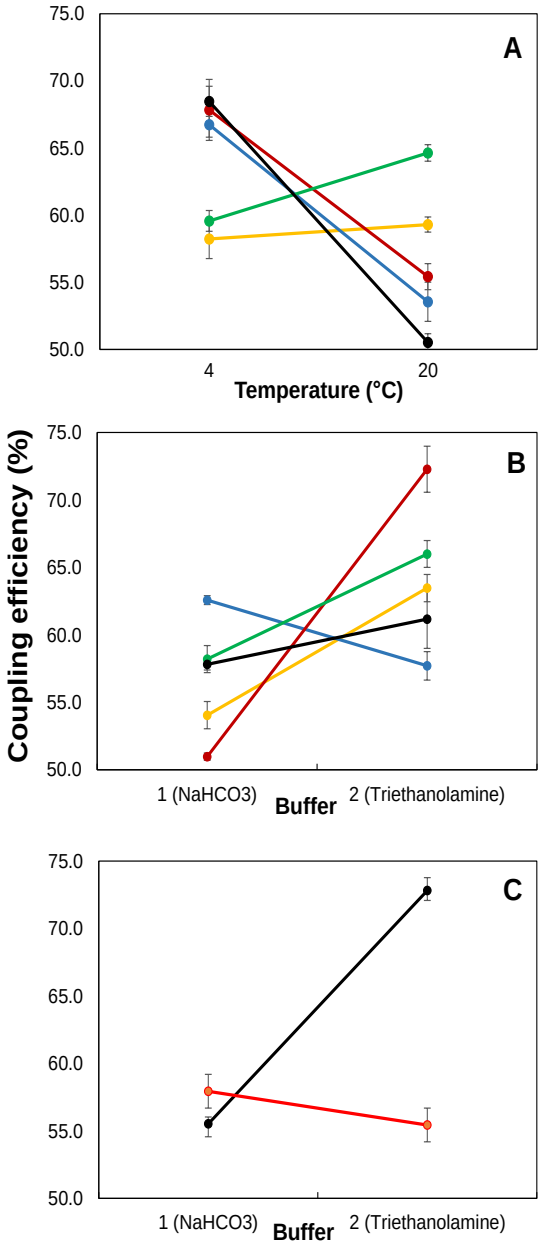

Figure 5.3 *Interaction plots of coupling efficiency (CE) percentages (%) for human Ig G immobilization on NHS activated Sepharose 4 Fast Flow.* ***(A)*** *Reaction time and temperature,* ***(B)*** *reaction buffer and time and* ***(C)*** *reaction buffer and temperature. (A) and (B): 4 h (●, yellow), 9 h (●, blue), 14 h (●, red), 19 h (●, green) and 24 h (●, black). (C): 4 °C (●, black) and 20 °C (●, red).*

Based on these results, the optimal conditions to statistically achieve the highest Ig G CE are reactions of 14 h at 4 ºC while using the triethanolamine buffer. For these conditions, the optimum coupling efficiency or OCE can be estimated at 83.4 ± 8.7% with an α (significance level or type I error) = 0.03. This value is in agreement with the 80% CE reported (**Table 5.2**) by Matson

and Little[24] for murine anti-tPA (tissue plasminogen activator) Ig G on NHS activated Affi-Prep 10 resin and by Salvador and collaborators[25] for Ab145 antibody immobilized on NHS-activated Sepharose 4FF. It should be noted however that in other reports[26, 27] CEs of antibodies up to 93% were reached in this resin. In the case of immunoaffinity resin for follicle stimulating hormone (FSH) the CE of 100% could be easily overestimated since the coupling was semiquantified through SDS-PAGE. Strict comparison is difficult to achieve with our experimental design since in most of these works details about coupling conditions at different stages are not given. From **Table 5.2** it is important to mention that the room temperatures (20-25 °C) and the short times (0.17-6 h) that were used in the other research reports differ with our study, nonetheless the initial antibody concentration used in these publications is high (2-10 mg mL^{-1}), while in this work antibody concentration was kept at 0.5 mg mL^{-1} representing an advantage from the standpoint of working with difficult to produce or expensive antibodies as ligands. Furthermore, as it was concluded with the anti-tPA immobilization study, the incremental ligand-matrix density may decrease the antigen binding efficiency.[24]

Table 5.2 *Immobilization conditions for different Ig G isotype antibodies on NHS activated Sepharose 4FF*

Coupled antibody	CE (%)	Antibody concentration (mg mL^{-1})	T (h)	T (°C)	Coupling buffer	Ref.
Anti-tPA (Ig G)	80	4	0.17	20†	0.1 M 3-(N-morpholino)-propanesulfonic acid pH 7.5 + 0.5 M NaCl	24
Ab145 (Ig G)	80	10	3	20†	0.2 M $NaHCO_3$ pH 8.3 + 0.5 M NaCl	25
M5B4 (Ig G)	93-95	10	4	25	0.2 M $NaHCO_3$ + pH 8.3 + 0.5 M NaCl	26
Anti-human-FSH (Ig G)	100	2*	6	20†	Phosphate buffer saline (PBS) pH 7.4	27
Human Ig G	74.7-92.1	0.5	14	4	0.15 M triethanolamine pH 8.3 + 0.5M NaCl	Current work

*Estimated value from data

†Inferred from literature.

5.4.2 Effect of antibody isotype on immobilization

The effect of antibody isotype on CE was studied as a nested factorial design. Nested factorial or hierarchical designs are those in which each level of a given factor (nested factor) appears in only a single level of any other factor.[28] For isotype comparison, the main levels are G and M isotype, the nested levels in the G isotype corresponded to the anti CD-133 and human Ig G antibodies, because these antibodies belong to G isotype. The anti-PEG Ig M antibody was used as representative antibody of the M isotype. Results of this nested design were analyzed by the General Linear Model option in Minitab. The R-squared for this analysis (91.5 %) is greater than the rule of thumb (≥ 75%),[17] achieving a good fit of the data. According to these results, antibody isotype presented significant coupling differences (p-value < 0.05) while different antibody kinds inside the isotype G showed no difference whatsoever (**Table 2.1B**). In this sense, the selected anti-PEG Ig M had the lowest CE (76.8 ± 1.7%) with respect to the antibodies of the G isotype that had a CE of about 83% (83.5 ± 0.8% for human Ig G and 83.3 ± 1.3% for the anti CD-133 antibody, respectively). Ig M has a pentameric mushroom-shaped structure with a central region that projects out from the plane while Ig Gs show a "Y" shaped structure that can be considered to be on a single plane. The immunoglobulin M isotypes present molecular weights around 950 kDa[29] while the G isotype has molecular weights of about 150 kDa.[30] Therefore, the observed differences in CEs may be a consequence of the isotype molecular size. It is believed that the larger size of Ig M molecules cause a higher steric hindrance that leaves a smaller number of available sites for antibody bonding to the activated resin. This might however be compensated in the IAC runs by its pentameric structure since larger amounts of the target biomolecule can be potentially bound. Ig Ms currently present a limited usage as chromatographic ligands prepared by covalent attachment,[31] but the use of Ig M isotypes in IAC may represent a viable option for the creation of IAC matrixes,[32] particularly when Ig Gs are not available or its production is expensive.

The performed Tukey test (**Figure 5.4**) does not present differences between the means of the experimental CE between the tested G isotype antibodies. Furthermore, the results in this work allow the use of the optimized coupling conditions found for human Ig G to other antibodies of the same isotype. This is important in the case of the anti CD-133 antibody since an immunoaffinity matrix for the chromatographic separation for stem cells can be potentially created. The same conditions may be extrapolated to other isotypes like the case of the selected anti-PEG Ig M in this study. Even when CE are not as high as for the Ig Gs, these are still good considering

that they surpass CEs of 70%. Anti-PEG Ig M can potentially be used in the recovery of polymer-modified molecules or in negative chromatography to remove PEG from process streams where this polymer needs to be added.

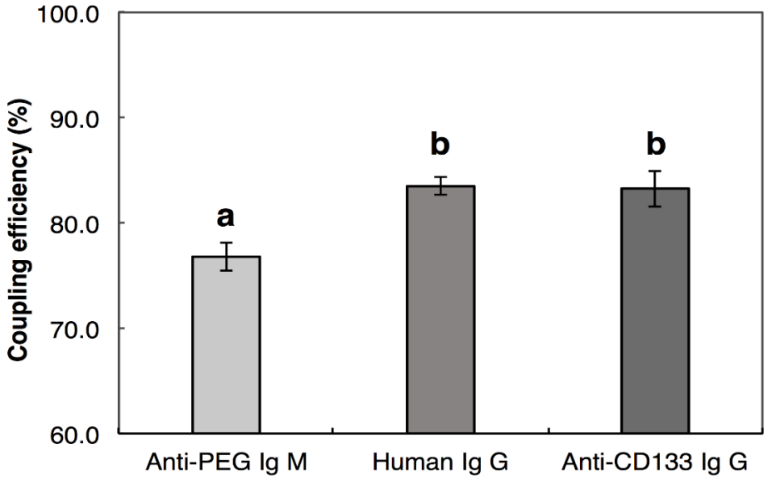

Figure 5.4 CE percentage comparison between selected antibody isotypes and kinds. Graph bars with different colors and letters are significantly different.

5.5 Conclusions

The covalent immobilization of Ig G antibodies on NHS activated Sepharose 4 Fast Flow can be optimized to reach a CE of up to 83.48 ± 0.80% with reaction conditions of 14 h, 4 ºC and using triethanolamine buffer. The tested Ig M isotype had a lower coupling efficiency compared with the anti G isotype antibodies, which can be related to its larger molecular size which causes steric hindrance. It was found however that antibodies of both isotypes can be covalently attached to the NHS groups in the resin using the same immobilization method and conditions as for the model human Ig G with excellent CEs. This is especially useful when dealing with expensive or difficult-to-manufacture antibodies intended to be used in IAC where large amounts of these molecules are needed to achieve the modification of industrial quantities of resin. This is the case of the selected anti CD-133 and anti-PEG antibodies selected which presented reactions with CEs of 83.3 ± 1.3% and 76.8 ± 1.7%, respectively. Future studies in this path will have to evaluate and correlate the binding capacity and antibody orientation of these immobilized antibodies with the achieved percentage of coupling in route of finding conditions to optimize biomolecule binding in IAC operation.

5.6 Funding

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Chapter 6. Conclusions, contributions and perspectives

After the results presented in this work, we found that PEGylation reaction media is suitable to perform an ATPS to recover the mono-PEGylated and di-PEGylated conjugated in (approximately 60% and 100%) in the top phase while most native lysozyme is partitioned towards the bottom phase. These results suggest that *in situ* ATPS might serve as an integrated unit operation not only for the recovery but also for the concentration of PEGylated proteins. Alternatively, this *in situ* ATPS procedure is a likely option to the re-use or the recycling of unmodified protein.

Regarding the AC research, it was concluded that PEGylation decreases the affinity of native lysozyme to Heparin Sepharose 6 Fast Flow resin while the free reactive mPEG propionaldehyde is not bound to the adsorbent. Optimized HAC is an advantageous technique in the purification of mono-PEGylated lysozyme in comparison to other chromatographic modes in packed-bed format, with high yield, purity and productivity. At the same time, the general rate theory is able to explain the mass transfer mechanisms involved in the separation, which showed to be controlling by the adsorption/desorption rate and convection processes. On its part, the covalent coupling of antibodies is mainly affected by temperature and the type of reaction buffer. However, reaction times also influence the degree of modification of the supports while the use of G isotype antibodies increases the coupling efficiency.

As general conclusion, *in situ* ATPS and heparin affinity chromatography are novel strategies in the recovery and purification of PEGylated proteins, which experiment great benefits (operation integration, time and reactive savings, selectivity, predictability, improved yields, purities and productivity, and easy) when these are combined with optimization tools such as modelling and design of experiments.

Among the main contributions of this thesis, a framed strategy to recover PEGylated proteins directly from the reaction media, considering the operation as part of a downstream processing strategy for PEGylated proteins was proposed. This aspect has not been fully addressed in previous works about ATPS with PEG-modified proteins. So, the incorporation of the complete PEGylation mixture and phase formation in a single operation reduces the number of purification stages. After the *in situ* ATPS implementation, the recovery of proteins from each phase through an ion exchange monolith is highlighted because this has also not been considered in earlier works. Moreover the proposed system studied here represents a green-environment solution due to ammonium sulphate rich-phase may be used as fertilizing solution for soils in comparison to the phosphate formed ATPS, which are responsible for eutrophication problems when these residues are discharged into bodies of water.

A robust affinity chromatographic method is suggested to produce mono-PEGylated lysozyme achieving market purity requirements and it also establishes a methodology to create a specific AC method to purify other therapeutic PEGylated proteins. It should be noted that DoE and the joint optimization of several response variables is scarcely treated in protein chromatography and in other newly developed methods. Another contribution is a mathematical algorithm to predict the separation of the modified PEG-proteins in HAC with application at laboratory, pilot or even industrial scales. A protocol to generate immunosorbents by covalent attachment of antibodies is suggested, minimizing time and antibody losses. From the point of view of knowledge generation, it is the first work dealing with AC applied to the purification of PEGylated proteins. All the results have either been published or accepted in indexed journals related to novel bioseparation procedures for PEG-proteins and the scientific disclosure of these contributions has been reinforced in two international conferences.

The perspectives or future work derived from this thesis have been classified in short-medium and long-term perspectives. The first type has been defined as those experiments or projects to be developed in a period not exceeding 2 years after the presentation of this dissertation and those are focused on the publication of scientific papers giving continuity to the generated knowledge. The second group refers to tentative projects which will aim for the generation of research products (patents, manuscripts or chapter books) and the formation of human resources (undergraduate, master and doctoral students).

In the short-medium term perspectives related to *in situ* ATPS, comparison between this operation and non-integrative ATPS (where the system is built in an independent operation adding extra PEG) can be made to validate the economic impact of the proposed strategy. In addition, the recovery and concentration of native lysozyme in the bottom phase by ultrafiltration and its use as reactant in other PEGylation reactions may be an alternative to reduce costs. The economic analysis using specialized software is an interesting option to demonstrate the advantages of this novel strategy.

For HAC, frontal analysis experiments to determine binding capacity and stability studies of the heparin adsorbent are planned to demonstrate extra advantages of this chromatography against other modes. The upcoming task derived from the simulation is the scale-up of this chromatography in a bigger column than that used in our studies, because a column with larger dimensions will increase the production volume of the PEG-conjugate protein and could represent an advancement towards its transference to an industrial scale.

After the optimization of the covalent immobilization of antibodies, the next task consists in the generation of immunoaffinity supports with immobilized anti-PEGylated protein antibodies and its evaluation in the separation these conjugates. In this way, some work has been started regarding the immobilization of these anti-PEGylated protein antibodies and heparin on chromatographic monoliths with the goal to produce affinity supports to purify these molecules. Finally, a review manuscript describing the current developments, trends and challenges in the application of AC to PEGylated protein separation is also visualized.

On the other hand, the long-term perspective projects derived from this work are:

- Physicochemical stability of *in situ* ATPS. This line will focus in studying the effect of temperature, pH and PEGylated proteins modified with different polymer sizes on the recovery of the molecules in *in situ* ATPS.
- Phase transfer PEGylation. It will consist on performing PEGylation reactions in a pre-formed PEG-phosphate system and analyzing the conversion yields and reaction rates *versus* conventional PEGylation.
- Design of affinity ligands for PEGylated-proteins. Considering the mentioned problems related to ligand availability (cost and production) as a limitation in the use of affinity chromatography, the generation of specific ligands to PEGylated proteins may represent

a promising future research line. At first, the characterization of the interactions between PEGylated proteins and the related affinity ligands by atomic force microscopy, microcalorimetry, protein-protein interactions and structural techniques (e.g. mass spectrometry, x-ray, circular dichroism) should be performed. Afterwards, structures with new chemistries would be designed and evaluated through molecular docking simulations with platforms such as PyMOL, AutoDock or QSAR. The production of these ligands can be achieved in two ways, by chemo-enzymatic synthesis for molecules or by peptide ligand design produced in simple recombinant systems.

- Development of *in situ*-affinity ATPS based bioseparations. Because of this thesis, this strategy would combine the *in situ* ATPS concept with the use of affinity ligands not only to recover PEGylated proteins but also to recover other biomolecules or cells.

Appendices

Appendix A – Material presented in Congresses

- **Abstract sent to Affinity 2015 (September 2015)**

Optimizing antibody immobilization for the preparation of immunoaffinity chromatography supports

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Abstract
Immunoaffinity chromatography (IAC) exploits the immobilization of antibodies on chromatographic supports for the separation of target molecules with high selectivity and specificity [1]. One of the most used antibody coupling techniques is the covalent attachment through primary amines in the antibody and an activated agent in the resin such as N-hydroxysuccinimide (NHS) [2].In this work, the coupling of human Ig G as a model antibody was optimized evaluating reaction times (5, 9, 14, 19 and 24 h), temperatures (4 or 20 ºC) and buffers (sodium bicarbonate or triethanolamine) as factors. The maximum coupling efficiency (83.43 ± 8.69%) was achieved with triethanolamine buffer at 14 h and 4 ºC. At these optimal coupling conditions, the coupling of two other potential IAC antibodies of different isotype and subtype (see Figure 1) was compared in a nested factorial analysis. Results indicate (Figure 1) that the Ig G isotype (83.38 ± 1.10%) had a better coupling efficiency with respect to Ig M (76.79 ± 1.72%). However, no significant difference between Ig G subtypes can be observed. Even when all tested antibodies presented good coupling efficiency (> 75%) significant differences can be observed between Ig isotypes, nonetheless, the obtained optimal conditions can be used in these procedures to prepare IAC supports.

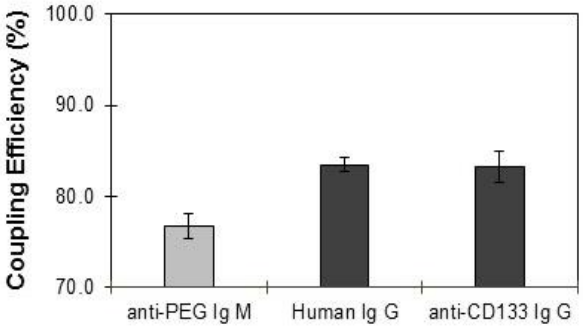

Figure 1. Coupling efficiency (%) for isotype antibody. Graph bars with different colors are significantly different.

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Keywords: Immunoaffinity Chromatography, Antibody Immobilization, Optimization.

- **Abstract sent to ESBES 2016 (September 2016)**

Innovative process for the production and recovery of PEGylated lysozyme through *in situ* aqueous two phase systems and monoliths

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Abstract

The development of novel, high-yield, fast and economical downstream processes is a current paradigm for biopharmaceutical proteins. The choice of appropriate techniques and logical combination are the key for a successful and efficient purification [1] of these products. As well is known, product of N-terminal amine PEGylation is a heterogeneous mixture of proteins with varying number of polyethylene glycol (PEG) moieties [2]. Aqueous two phase systems represents a valuable and attractive recovery option for proteins [3] and also for PEG modified proteins. In this work, ATPS formation immediately after lysozyme PEGylation reaction was researched with different polymer (UCON, ficoll, dextran) and salt (sodium phosphates, potassium sulphates, sodium sulphate, ammonium sulphate, sodium carbonate) solutions, using the reaction media as part of the phases system. The best option for the ATPS formation was the addition of 4M ammonium sulphate. This approaching has the advantages of PEG saving, quick processing and concentration stage emerges being called here *in situ* ATPS. The phases were analyzed using a cation exchange monolithc disk. The results shown a preferential distribution of PEGylated conjugates in top phase (PEG) and native lysozyme for bottom (salt). SDS-PAGE electrophoresis of eluted fractions from chromatography helped to identify the proteins and their relative purity. Implementation of monolithic chromatography in PEGylated protein purification offers fast separation at large scale due to the convective mass transference properties [4]. As a final conclusion an integral bioprocess is suggested in the purification of monoPEGylated lysozyme and potential recovery of non-reacted native lysozyme.

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Keywords: *in situ ATPS,* monolithic disk, PEGylated lysozyme, PEGylation

- **Abstract sent to Affinity 2017 (June 2017)**

Optimized purification of mono-PEGylated lysozyme in Heparin Affinity Chromatography using Response Surface Methodology

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Abstract

Therapeutic proteins covalently modified with polyethylene glycol (PEG) have improved biodistribution in the organism due to the positive properties that the PEGylation confers to them [1, 2]. The purification of these proteins is still a challenging task in chromatography [3]. Affinity chromatography (AC) is an attractive option due to its selectivity, high purification level and mild elution conditions [4]; however, it has been scarcely reported in purification of PEGylated proteins. Heparin Affinity Chromatography (HAC) has been used for the fractionation of proteins, coagulation factors in serum, proteases, lipoproteins, polymerases and growth factors [5, 6]. In this work we characterized the binding of di-, mono-PEGylated and native lysozyme to a heparin adsorbent. Then, we developed a chromatographic method for the separation of mono-PEGylated lysozyme from a PEGylation reaction in a linear NaCl elution gradient. Three response variables (yield, purity and productivity) were jointly optimized for three factors (flow, protein load and gradient length) by Response Surface Methodology considering minimum and target values for each variable. The optimal conditions allowed to find a better separation with a step gradient method. The results showed that PEGylated lysozymes have less affinity for the heparin support with respect to the native lysozyme, and their adsorption followed a Langmuir behavior. Purity (~100 %), yield (~100 %) and productivity (0.048 mg/mL min) achieved in the step gradient of HAC (Fig. 1) were better than those found in other chromatographic modes, particularly with hydrophobic interaction chromatography (HIC).

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Vita

Luis Alberto Mejía Manzano was born in Pachuca, Hidalgo, México, on October 26, 1986. His parents are José Isabel Mejía Zaragoza and Rita Yolanda Manzano Rivera. T**hey ha**ve **two sons: Luis Alberto, the eldest, and Norma Angélica. He currently lives in Monterrey, Nuevo León, Mexico where he studied his PhD courses.** He has a bachelor´s degree in Biotechnology Engineering at the Universidad Politécnica de Pachuca in Zempoala, Hidalgo through an excellence academic scholarship where he graduated in October 2009. He was teacher of basic sciences (Physics, Chemistry and Maths) in several institutions and professor of Organic Chemistry at Universidad Politécnica de Pachuca. During his Master's degree at Tecnológico de Monterrey he worked in the isolation and purification of an anticancer triterpene (pristimerin) from a Celastraceae endemic plant of the state of Nuevo León graduating in May 2012. After working for some months in Análisis Técnicos, Agrolab as a liquid chromatography and heavy metal analyst he started his Ph.D. program at Tecnológico de Monterrey. There, he worked in research related to the purification of PEGylated proteins using chromatography and aqueous two-phase systems. He also had a one-year research stay in the Centre for Biotechnology and Bioengineering (CeBiB) at Universidad de Chile in Santiago, Chile. To date, he has 4 published articles in indexed journals. This thesis presents the results of his doctoral work.

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