UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Effect of salt type on PEGylated lysozyme adsorption in hydrophobic interaction chromatography: a calorimetric study

Ana Isabel da Silva Gonçalves

Dissertação para obtenção do Grau de Mestre em
Bioquímica
(2º ciclo de estudos)

Orientador: Prof. Doutora Ana Cristina Mendes Dias Cabral

Covilhã, outubro de 2014

To my mum, dad and sister

Acknowledgments

First and foremost I offer my sincerest gratitude to my supervisor Professor Cristina Cabral, for all the help and support she gave me during the execution and writing of this MSc dissertation. Without her knowledge and scientific insights, this dissertation would not have been possible.

I also would like to thank to FCT (Portuguese Foundation for Science and Technology), project number FCOMP-01-0124-FEDER-014750 (Ref. FCT PTDC/EBB-BIO/113576/2009) for supporting this work.

To my laboratory colleagues, especially from my work group, thank you for the companionship and the help. Also a special mention to Patrícia, for the friendship we created. Thank you for sharing good times, help and stimulus in my times of discouragement. To Gonçalo, a special thank for all the availability that he always showed, for his encouragement and support. I also would like to thank Fatima and Silvia for their valuable collaboration in this work.

To my friends, thank you for always being present in my life. Thank you, for your patience, for your support, for everything…

To my love, thank you for supporting my faults, tolerate my moods and, especially to understand me. An enormous thank, with love, to João.

Last but not least, an enormous thank to all my family, especially my mom, dad and sister, for all the unconditional support and for always believing in my effort and commitment. Thank you for all the motivation, affection and understanding. To them, I dedicate all this work.

Resumo

A utilização terapêutica de péptidos e proteínas tem adquirido cada vez mais importância nos últimos anos. No entanto, a administração destas macromoléculas, pela sua natureza biológica, acarretam alguns problemas, como a suscetibilidade para a degradação enzimática, pouco tempo de circulação e baixa solubilidade e imunogenicidade, o que leva a uma redução na eficácia terapêutica. Uma maneira de ultrapassar este problema é ligar moléculas de polietileno-glicol (PEG) de modo covalente a proteínas terapêuticas. Este processo, denominado PEGuilação, normalmente resulta numa população de espécies de conjugados, além da proteína não modificada. As espécies combinadas podem diferir em termos do número de cadeias de PEG ligadas e da sua localização, o que influencia todo o processo de purificação. Várias técnicas cromatográficas foram utilizadas para resolver as misturas resultantes de reações de PEGuilação, tais como a cromatografia de exclusão molecular e a cromatografia de troca iónica. Comparativamente, pouco trabalho tem sido feito com a cromatografia de interação hidrofóbica. Durante as últimas décadas esta técnica tem sido desenvolvida por um certo número de investigadores e, é hoje considerada muito poderosa para a separação e purificação de biomoléculas. Um estudo mostra inversão na ordem de eluição das isoformas de lisozima PEGuilada, dependendo do tipo de sal usado como modulador (sulfato de amónio ou cloreto de sódio). Apesar de o cloreto de sódio apresentar melhores resultados, o mecanismo de separação ainda é desconhecido. A análise termodinâmica dos processos de adsorção através da Microcalorimetria de Fluxo (FMC) tem vindo a ser utilizada para obter uma melhor compreensão das forças motrizes, dos mecanismos e das cinéticas envolvidas no processo de adsorção de biomoléculas em diferentes sistemas cromatográficos. Por isso, neste trabalho, usando o FMC como técnica central, pretende-se compreender o efeito do tipo de sal no mecanismo de adsorção da lisozima (PEGuilada e nativa) sobre um suporte cromatográfico de interação hidrofóbica (Toyopearl Butyl–600M). A lisozima foi PEGuilada com mPEG-ALD (20 kDa), mostrando a lisozima PEGuilada em função do tipo de sal, diferentes perfis de adsorção comparativamente com a sua forma nativa.

Palavras-chave

Lisozima, PEGuilação, Adsorção, Cromatografia de Interação Hidrofóbica, Microcalorimetria de fluxo, Sulfato de amónio, Cloreto de sódio.

Resumo alargado

As proteínas estão presentes em células vivas, cujas funções são cruciais em todos os processos biológicos. Atuam como catalisadores, transportam e armazenam outras moléculas, fornecem apoio mecânico e proteção imunitária, geram movimento, transmitem impulsos nervosos e controlam o crescimento e a diferenciação celular. A aplicação das proteínas e péptidos como moléculas terapêuticas tem adquirido cada vez mais importância nos últimos anos. Contudo, essas macromoléculas, devido às suas características intrínsecas, acarretam alguns problemas, como a suscetibilidade para a degradação enzimática, pouco tempo de circulação e baixa solubilidade e imunogenicidade, o que leva a uma redução na eficácia terapêutica. Uma maneira de ultrapassar este problema é através da PEGuilação em que moléculas de polietileno-glicol (PEG) se ligam de modo covalente a proteínas. A PEGuilação melhora as propriedades físicas e químicas das proteínas, nomeadamente a conformação, a ligação electroestática e as características hidrofóbicas. Em geral as vantagens incluem; o aumento do tempo de permanência das proteínas no sangue, a diminuição da taxa de eliminação plasmática por reduzir a degradação metabólica e a endocitose da proteína da corrente sanguínea mediada por recetores e a redução da imunogenicidade.

A PEGuilação normalmente, origina uma população heterogénea de espécies conjugadas e proteínas não modificadas. As espécies combinadas podem diferir em termos do número de cadeias de PEG ligadas e da sua localização, o que influencia todo o processo de purificação. Várias técnicas cromatográficas têm sido utilizadas tais como, a cromatografia de exclusão molecular e a cromatografia de troca iónica, para purificar e separar os produtos PEGuilados. Neste trabalho a lisozima, uma proteína modelo, foi PEGuilada com mPEG-ALD de peso molecular médio de 20kDa. Um método de purificação para proteínas PEGuiladas usando um permutador de catiões TSKgel SP-5PW foi utilizado, e a identificação das espécies foi alcançada por análises MALDI-TOF. Diferentes espécies PEGuiladas (mono- e di-PEGuilada) foram isoladas. No entanto, pouco trabalho tem sido feito com a cromatografia de interação hidrofóbica. Durante as últimas décadas esta técnica tem sido desenvolvida por um certo número de investigadores e, é hoje considerada muito poderosa para a separação e purificação de biomoléculas. Um estudo mostra inversão na ordem de eluição das isoformas PEGuiladas de lisozima, dependendo do tipo de sal usado como modulador (sulfato de amónio ou cloreto de sódio). Apesar de o cloreto de sódio apresentar melhores resultados na separação das diferentes isoformas PEGuiladas, o mecanismo de separação ainda é desconhecido.

A análise termodinâmica dos processos de adsorção através da Microcalorimetria de Fluxo (FMC) tem vindo a ser utilizada para obter uma melhor compreensão das forças motrizes, dos mecanismos e das cinéticas envolvidas no processo de adsorção de biomoléculas em diferentes sistemas cromatográficos. Por isso, neste trabalho, usando o FMC como técnica central, pretende-se compreender o efeito do tipo de sal (sulfato de amónio e cloreto de sódio) no mecanismo de adsorção da lisozima (PEGuilada e nativa) sobre um suporte cromatográfico de interação hidrofóbica (Toyopearl Butyl–600M). Para além do estudo do processo de adsorção tendo em conta a interação entre a biomolécula e o suporte cromatográfico, foi também considerada e avaliada a presença e a influência de efeitos não específicos como por exemplo a reorientação da biomolécula. A injeção das amostras foi realizada através da introdução de um pulso de amostra na célula do calorímetro utilizando um loop de 30 μL. Com exceção do termograma da lisozima mono-PEGuilada (fase móvel contendo sulfato de amónio) em que se obtiveram dois picos exotérmicos e dois picos endotérmicos, todos os outros termogramas obtidos são compostos por um primeiro pico exotérmico seguido de um pico endotérmico, o qual, resulta de picos sobrepostos. A análise calorimétrica da lisozima pura, na presença de sulfato de amónio, um sal anti-caotrópico, mostrou que o calor resultante da adsorção proteína foi ligeiramente exotérmico, o que indica que o processo é conduzido entalpicamente. Nas experiências com cloreto de sódio, um sal caotrópico, a energia total foi ligeiramente endotérmica, sendo neste caso, a entropia a força motriz durante a adsorção. Nas experiências com lisozima mono-PEGuilada, tanto na presença de sulfato de amónio como cloreto de sódio, é visível que a adsorção foi endotérmica, devido à libertação de água a partir da camada de hidratação da proteína e da resina, resultando em grande entropia associada, que conduziu o processo. No caso da lisozima di-PEGuilada, as diferenças em termos de calor global do processo são mais acentuadas, sendo este endotérmico na presença de um sal anti-caotrópico e exotérmico na presença de um sal caotrópico. Estas diferenças resultam da maior área hidrofóbica efetiva da biomolécula PEGuilada e da redução da repulsão entre as biomoléculas adsorvidas em presença de cloreto de sódio. Verificou-se que em experiências com a lisozima pura, ocorre reorientação molecular após a adsorção. Por outro lado, quando a lisozima está PEGuilada isso não se verifica. Sugerindo assim que o PEG quando ligado covalentemente à proteína impede que esta se reoriente.

Abstract

The therapeutic use of peptides and proteins has gained increasing importance in the recent years. However, the administration of these macromolecules, due to their biological nature has some problems, such as susceptibility to enzymatic degradation, a low circulation time and a low solubility and immunogenicity, which diminish the therapeutic efficiency. One way to overcome this problem is to covalently bind molecules of polyethylene glycol (PEG) to the therapeutic proteins. This process, called PEGylation, usually results in a population of conjugated species in addition to the residual unmodified protein. The combined species may differ in terms of the number of attached PEG chains and its location, influencing the whole purification process. Several chromatographic techniques have been used to resolve mixtures resulting from PEGylation reactions such as size exclusion chromatography, and ion exchange chromatography. Comparatively little work has been done with hydrophobic interaction chromatography. During the last decades this technique has been developed by a number of researchers and is considered today quite powerful for the separation and purification of biomolecules. A study shows an inversion of lysozyme isoforms PEGylated elution order, depending on the type of salt used as modulator (ammonium sulfate or sodium chloride). Although, sodium chloride presented better results, the separation mechanism is still unknown. Flow Microcalorimetry (FMC) has proven its ability to provide an improved understanding of the driving forces, mechanisms and kinetics involved in the adsorption process of biomolecules onto several chromatographic systems. Thus, using FMC as a central technique, this study aims to evaluate the effect of salt type on the binding mechanism of native and PEGylated lysozyme adsorption onto a HIC resin (Toyopearl Butyl-600M). In this work, the lysozyme was PEGylated with mPEG-ALD (20 kDa). PEGylated lysozyme showed a different adsorption profile compared to the native form and each salt seemed to have a different effect on the PEGylated isoforms adsorption.

Keywords

Lysozyme, PEGylation, Adsorption, Hydrophobic Interaction Chromatography, Flow Microcalorimetry, Ammonium sulfate, Sodium chloride.

Index

Figures List

Tables List

Acronyms lists

PEG	Polyethylene-glycol.
mPEG-ALD	Methoxy-PEG-aldehyde.
BSA	Bovine serum albumin.
NHS	N- hydroxysuccinimidyl.
GalNAc	N-acetylgalactosamine.
G-CSF	Granulocyte-colony stimulating factor.
IFN	Interferon.
Tgase	Transglutaminase.
TFA	Trifluoroacetic acid.
IEC	Ion-exchange Chromatography.
HIC	Hydrophobic-interaction Chromatography.
SEC	Size-exclusion Chromatography.
ITC	Isothermal titration Calorimetry.
FMC	Flow microcalorimetry.
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization – Time of Flight.

Chapter 1- Introduction

1.1. Proteins- polymer conjugates

Proteins present in the organism appear in conjugated state in order to accomplish several important biological functions. They act as catalysts, transport and store other molecules, give mechanical support and immunulogical protection, generate movement, transmit nerve impulses and control the cell growth and differentiation. Diverse are the chemical reactions that modify their primary sequence: phosphorylation, acylation, methylation, glycosylation and sulfation, among others. Each of these modifications can involve different sites of a protein and they are exploited by nature to obtain different functions in vivo, where even a small modification creates a new entity that is recognized and might perform a different function compared with the native protein. Thus, a simple change in the protein structure can trigger functions, such as signaling, targeting, catalysis, catabolism, modification of circulation time in the body and immunogenicity (Veronese and Pasut, 2005; Pasut *et al.*, 2004).

PEGylation is a procedure of growing interest for enhancing the therapeutic and biotechnological potential of peptides and proteins (Veronese, 2001). Over the years, several strategies have been devised to yield conjugates with more-favourable behaviour. Among these were the entrapments of peptides into particles, gels or liposomes, genetic substitution of amino acids to stabilize the structure or reduce immunogenicity, and modification of the surface by covalent conjugation with low-or high-molecular-weight compounds such as simple acylating agents, sugars, or polymers (Veronese and Mero, 2008). The most successful strategy employs polyethylene-glycol (PEG) as modifying polymer, a strategy termed PEGylation, which led to important results in therapy, organic biocatalysis and diagnosis (Harris and Chess, 2003; Veronese and Pasut, 2005; Pasut and Veronese,2007).

1.1.1. Protein PEGylation

PEGylation was first reported by Davies and Abuchowsky in the 1970s, for the modification of albumin and catalase (Li *et al.*, 2013). Since then, this chemical modification of proteins has been of great interest to the pharmaceutical and biotechnological industry once protein's physical and chemical properties can be modified and improved, namely its conformation, and its electrostatic binding and hydrophobic characteristics (Mayolo-Deloisa *et al.*, 2012; Moosmann *et al.*, 2010; Veronese and Harris, 2008). The process of PEGylation involves formation of a stable covalent bond between activated PEG polymers and the biological macromolecules (Mayolo-Deloisa *et al.*, 2012).

In general, the advantages of PEGylation, some shown in figure 1.1.1.1, include: a) the increase in hydrodynamic volume conferred to conjugated molecules, thus reducing their kidney excretion and prolonging *in vivo* half-life, b) the protection of amino acid sequences sensitive to chemical degradation, c) the masking of critical sites sensitive to metabolic enzyme degradation or to antibody recognition, d) the possibility to solubilize proteins in organic solvents allowing new enzyme applications as biocatalysts, e) the solubilization of water insoluble drugs in a physiological medium, f) the reduction of either protein opsonization of liposomes and microparticles or protein adhesion of surfaces coated with PEG, thus increasing their biocompatibility and, g) the reduction of protein aggregation (Veronese and Pasut, 2005; Veronese, 2009)

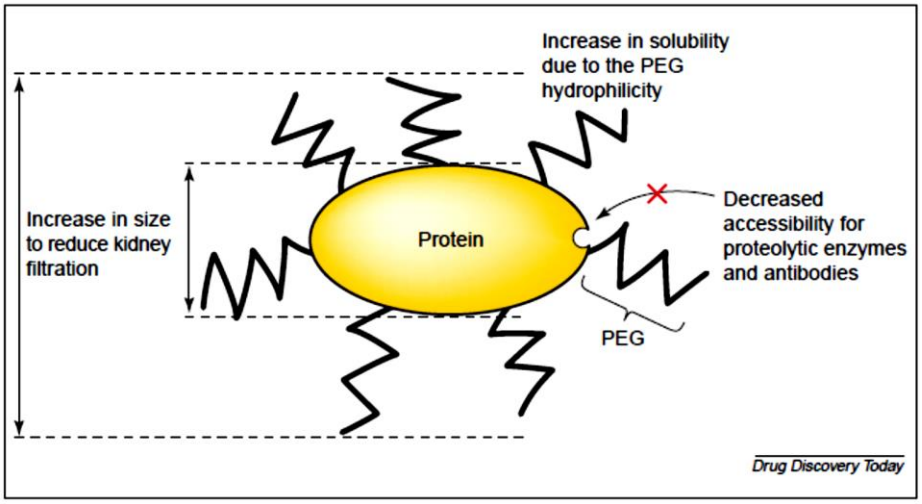

Figure 1.1.1.1- Main advantages of PEGylated protein adapted from Veronese and Pasut, 2005.

A number of different parameters must be taken into consideration when producing a PEG-protein conjugate. The appropriate molecular weight of PEG can be easily estimated based on the size of the given protein and the desired *in vivo* half-life increase, by linking either a long strand or several small PEG oligomers (Pasut and Veronese, 2012). In addition to PEG length, its shape greatly influences absorption and elimination half-life. Various sources have confirmed that branched PEGs (figure 1.1.1.2); characterized by two chains of polymer linked to one activated moiety, extend elimination half-life more than linear PEGs of the same nominal molecular weight. The importance of this product stems from the fact that this form allows for higher masking and protection of the protein surface based on the so-called "umbrella-like effect" (Jevsevar *et al.*, 2010; Veronese and Mero, 2008).

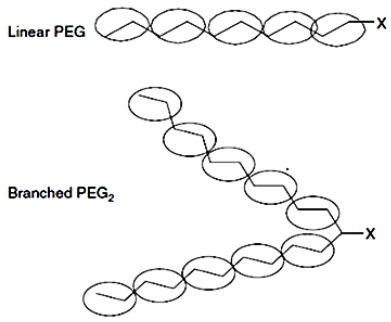

Figure 1.1.1.2- Schematic representation of linear polyethylene glycol (PEG) and the branched form (PEG_2) adapted from Veronese and Mero, 2008. The circles around the PEG chain represent the bound water and X represents the protein reactive groups.

Another parameter to take into account is the net charge of conjugate. Once protein amino groups are the most exploited sites for polymer modification, at physiological conditions these groups are protonated allowing protein solubility, and may be involved in recognition process with receptors. The appropriate PEGylation strategy must be chosen and optimized for any protein to improve conjugation yield, conjugate purity and biological activity (Pasut and Veronese, 2007). There are a large number of PEGylation strategies, targeting different residues of surface amino acids. The most common PEG conjugation technologies are listed below and are illustrated in Figure 1.1.1.3.

— Amino residue modification

The most commonly used PEGylation reactions for pharmaceutical therapeutics modify the amino residues of the target molecule including the α-amino residue (N-terminus) and the ε-amino residues, such as lysine. For amino residue modification, different PEG molecules can be applied, including PEG-aldehyde or N-hydroxysuccinimidyl (NHS) activated PEGs. In addition to lysine residue PEGylation, PEG-NHS is also known to react with histidine and tyrosine residues. Additionally, it is worth noting that acylating PEGs such as PEG-NHS can alter the charge of the target molecule by neutralizing the positive charge of a lysine residue due to amide or urethane formation. Alkylating PEGs including PEG-aldehyde maintain the charge. As lysine residues are generally well represented in proteins, these kinds of reactions often result in multi-PEGylation, requiring additional purification. A positive aspect of PEG-aldehyde is given with the possibility of a selective N-terminal PEGylation. Using mild acidic pH values of about 5-6, the N-terminal amino group can be PEGylated selectively due to its lower pK_a value, compared to the ε-amino residues (Veronese and Pasut, 2005; Veronese and Mero, 2008; Roberts *et al.*, 2002).

— Enzyme mediated modification

Enzyme mediated PEGylation can be provided, targeting different surface amino acids. Two main conjugation methods are established and are partially used for products in clinical phases. To mimic natural glycosylation, the so called GlycoPEGylation can be applied. For this, a two-step conjugation is necessary, where the first step involves a glycosylation of serine and threonine residues (O-glycosylation) with N-acetylgalactosamine (GalNAc). The second step includes a subsequent PEGylation of glycosylated residues with sialyl-PEG, using sialyltransferase. This method was successfully applied for mono-PEGylated G-CSF (Granulocyte-colony stimulating factor) and IFN (interferon). Another possibility of enzyme PEGylation enables a glutamine residue modification using transglutaminase (Tgase), which catalyzes the addition of a primary amine to an acyl residue. Consequently, amino- PEGs (PEG-NH2) can be used as PEGylation reagents for this reaction. As Tgase only recognizes glutamine residues that are located in exposed and flexible regions of the target molecule, this approach can thus be used to reduce the number of positional isoforms. Studies with G-CSF, consisting of 17 glutamine residues resulted in a site-specically mono-PEGylation, by applying this method (Sato, 2002; Pasut and Veronese, 2012).

— Thiol residue modification

PEGylation of unpaired cystein residues can be conducted with PEG-maleimide, which is already used in clinical practice for example to produce PEGylated anti-TNF antibody fragments (Cimzia®). However, free cystein residues are generally not exposed in natural proteins, as they are often involved in disulfide bridges. Thus, reducing conditions are needed in case of antibody fragment PEGylation to prevent diol formation and to enable a PEG conjugation. The integration of free cystein residues into the amino acid sequence of a target molecule using genetic engineering can provide the possibility of a site specific PEGylation. However, this approach often leads to low yields due to dimerization and incorrect protein folding (Basu *et al.*, 2006).

— Disulfide bridging

A novel approach providing a selective PEGylation of interferon α-2b via a disulfide bridge was first described by Shaunak *et al.* (Shaunak *et al.*, 2006). For this, a specific mono-sulfone PEG reagent was used to perform a site selective PEGylation of both cystein sulfur atoms via a so called three carbon PEGylation bridge. The disulfide bridge involved in the conjugation was preserved, resulting in a correct conformation of the PEG-protein conjugate. An advantage of this approach is related with the small number of disulfide bridges that are present in proteins of pharmaceutical interest.

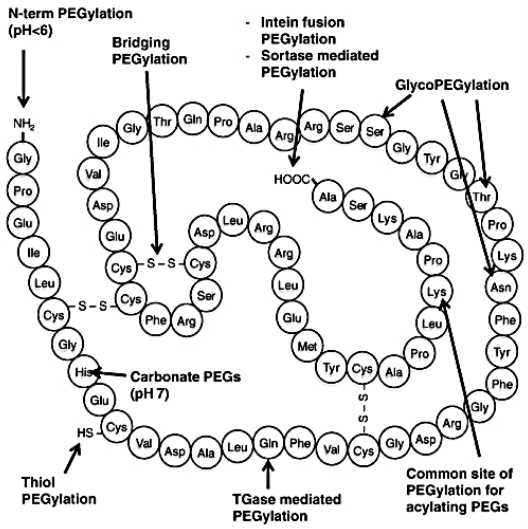

Figure 1.1.1.3-Different possibilities for protein PEG modication and corresponding PEG reagents. Illustration adapted from (Pasut and Veronese, 2012).

To date, many PEGylated biomolecules have been approved by the FDA and are on the market, some details are shown in table 1.1.1.1 (Fee and Alstine, 2006; Jevsevar *et al.*, 2010).

Table 1.1.1.1- FDA-approved PEGylated drugs adapted from Li *et al.*, 2013.

Commercial name	Drug name	Company	PEG size (Da)	Indication	Year of approval
Adagen®	Pegadamase	Enzon	Multiple linear 5000	SCID	1990
Oncaspar®	Pegaspargase	Enzon	Multiple linear 5000	Leukemia (ALL, CML)	1994
PEG-INTRON®	Peginterferon-α2b	Schering-Plough	Linear 12,000	Hepatitis C	2000
PEGASYS®	Peginterferon-α2a	Hoffman-La Roche	Branched 40,000	Hepatitis C	2001
Neulasta®	Pegfilgrastim	Amgen	Linear 20,000	Neutropenia	2002
Somavert®	Pegvisomant	Pharmacia & Upjohn	4–6 linear 5000	Acromegaly	2003
Macugen®	Pegaptanib	Pfizer	Branched 40,000	Age-related macular degeneration	2004
Mircera®	mPEG-epoetin-β	Hoffman-La Roche	Linear 30,000	Anemia associated with chronic renal failure	2007
Cimzia®	Certolizumab pegol	UCB	Branched 40,000	Reducing signs and symptoms of Crohn's disease	2008
Puricase1®/Krystexxa®	PEG-uricase	Savient	10,000	Gout	2010

ALL: acute lymphoblastic leukemia; CML: chronic myeloid leukemia; GH: growth hormone; SCID: severe combined immunodeficiency disease.

The first few PEG-protein products, now on the market (Adagen®, Oncospar®, and PEG-Intron®), were developed using first generation PEG chemistry. One characteristic of first generation PEG chemistry is the use of low molecular weight linear PEGs (≤ 12 kDa) with chemistry that may result in side reactions or weak linkages upon conjugation with polypeptides. The next generation of PEG-protein therapeutics, which will come to market in the next several years, uses second-generation PEG chemistries. Second-generation PEGylation was designed to avoid the problems of first generation chemistry, namely deactivated PEG impurities, restriction to low molecular weight mPEG, unstable linkages and lack of selectivity in modification (Roberts *et al.*, 2002; Pasut *et al.*, 2004). Successful

protein biopharmaceuticals include PEGylated α-interferons for use in the treatment of hepatitis C (PEGasys® and PEG Intron®), PEGylated growth hormone receptor antagonist (Somavert®), PEGasparaginase (Oncospar®), adenosine deaminase (Adagen®), and granulocyte colony stimulating factor (Neulasta®). Many other PEGylated proteins are currently under development.

Theoretically, in a conjugation reaction conducted with an excess of PEG, one may expect that all the reactive groups of the protein are modified thus yielding mainly a single product. However, to prevent loss of biological activity, a lower amount of PEG is generally employed which usually yields to a mixture of positional isomers as well as products with different extent of PEGylation (i.e., mono-, di-, tri-, tetra-, and more PEGylated proteins) (Pasut and Veronese,2007). PEGylation of proteins creates two basic challenges for purification. The first involves separation of PEGylated proteins from other reaction products including non-reacted PEG and protein. The second is the sub-fractionation of PEGylated proteins on the basis of their degree of PEGylation and positional isomerism (Mayolo-Deloisa *et al.*, 2011; Cisneros-Ruiz *et al.*, 2009; Mayolo-Deloisa *et al.*, 2012; Blaschke *et al.* , 2011). The change of physicochemical properties of PEGylated proteins influences their behaviour throughout the purification process leading to a change of behaviour in practically all modes of chromatographic separation such as ion-exchange chromatography (IEC) and hydrophobic-interaction chromatography (HIC). Other important separation methods frequently used for downstream processing are also affected, e.g. filtration. Changes of protein behaviour during all kinds of electrophoretical separations can also be expected, e.g. SDS-PAGE (Müller *et al.*, 2010).

Several researchers have used standard proteins to study PEGylation. For example, Pabst *et al.* (Pabst *et al.*, 2007) have studied the effect of protein PEGylation on ion-exchange adsorption using bovine serum albumin (BSA) as a model system. Moosmann and co-workers (Müller *et al.*, 2010) have studied the solubility and binding properties of PEGylated lysozyme derivatives on hydrophobic-interaction chromatographic resins. In this work, Lysozyme was chosen as model protein for PEGylation (see section 1.1.3).

1.1.2. PEG composition and properties

PEG, as obtained by ethylene oxide polymerization, presents one or two terminal hydroxyl groups (mPEG-OH or HO-PEG-OH) depending upon the initiator of polymerization: methanol or water, respectively. A branched form may also be obtained, as well as products with multiple reactive groups, at one or at both edges (Figure 1.1.2.1) (Pasut *et al.* ,2004).

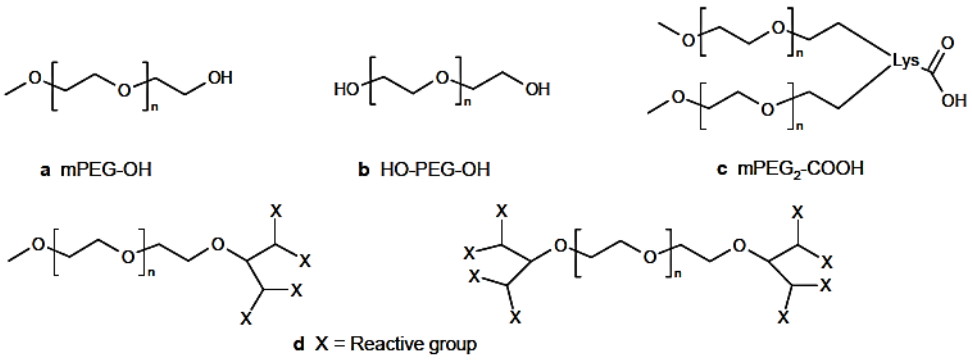

Figure 1.1.2.1- Different PEG structures. a) Linear monomethoxy PEG; b) Linear diol PEG; c) Branched PEG; and d) Multifuntional PEGs, adapted from Pasut *et al.*, 2004.

The repeated ethylene oxide units along the PEG chain gives unique properties to this polymer: the ethylene moiety confers hydrophobicity, while the oxygen allows strong interactions with water. The polymer is neutral, hydrophilic and soluble in various aqueous solutions and may also be hydrophobic and soluble in organic solvents, behaving as an amphipathic molecule. Furthermore, the carbon–carbon and carbon–oxygen bonds give great flexibility to the overall structure and allow repulsion of incoming molecules (Fee and Van Alstine, 2006; Müller *et al.*, 2010; Veronese, 2009).

PEG is generally considered as a non-toxic, non-immunogenic, uncharged and nondegradable polymer. It can be used as a precipitant and crystallization agent for proteins and also tends to behave like a weak detergent. Some studies indicate that it can be oxidized by various enzymes, such as alcohol, aldehyde dehydrogenases and cytochrome P450 dependent oxidases (Fee and Van Alstine, 2006; Müller *et al.*, 2010; Jevsevar *et al.*, 2010).

PEG reagents are commercially available in different lengths, shapes and chemistries, allowing them to react with particular functional groups of proteins for their covalent attachment. Monodisperse PEG has recently become commercially available, but, unfortunately, so far at low molecular weights only, between 500–800 Da. A monodisperse high molecular weight PEG would be very welcome to overcome the subtle differences in biological properties of polydisperse conjugates, but its synthesis and purification using current technologies would be too difficult and expensive for a commercial product (Jevsevar *et al.*, 2010; Veronese and Mero, 2008).

1.1.3. Lysozyme PEGylation

Lysozyme (E.C.3.2.17) also called N-acetylmuramidase or muramidase is a hydrolyse-type enzyme, acts by catalyzing the hydrolysis of 1,4-beta-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in peptidoglycans (Callewaert and Michiels, 2010;

Benkerroum, 2008). Lysozyme is a well defined and characterized standard protein. This protein is widely used in chromatography and has been studied by many authors.

Lysozyme is commonly found in nature and was first discovered by Alexander Flemming in 1922 (Cegielska-radziejewska *et al.*, 2008; Benkerroum, 2008). It is a globular basic protein characterized by M_W of approx. 14.4 kDa. Its structure is a single polypeptide chain of 129 amino acids, in which lysine is the N-end amino acid and leucine is the C-end one (Cegielska-radziejewska *et al.*, 2008; Mir, 1977). The predominant form in the tertiary structure of lysozyme is alpha helix, as shown in Figure 1.1.3.1 (Benkerroum, 2008).

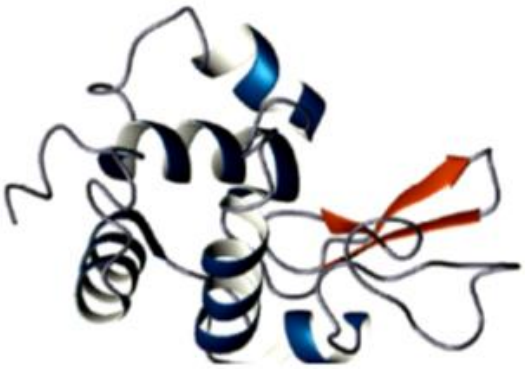

Figure 1.1.3.1- The tertiary structure of egg white lysozyme adapted from Benkerroum, 2008.

Lysozyme has been classified in different classes/types according with amino acid sequences, biochemical and enzymatic properties. The most studied and the best known is the chicken-type (i.e., c-lysozyme), with the lysozyme derived from the egg white of domestic chicken. Other types of lysozyme are also known; and they include the *g*-type derived from the egg white of domestic goose, h-type lysozyme from plant, i-type from invertebrates, b-type from bacteria and *v*-type from viruses (Callewaert and Michiels, 2010; Benkerroum, 2008).

Lysozyme can be PEGylated at its lysine residues, methoxy-PEG-aldehyde (mPEG-ALD) reacts with the amino group of the lysine residue and builds a so called Shiff base which is reduced selectively to the desired product with the use of sodium cyanoborohydride as reducing agent. Only three of the residues: 33, 97 and 116 can be PEGgylated. Different isoforms are expected; three mono-PEGylated, three di-PEGylated and one tri-PEGylated. As the protein is rather small the number of PEGylation products also is relatively small, and individual species can be easily separated and isolated by chromatography (Müller *et al.*, 2010).

1.2. Chromatography

After PEGylation, the reaction mixture has to be purified in order to remove non-reacted protein and undesired reaction products. Chromatography is an important separation technique for the separation and purification of biomolecules being widely employed in the downstream processing of proteins. All chromatographic modes used in downstream processing show altered behaviour of the PEGylated proteins in comparison to non-modified ones (Moosmann *et al.*, 2010; Bellot and Condoret, 1993)

Chromatography, by definition, is a differential migration process, where the components of a mixture are separated based on the rates at which they are carried by a mobile phase through a stationary phase. The stationary phase remains fixed in a column while the mobile phase works as an eluent that carries the sample to be purified. Substances to be separated should have different relative affinities for the stationary and the mobile phases. Thus, a substance with relatively higher affinity for the stationary phase moves with a lower velocity through the chromatographic system than a substance with lower affinity. This difference in migration velocity ultimately leads to physical separation of the components in a sample, which are recovered at the bed exit, once they elute with different residence times (figure 1.2.1) (Hedhammar *et al.*, 2006; Carta and Jungbauer, 2010).

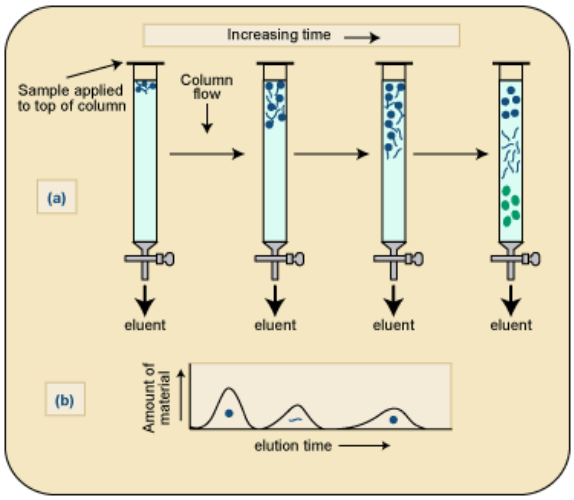

Figure 1.2.1- (a) Diagram of Madison Area Technical College showing the separation of a mixture of components of one sample by column elution chromatography. (b) The output of the signal detector at the various stages of elution shown in (b).
(http://serc.carleton.edu/microbelife/research_methods/biogeochemical/ic.html).

There are different chromatography techniques according exploit specifics proprieties, such as, charge, hydrophobicity, and size or biospecific interaction, some examples ore shown in figure 1.2.2 (GE Healthcare, 2006).

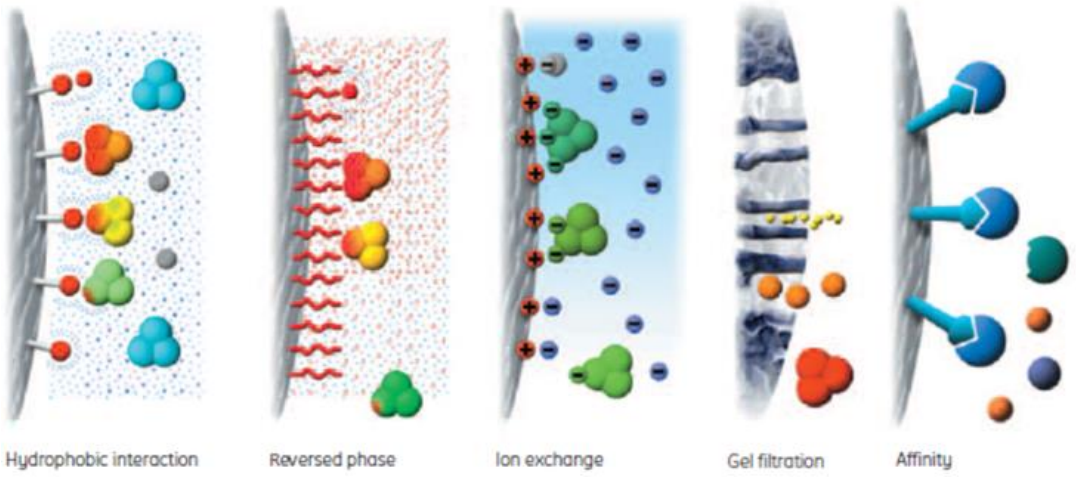

Figure 1.2.2- Separation principles in chromatographic purification adapted from (GE Healthcare, 2006).

IEC is based on the charge-charge interactions between the charged amino acids on the protein surface and electrically charges in resins (Staby *et al.*, 1998; Lin *et al.*, 2001). This chromatography technique can be subdivided into cation exchange chromatography, in which positively charged ions or molecules bind to a negatively charged resin; and anion exchange chromatography, in which the binding species are negative, and the immobilized functional group is positive (Heftmann, 2004; Hedhammar *et al.*, 2006; Chung *et al.*, 2009). The binding strength of the protein to the ion exchange resin can be affected by numerous factors, including pH value, salt type and concentration, and temperature (Lin *et al.*, 2001). The increasing ionic strength affects the elution in IEC. Salt ions compete with target molecules for opposite charges on the resin, and depending on the strength of interactions, molecules are removed or retained (Heftmann, 2004).

Size exclusion chromatography (SEC), also called gel permeation chromatography in the case of lipophilic macromolecules or gel filtration chromatography in the case of hydrophilic macromolecules, is a separation technique based on the molecular size (Heftmann, 2004). Therefore, the molecules are separated according to their hydrodynamic volume once the stationary phase, due to its porosity, allows or hinders the entry of molecules to be separated. Thus, smaller molecules are retained in the pores since they take a longer way. Heavier or bigger molecules are the first to elute (Berek, 2010; Hong *et al.*, 2012). Unlike many other chromatographic procedures, size exclusion is not an adsorption technique. The mobile phase should be considered as a carrier phase since it does not have a large effect on the chromatographic process. However, the sample may require a buffer solution with a well-defined pH and ionic composition to preserve the structure and biological activity of substances of interest (Heftmann, 2004).

Fee and van Alstine have reported that SEC and IEC are the predominant methods for purification of the PEGylated products (Mayolo-Deloisa *et al.*, 2011; Mayolo-Deloisa *et al.*, 2012).

Another technique used for the separation and purification of biomolecules is affinity chromatography. The binding of proteins can involve a combination of electrostatic or hydrophobic interactions as well as molecular interactions such as van der Waals forces and hydrogen bonds (Hedhammar *et al.*, 2006). The protein binds to a solid support matrix in a selective and reversible way. The immobilized ligand in the matrix is the key factor that determines the success of any affinity chromatographic method. Thus the ligand, which can be as small and specific biomolecule as an antibody is immobilized and bound to a column matrix such as polyacrylamide or cellulose. The target protein flows through a column and binds to the ligand, while other molecules not adsorbed will be first eluted (Hage, 1999).

HIC developed in the following section is also a powerful adsorptive separation technique used in the purification of biomolecules. This technology is based on the hydrophobic interactions between hydrophobic ligands and non-polar regions on the surface of biomolecules (Bonomo *et al.*, 2006).

Reversed Phase Chromatography, results from the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. Decreasing the mobile phase polarity by adding organic solvents reduces the hydrophobic interaction between the solute and the solid support resulting in desorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote desorption (Heftmann, 2004; Hedhammar *et al.*, 2006, Haimer *et al.*, 2007).

1.2.1. Hydrophobic Interaction Chromatography

It has been shown that HIC is a promising technique for the separation of PEGylated from unPEGylated proteins (Müller *et al.*, 2010). However, yet there is a lack of fundamental understanding of the hydrophobic interactions between the proteins and resin (Lin *et al.*, 2000; Werner *et al.*, 2012).

HIC is used to separate proteins in their native state; on the basis of their relative hydrophobicity. It is a powerful adsorptive separation technique because of the fast separations achieved with little product degradation, low solvent requirements and very good purification levels (Haidacher *et al.*, 1996; Bonomo *et al.*, 2006).

It was described for the first time, in 1949, by Shepard and Tiselius as “salting-out chromatography”. In 1973, Shaltiel and Er-el introduced the term “hydrophobic chromatography”. Since then, great improvements have been made in developing the technique, which led Hjertén suggesting the now generally accepted name of the technique: Hydrophobic Interaction Chromatography (Hedhammar *et al.*, 2006; Mahn, 2008). With this technique successful isolation of enzymes, purification of therapeutic proteins, and the removal of viruses from human plasma has been achieved (Diogo *et al.*, 2000) .

The principle of protein adsorption to HIC media is based on hydrophobic interactions between the hydrophobic resin moieties and the non-polar groups on the protein surface (Xia *et al.*, 2005). From a thermodynamic point of view, this interaction is an entropy-driven process, based on the second law of Thermodynamics. As the protein mixture flows through a column, hydrophobic patches on the surface of the proteins contact with the hydrophobic ligands on the support. The ordered water molecules will be excluded and will adopt the less ordered bulk water state which is equivalent to an increase in entropy. As free energy value ΔG of a hydrophobic interaction is a function of ΔH and ΔS, according to equation :

$$\Delta G = \Delta H - T\Delta S \qquad (1)$$

and ΔH is small when compared to TΔS value, the process is fundamentally determined by the change in entropy without significantly changing its enthalpy, leading the free energy to decrease on adsorption. Hydrophobic adsorption of proteins is thus an entropy-driven, thermodynamically favorable process where the driving force is the reduction of surface area (Korfhagen *et al.*, 2010; Esquibel-King *et al.*, 1999; Hedhammar *et al.*, 2006; Mahn, 2008).

For selective elution (desorption), the salt concentration is gradually lowered and the sample components elute in order of hydrophobicity (Figure 1.2.1.1). So, when using high salt concentrations, protein adsorption is dominated by hydrophobic interactions, and lowering the salt concentrations causes the protein retention time to decrease (Korfhagen *et al.*, 2010; Mahn, 2008; Queiroz *et al.*, 2001).

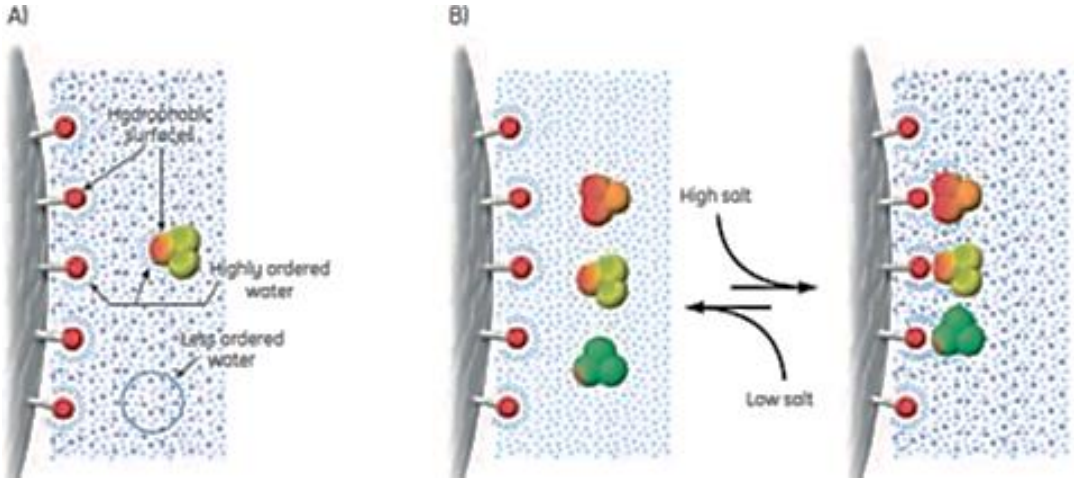

Figure 1.2.1.1- Protein binding in HIC systems. A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration (GE Healthcare, 2006).

Besides, factors such as type/concentration of salt and the stationary phase (ligand type and degree of substitution, and type of base matrix), developed in section 1.2.1.1 and 1.2.1.2, respectively, temperature, pH and buffer additives also affect protein binding in HIC systems (Xia *et al.*, 2005). Increasing temperature enhances protein retention and decreased temperature generally promotes protein elution (Bonomo *et al.*, 2006). Chen *et al.* (Chen *et al.*, 2003) demonstrated that the exposed hydrophobic regions of the protein increased with temperature, resulting in the binding mechanism changing from adsortion to partition in some cases. Researchers have traditionally developed thermodynamic analyses based on the van´t Hoff dependencies to study the interaction between proteins and hydrophobic solid surfaces. Enthalpy and entropy changes at different temperatures can se obtained by calorimetric techniques, being important to estimate the significance of sub processes in the adsorption mechanism (Bonomo *et al.*, 2006; Ueberbacher *et al.*, 2010; Dias-Cabral *et al.*, 2002). The pH of the medium changes the charge of ionisable groups of protein molecules. This effect will obviously affect the separation based on hydrophobic interactions (GE Healthcare, 2006). It is found that, in general, the retention of protein samples changes dramatically below pH 5 and above pH 8.5, thus the states close to the zwitterionic state are advantageous for HIC. (Hjertén *et al.*, 1986) Water-miscible organic solvents (alcohols, acetonitrile, dimethyl formamide, etc.) or added detergents can reduce the binding of proteins to be separated, even when present at low concentrations. These compounds “compete” with the protein for the adsorption sites on the matrix surface. Therefore, when added in low concentrations, these substances can increase the efficiency of the elution (GE Healthcare, 2006).

1.2.1.1. Type and concentration of salt

In HIC media the hydrophobic interaction depends on the ionic species present and on the concentration of a particular salt. The correct choice of salt type and concentration are the most important parameters that influence capacity and final selectivity. As already mentioned, high salt concentration in the mobile phase induces hydrophobic interactions between the immobilized hydrophobic ligands and the non-polar regions at the protein surface. Similarly, salt-induced protein precipitation is promoted by high salt concentration in the same way. The elution/precipitation strength of an ion is described by Hofmeister who ranked the anions and cations according to their ability to precipitate proteins, which is generally known as the Hofmeister or lyotropic series, schematised in figure 1.2.1.1.1. The ions at the beginning of this series, called cosmotropes or anti-chaotropes, are considered to exhibit stronger interactions with water molecules and thereby also be water structuring and promote hydrophobic interactions. The most commonly used salts are $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl and CH_3COONH_4 (Nfor *et al.*, 2011; G.E. Healthcare, 2006; Carta and Jungbauer, 2010; Lund and Jungwirth, 2008).

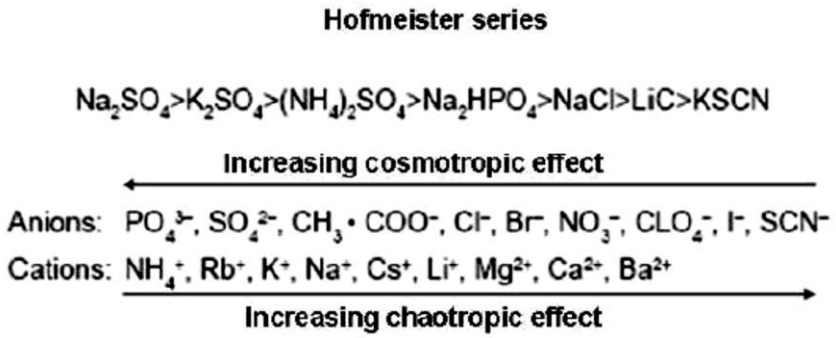

Figure 1.2.1.1.1- Hofmeister series adapted from Mahn, 2008.

Some explanations have been proposed for the effect of salts in protein adsorption. One approach is based in the solvophobic theory and the other is based in the preferential interaction (PI) theory. The solvophobic theory, first described by Green (Green and Hughes, 1955), examines the effect of salt in the properties of the solvent, describing surface tension effects. Under high salt conditions of interest in HIC, this theory indicates that the variation in the log of the retention time with salt concentration should be proportional to the molal surface tension increment of the salt. Thus, salts that increase the surface tension of the solvent will favor the reduction in the surface area that accompanies adsorption, resulting in increased retention times. The PI theory describes salt-protein interactions. A model-independent thermodynamic analysis is used to relate the effect of salt on the observed equilibrium constant to the change in the distribution of salt ions and water molecules. This analysis predicts that processes that reduce wetted surface area are favored by solutes that are preferentially excluded from the surfaces. Conversely, these processes are impeded by

solutes that are accumulated near the surfaces. According to the PI theory, lyotropic salts promote protein salting out by being preferentially excluded from the protein surface, thereby promoting hydration of the surface and enhancing the protein stability. Chaotropic salts, on the other hand, undergo specific interactions with proteins at high salt concentration, increasing protein solubility and also destabilizing the protein (Perkins *et al.*, 1997; Nfor *et al.*, 2011; Huang *et al.*, 2000; Mahn, 2008; Ueberbacher *et al.*, 2010).

1.2.1.2. The Stationary Phase

Ligand type and size have a great impact on the properties of HIC and contribute significantly to the degree of hydrophobicity of a medium; likewise the matrix can also influence the final selectivity (Bonomo *et al.*, 2006). There is a variety of stationary phases and the supports can be further modified by linking hydrophobic ligands. The ligand is linked to the support through a spacer arm, so that there is no steric impediment for macromolecule-ligand interaction, and avoiding hydrophobic interaction between the ligands. Figure 1.2.1.2.1 illustrates the retention of a protein to a HIC stationary phase (Mahn, 2008).

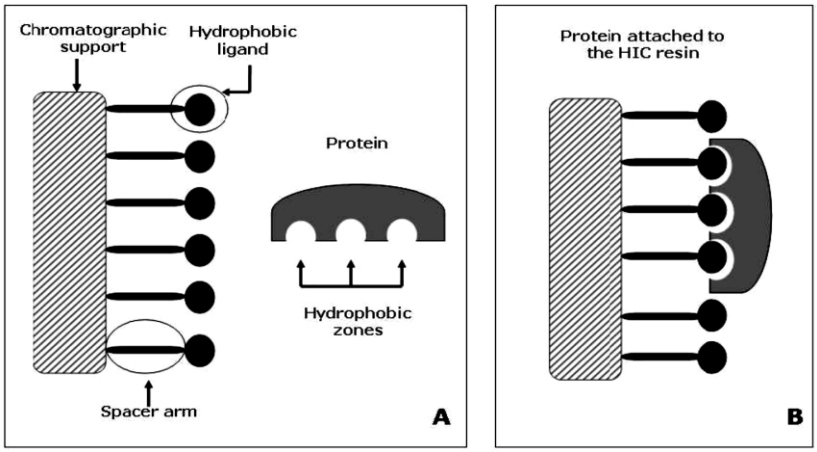

Figure 1.2.1.2.1 - Protein retention mechanism in HIC. (A) The basic structure of a HIC resin is depicted, and a protein is schematized highlighting the hydrophobic zones on the protein surface. (B) The protein gets in contact with the hydrophobic ligands of the resin, suffering a spatial reorientation. The hydrophobic ligands of the matrix interact with the exposed hydrophobic zones of the protein, and thus the protein is reversibly attached to the resin. Adapted from (Mahn, 2008).

— Ligand Type and degree of substitution

A protein's adsorption behaviour is determined by the type of immobilized ligand. The most widely used ligands for HIC are linear chain alkanes and aryl ligands. In general, straight chain alkyl ligands (butyl, octyl, ether, isopropyl) demonstrate hydrophobic character while aryl ligands (phenyl) show a mixed mode behaviour where both aromatic and hydrophobic interactions are possible. The protein binding capacity increases with an increased degree of substitution of the immobilized ligand. With a high level of ligand substitution, the binding

capacity remains constant; however, the affinity of the interaction increases. Proteins bound under these conditions are difficult to elute due to multi-point attachment (Heftmann, 2004).

— Type of base matrix

Chromatography media for hydrophobic interaction are made from highly porous matrices, chosen for their physical stability, their chemical resistance to stringent cleaning and sanitation conditions and their low level of non-specific interaction. There are a variety of materials available that meet these requirements. The base of the resins is divided into natural polymers, such as cellulose, agarose, dextrano, and chitosan, and synthetic polymers detailed in table 1.2.1.2.1 (GE Healthcare, 2006; Carta and Jungbauer, 2010).

Table 1.2.1.2.1- Examples of protein chromatography media based on synthetic polymers (Carta and Jungbauer, 2010).

Matrix material	Examples	Manufacturer
Acrylamido and vinyl co-polymers	UNOsphere Macroprep Bio-Gel	Bio-Rad Laboratories
Acrylic polymers	Toyopearl Fractogel	Tosoh Bioscience E. Merck
Poly(methacrylate)	CIM disks and tubes	BiaSeparations
Poly(styrene-divinyl benzene) co-polymers	Source, Resource POROS	GE Healthcare Applied Biosystems.

Common to all synthetic polymers is their resistance to extreme chemical conditions such as pH or oxidizing environment and autoclaving (Carta and Jungbauer, 2010). Toyopearl® chromatography resins are hydrophilic, macroporous, bulk processing media designed especially for large-scale chromatography application. In this work was used the Toyopearl Butyl 600M, an acrylic polymer with rigid structure (figure 1.2.1.2.2), shows excellent pressure/flow properties, resulting in faster process throughput. This resin of HIC has high mechanical stability, high binding capacity for mAb's and efficent recovery and impurities removal (Tosoh Bioscience, 2007).

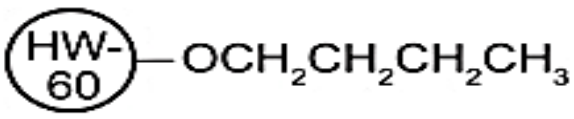

Figure 1.2.1.2.2- Structure of Toyopearl Butyl-600, adapted from (Tosoh Bioscience).

1.2.2. Chromatographic applications for the separation and purification of PEGylated proteins

As already mentioned the purification of PEGylated proteins is required to obtain the final products from the complexity of PEGylation mixtures. SEC separates these molecules reducing their accessibility through the porous support and altering their elution times. However, the differences between the sizes of species are not enough to achieve positional isomer separation (Mayolo-Deloisa *et al.*, 2011). Thus, for isolation of the target PEG-protein, researchers have been using different chromatographic techniques (Jevsevar *et al.*, 2010). Many approaches showed that IEC is an effective tool and currently the method of choice for conjugate and positional isoform separation, by means of salt gradients (Fee and Alstine, 2006). Little work has been done to explore the hydrophobic interaction mode (Cisneros-Ruiz *et al.*, 2009).

Pabst *et al.* (Pabst *et al.*, 2007) have studied the influence of the degree of PEGylation on the adsorption of PEGylated lysozyme on strong anion exchange resin. They have presented a method to produce mono-PEGylated BSA. That means that only a single PEG chain is coupled to the BSA and a well defined PEGylation product is obtained after purification. For this, they studied the chromatographic purification of this mono-PEGylated BSA on different anion-exchange resins. Cisneros-Ruiz *et al.* (Cisneros-Ruiz *et al.*, 2009) have investigated the use of a mildly amphiphilic support as an alternative for separating PEGylated proteins from their unmodified counterparts. The effects of parameters such as pH, salt type and salt concentration upon the chromatographic behaviour of native, mono-PEGylated and di-PEGylated ribonuclease A on media were characterized. The separation of the native protein from the PEGylated species was achieved using a gradient elution. The pH of the mobile phase as well as the addition of a mobile phase modifier to the low ionic strength phase had no significant influence on chromatographic behaviour of the species. Moosmann *et al.* (Moosmann *et al.*, 2010) have studied the effect of PEGylation on IEC with PEG of different chain lengths using lysozyme as a model system. Different cation exchange resins were tested and also the influence of the PEG chain length was investigated. In this work, the selectivity for separation of PEGamers was shown to be dependent on the particle size of chromatographic resins. The dynamic binding capacity for PEGylated lysozyme decreased in comparison to native lysozyme and it also decreased with the length of the PEG chains attached to the native molecule. They concluded that this decrease was due to the increased size and the accompanying increase of mass transfer resistance and the modified electrostatic characteristic of lysosyme because of the covalently linked neutral PEG. Muller *et al.* (Müller *et al.*, 2010) have used HIC resins for solubility and binding properties studies of PEGylated lysozyme on HIC resins. It was found that PEGylation decreases lysozyme solubility in ammonium sulfate as function of attached PEG chains lenght. On the other hand the

PEGylated lysozyme has an increased solubility in sodium chloride. Compared to the separation in ammonium sulfate, the protein selectivity was reversed in sodium chloride. A good selectivity, with PEGamer separation, was observed only in sodium chloride as a result of a still unknown supporting separation mechanism.

1.3. Calorimetry

Calorimetry is one of the oldest field of study in physical chemistry, emerged in the decade of 18 and since then many new methods have been developed and the measuring techniques have been improved. At present, numerous laboratories and companies worldwide continue to focus attention to the development and applications of this science of heat, and in the production of calorimeters (GE Healthcare, 2006).

Calorimetry is a valuable tool to study the underlying mechanism of protein adsorption, it allows understanding protein–adsorbent surface interactions, due to its capability to detect minute thermal changes (Katiyar *et al.*, 2010). The Gibbs free energy change of adsorption determines the equilibrium capacity for a protein in a surface. This free energy change, in turn, is dependent on the enthalpy change of adsorption. Thus, the enthalpy change that can be measured calorimetrically reflects the overall reaction process. The protein adsorption process can be exothermic or endothermic. Attractive forces between the surface and the adsorbing protein, *i.e.* adsorption, result in exothermic interactions. On the other hand, endothermic adsorption is often attributed to changes of protein conformation or orientation upon adsorption or to the associated release of solvent from the surface; in this case the adsorption process is entropy-driven (Bowen and Pan, 1997; Katiyar *et al.*, 2010; Thrash and Pinto, 2001; Thrash *et al.*, 2004). An example of an endothermic process is shown in Figure 1.3.1. for a calorimeter operated in the heat conduction mode.

Various classifications of calorimeters have already been developed to measure the thermodynamic effect of adsorption from solutions, even in complex experimental situations such as at high protein concentration (overload conditions), highly non-ideal mobile phases, and strong interaction between proteins adsorbed on the surface. The two main types of calorimeter used for this type of analyses are the isothermal titration calorimetry (ITC) and the flow microcalorimetry (FMC), highlighted in section 1.3.1 and 1.3.2, respectively (Lantenois *et al.*, 2007; Kim *et al.*, 2011; Ueberbacher *et al.*, 2010*)*.

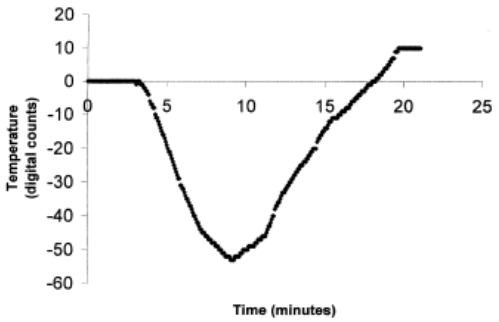

Figure 1.3.1- Typical endothermic BSA-Polyethyleneimine temperature profile adapted from Thrash and Pinto, 2002.

1.3.1. Isothermal Titration Calorimetry

ITC is a important tool, consisting in an adiabatic method, that allows studing the heat resulting from the interaction of a protein with a ligand (Ueberbacher *et al.*, 2010). Measurement of this heat leads to accurate determination of binding constants, reaction stoichiometry (n), enthalpy (ΔH) and entropy (ΔS), thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment (Wiseman *et al.*, 1989).

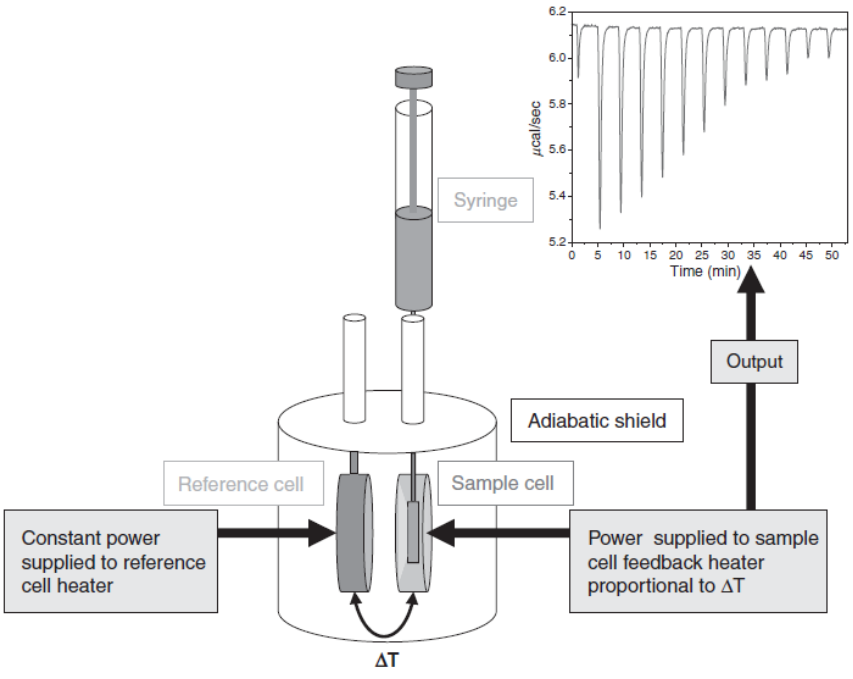

Figure 1.3.1.1- Representative diagram of a typical power compensation ITC. Major features of this type of instrument such as the reference and sample cells, syringe for adding titrant, and the adiabatic shield are noted in the figure. This diagram shows an oversimplification of how the power applied by the instrument to maintain constant temperature between the reference and sample cells is measured resulting in the instrument signal (Freyer and Lewis, 2008)

A typical power compensation ITC is shown schematically in figure 1.3.1.1. The reference cell contains water or buffer, and the sample cell has substrate, which is titrated at constant temperature with the ligand in a syringe. Prior to injection of the titrant, a constant power (< 1 mW) is applied to the heater of the reference cell. This, in turn, directs a feedback circuit activate the heater on the sample cell. During the injection of the ligand into the sample cell, the generated heat can be endothermic or exothermic, resulting in an activation or deactivation of the feedback power respectively in order to maintain equal temperature between the two cells. The heat absorbed or released (in terms of molar enthalpy ΔH) upon injection is monitored over time. Thus, the raw signal in the ITC is the power (mcal/sec or mJ/sec) applied to the control heater that is required to keep the calorimeter cell from changing temperature as a function of time. The heat change is then simply calculated by integrating the heater power over the time (sec) of the measurement (or, more specifically, the time required for the control heater power to return to a baseline value) (Freyer and Lewis, 2008; Wettig and Kamel, 2013).

Several applications of this technique have been published for the study of biological and bio-molecular recognition interactions, ligand binding, enzyme activity, drug discovery, design and development, protein–protein, protein–receptor, lipid–lipid and lipid–small molecules interactions (Bouchemal, 2008). Wiseman *et al.* studied binding constants and heats of binding of cytidine 2'-monophosphate (2'CMP) to the active site of ribonuclease A (Wiseman *et al.*, 1989). Spink and Wadso in 1976 published one of the first calorimetric studies of enzyme activity. Ladbury has published a series of annual reviews on ITC, describing the newest applications and a year-to-year survey of the literature on ITC applications (Freyer and Lewis, 2008).

1.3.2. Flow Microcalorimetry

FMC is a very practical and versatile technique which measures the small thermal changes occurring during adsorption and desorption of molecular species carried in a fluid stream (Lin *et al.*, 2001).

The FMC, shown in figure 1.3.2.1, consists of a thermostated block containing a small column where the adsorbent is packed (FMC unit); and probes equipped with thermistor sensors connected to a measurement and control unit. The existence of a peristaltic pump and a switching valve allows to select between an open mode where all the material from the column output is wasted and a closed mode in which the total flow circulation is achieved. The FMC is a fixed-bed process. In the beginning, the system is stabilized by pumping a buffer solution through the column. After thermal stabilisation, the measurement begins by replacing the buffer with the substrate solution (Stefuca and Gemeiner, 1999; Kim *et al.*, 2011).

The adsorption of the sample onto an adsorbent surface causes a change in the cell temperature, which is converted to a heat signal by the FMC through an experimentally determined calibration factor (Esquibel-King *et al.*, 1999). Typical FMC thermograms comprise endothermic and exothermic peaks depending on the release or absorption of energy during interactions. This calorimeter has been constructed to be operated in the heat conduction mode. Endothermic events (reactions where energy is absorbed) result in negative peaks as the thermistors register a decrease in the cell temperature. On the other hand, exothermic events (reactions where energy is released) result in positive peaks as the thermistors register an increase in the cell temperature (Thrash and Pinto, 2002). The heat signal indicative of the adsorption/desorption processes is saved by an analog recorder in the computer memory to posterior analysis. These data, once processed by Caldos (a program that acquires, stores, presents, annotates, processes, calibrates, and manages data for the entire FMC system), allows to determine a number of parameters such change in enthalpy (ΔH), change in entropy (ΔS), and the quantity of material that reacted. These parameters can then be used to characterize a reaction in terms of the rate and mechanism (Korfhagen *et al.*, 2010; Esquibel-King *et al.*, 1999).

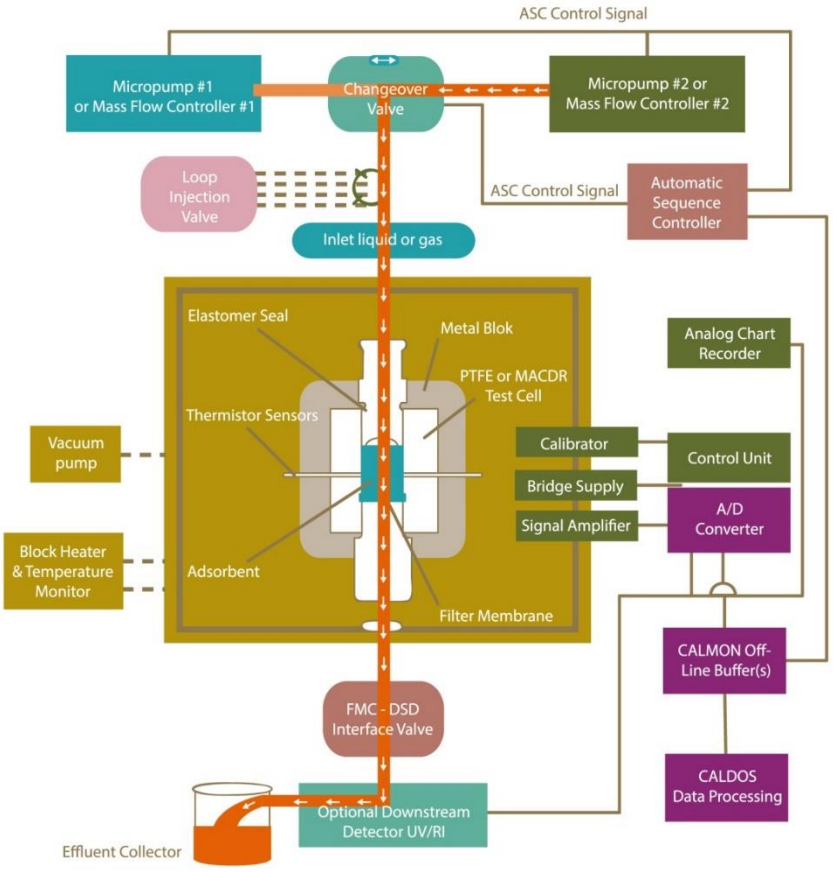

Figure 1.3.2.1- Representative scheme of the flow microcalorimeter.

1.3.3. Calorimetric studies in HIC

Several researchers have applied the calorimetry in HIC systems. Yamamoto and Ruaan and co-workers (Lin *et al.*, 2001; Huang *et al.*, 2000) studied the effects of salt, ligand hydrophobicity, and protein structure on the interaction between protein and ligand using microcalorimetric measurement. Dias-Cabral *et al.*, Korfhagen *et al.*, and Esquibel-King *et al.* (Dias-Cabral *et al.*, 2003; Esquibel-King *et al.*, 1999) studied the interaction thermodynamics associated with BSA adsorption on hydrophobic interaction sorbents under linear and overloaded conditions, presenting experimental data from equilibrium binding isotherms, linear chromatography, and calorimetry. All of these cited studies conclude that water release is a key component of the entropic driving force underlying protein adsorption/binding. In addition to the effects of water release, it was also shown that structural changes associated with protein adsorption on mild hydrophobic surfaces can be significant *(*Thrash *et al.*, 2004).

1.3.4. Calorimetric studies with PEGylated proteins

Blaschke *et al.* (Blaschke *et al.*, 2011) have studied the adsorption of PEGylated lysozyme on a strong cation exchange resin. For this, isothermal titration calorimetry and independent adsorption equilibrium measurements were applied. The adsorption of PEGylated lysozyme forms was found to be entropy driven although the adsorption of the native protein is enthalpy driven. This effect is mostly caused by the addition of flexibility to the protein molecule through the PEG chain. The flexibility of the PEG chain and its large hydration shell lead to structure rearrangements upon adsorption where water is released leading to an entropy gain. Recently, the same research group, have used the calorimetry for study the adsorption of native and mono- PEGylated BSA on anion-exchangers (Blaschke *et al.*, 2013). The adsorption of BSA on strong anion-exchangers was found to be exothermic, but the magnitude of the adsorption enthalpy and the entropy was dependent on the type and structure of the resin and its polymer modification. The adsorption on weak anion-exchangers is endothermic and driven by entropic contributions. The adsorption of PEG-BSA on strong anion exchangers is exothermic, again, the magnitude of the enthalpy of adsorption and the entropy strongly depend on the resin. Hasse and co-workers (Werner *et al.*, 2012) have investigated of the adsorption of PEGylated lysozyme and PEG on a mildly hydrophobic resin, the molar enthalpy of adsorption was also determined from the calorimetric and the adsorption equilibrium data. It is found to be endothermic in all experiments. The comparison of the adsorption of different PEGylated forms showed that the adsorption of PEGylated lysozyme is driven by the adsorption of the PEG chain.

1.4. Goal of Study

PEGylation modifies properties of proteins in order to improve its chemical stability, solubility, immunogenicity and *in vivo* half-life. PEGylation chemistry and strategies, as well as their influence on protein behaviour, have been described by several researchers. It is well known that, among other protein properties, PEGylation changes its hydrophobicity. Hydrophobic interaction chromatography is rather rarely applied for the separation of PEGylated proteins. However, some studies have shown that this type of chromatography can be a promising technique for the separation of PEG conjugates. Still, little work has been done to explore the hydrophobic interaction mode. One of these studies using a butyl hydrophobic resin highlights the primordial influence of salt in the separation of PEGamers. Different elution order was found in presence of different salt types (ammonium sulfate and sodium chloride). Thus, the main goal of the present study is to understand if there is an alteration on the PEG conjugates adsorption interaction mechanism when different type of salts are used.

Flow Microcalorimetry has proven its ability to provide an improved understanding of the driving forces, mechanisms and kinetics involved in the interaction process of biomolecules adsorption onto several chromatographic systems. Thus, using FMC as a central technique, the adsorption mechanism of native and PEGylated lysozymes will be investigated on a hydrophobic resin (Toyopearl Butyl-600M) as function of salt type (ammonium sulfate and sodium chloride).

To prepare a PEGylated lysozyme, it is desirable that one PEG molecule is attached to a specific site (site-directed mono- PEGylation). However, as the PEGylation reaction is not completely understood, it is not easy to adjust or optimize the reaction process. The reaction mixture after PEGylation, from which the desired PEGylated protein must be purified, is higly complex. Since it is necessary for our study the use of individual PEGamers, ion exchange chromatography will be used to accomplish its separation. The cation exchanger TSKgel SP-5PW was chosen once it has been highlighted as an effective support for this kind of separation, using a salt gradient elution. MALDI-TOF analysis was used to identify PEGylation reaction products separated with the cation exchanger support.

Chapter 2 - Reagents, apparatus and methods

2.1. Reagents

Hen egg white lysozyme was obtained from Sigma-Aldrich (St.Louis, MO, USA). Methoxy-PEG-aldehyde with an average molecular weight of 20 KDa was purchased from Laysan Bio, Inc (Al, USA). The cation-exchange resin TSKgel SP-5PW and the hydrophobic resin Toyopearl Butyl-600M were supplied by Tosoh Bioscience GmbH (Stuttgart, Germany). The salts used for the buffer preparation, namely sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$), ammonium sulfate ($(NH_4)_2SO_4$) and sodium chloride (NaCl) were obtained from Panreac (Barcelona, Spain). The sodium acetate anhydrous (CH_3COONa), glacial acetic acid (CH_3COOH) and Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$) were purchased from Merck, Chem lab and Nzytech, respectively. The pH was adjusted with NaOH (J.T. Baker, Deventer, Netherlands) and HCl (Sigma-Aldrich, Steinheim, Germany). The reagents used for MALDI analysis, mainly sinapic acid and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (St.Louis, MO, USA). The acetonitrile was purchased from Panreac (Barcelona, Spain).

2.2. Apparatus

An Äkta Explorer System, illustrated in figure 2.2.1A (GE Healthcare, Uppsala, Sweden) was used for all preparative purifications.

Allegra X-22R Centrifuge (Beckman Coulter, USA) was used to concentrate the PEGylated lysozyme solutions and a ScanVac Coolsafe™ Lyophilizer (Labogene, Lynge, Denmark) was used to lyophilize the samples (figure 2.2.1B and C).

The 4800 Plus MALDI TOF/TOF™ Analyzer (AB SCIEX, MA, USA) allowed to identify the resulting products of the PEGylation reaction (Figure 2.2.2).

In figure 2.2.3 is represented the FMC (Microscal Ltd, London, UK). This was used for the investigation of lysozyme-PEG adsorption mechanism in HIC under different operating conditions.

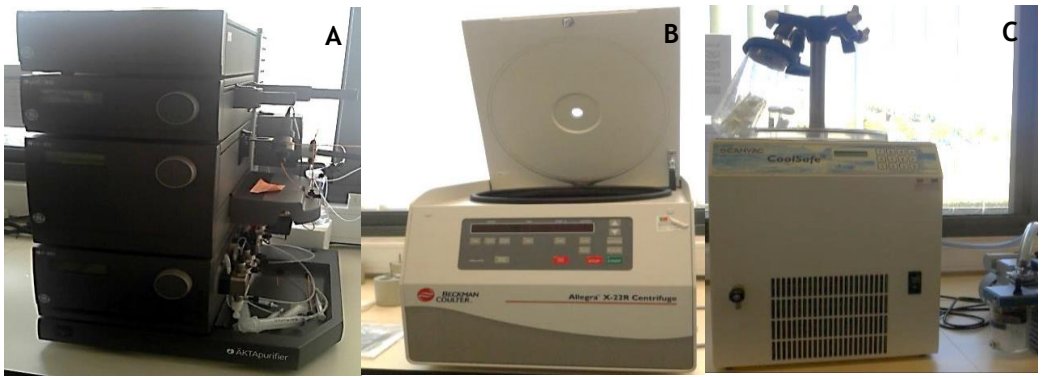

Figure 2.2.1- A: Äkta Explorer System, B: Allegra X-22R Centrifuge, C: ScanVac Coolsafe™ Lyophilizer.

Figure 2.2.2- 4800 Plus MALDI TOF/TOF™ Analyzer in a CICS-UBI laboratory.

Figure 2.2.3- Microcalorimeter in a CICS-UBI laboratory.

2.3. Methods

2.3.1. PEGylation of lysozyme

Lysozyme (2.75 mg/mL) was allowed to react with 20kDa mPEG-ALD reagent at different molar ratios of mPEG: protein (5:1 and 25:1) in 50mM acetate buffer, pH 5.0 in the presence of 3 mg/ml of $NaCNBH_3$ for the reduction of Schiff's bonds, at 4 °C. The reaction was quenched with 0.5 M Tris-HCl pH 7.4 after 18, 24 and 48 h. Removal of low-molecular weight reaction end-products and salts was performed using a PD10 desalting column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 25 mM sodium phosphate buffer pH 6.

2.3.2. Chromatographic purification

The chromatographic purification was similar to the one developed by Moosmann *et al*. and optimized for the present laboratory equipment (Moosmann *et al.*, 2010).

Preparative chromatographic purification was carried out on an Äkta Explorer System equipped with a single wavelength UV, conductivity and pH combi-monitor. The UV signal was used at a wavelength of 280 nm to detect protein signals; the conductivity detector monitors the eluting buffer salt concentration. EcoPlus glass column (05 mm x 125 mm, KronLab, Dinislaken) was packed with TSKgel SP-5PW to a total bed height of 8 cm which yields a total column volume (CV) of 1.71 mL. The amount of injected sample was approximately 100 µL. The chromatographic separation was carried out at a flow rate of 0.5 mL/ min. Buffer A consisted of 25mM sodium phosphate buffer pH 6. Elution buffer B was prepared by adding 1M of NaCl to buffer A. A gradient elution of buffer B was used for separation of non-reacted protein and differently PEGylated lysozyme variants.

Desalting and concentration of the purified fractions were performed using a Vivaspin™ sample concentrators (Sartorius Stedim Biotech S.A., Aubagne, France). Finally, the fractions were stored lyophilized.

2.3.3. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF)

MALDI-TOF analysis was used to determine the resulting products of the PEGylation reaction, separated by cation exchange chromatography. For sample clean-up, 25 µg of collected samples were prepared using 2-D Clean-Up kit from GE-Healthcare (Uppsala, Sweden) according to manufacturer's instructions. For MALDI analysis, 1 µL of prepared samples were mixed with 1 µL of a matrix (containing 10 mg/mL sinapic acid in 30% (V/V) acetonitrile and 0.3% TFA) and was applied and air dried on an Opti-TOF™ 384-well MALDI plate insert (AB Sciex, MA, USA).

The measurement was performed in a MS linear positive ion. The measured spectra are results of 900 profiles with 30 laser shots and 30 sub-spectra (laser intensity 6000). The MALDI-TOF was calibrated using CALMIX 3 (AB Sciex, MA, USA) according to manufacturer's instructions.

2.3.4. Flow Microcalorimetry (FMC)

The flow calorimetric adsorption experiments were performed using a methodology similar to the one developed by Dias-Cabral and co-workers optimized for the present research (Silva *et al.*, 2014).

The heat of adsorption experiments started with loading of the hydrophobic resin in a clean and dry flow cell, 171 µL of Toyopearl Butyl-600M was placed in the calorimetric cell. The system was left to equilibrate at a constant flow rate of 1.5 mL/h, controlled by precision syringe micropumps, with the carrier solution. This consists of 25 mM phosphate buffer at pH 6 prepared in different salts: 4 M of NaCl and 1 M of $(NH_4)_2SO_4$. Once the system has reached thermal equilibrium, the sample (dissolved in the carrier fluid) was loaded into the injection loop of 30 µL in a constant flow rate of 1.5mL. Phosphate 25 mM at pH 6 was used as washing solution between each injection.

Adsorption of sample components on the hydrophobic resin alters the thermal and mass equilibrium in the cell. The temperature change is recorded by the thermistor, which is converted to a heat signal by the FMC electronics through an experimentally determined calibration factor (the calibration factor was obtained using an electrical impulse of 0.030J). The adsorbed amount is calculated by a mass balance between the amount of solute in the effluent by UV detection and the fed amount.

CALDOS 4 data processing software (Microscal Limited, London, UK), was used to acquire, store, calibrate, process and present enthalpy data. Peak deconvolution was performed using the PEAKFIT software package (versão 4.12, Seasolve Software Inc., San Jos, EUA) using asymmetric Gaussian peaks. The integral heat of adsorption was calculated from the area of the deconvoluted peaks.

Chapter 3 – Results and Discussion

3.1. PEGylation of lysozyme

In this work we investigated the adsorption mechanism of native and PEGylated lysozymes as function of salt type on a hydrophobic resin. To this end, it was necessary to use individual PEGamers. The PEGylation reaction used in these studies took place between the aldehyde group of methoxy-PEG-aldehyde and the free amino acid group (NH_2-group) of lysine residues within lysozyme. A Schiff base with a reactive double bound is formed during that reaction. Sodium cyanoborohydride was added to the reaction buffer for covalent attachment. The reaction was a random PEGylation, which lead not only to mono-PEGylated lysozyme but also to poly-PEGylated lysozyme. For the separation of PEGamers the cation exchanger TSKgel SP-5PW was chosen, since it has been highlighted as an effective support for this kind of separation (Moosmann *et al.*, 2010).

Moosmann *et al.* (Moosmann *et al.*, 2010) developed a purification method for PEGylated proteins using cation exchange chromatography. As the mPEG-ALD, with an average molecular weight of 5 kDa, 10 kDa and 30 kDa, tested by them were different from the one used by us (20 kDa), it was necessary to make some optimizations.

The chromatographic separation was carried out at a flow rate of 0.5 mL/min. Buffer A consisted of 25 mM sodium phosphate buffer pH 6. Elution buffer B was prepared by adding 1M of NaCl to buffer A. Different elution gradients of buffer B were tested for separation of non-reacted protein and differently PEGylated lysozyme variants. As we can see in figure 3.1.1 (A), about 5 minutes after injection a linear gradient was performed (with a duration of 18 minutes), to achieve a percentage of 100% B. A step gradient was also used, as shown in figure 3.1.1 (B). Also a linear gradient of B, until 50%, for 18 min was used followed by about 2 minutes at 100% B, as represented in figure 3.1.1 (C). As it can be seen in figure 3.1.1, with elution profile (C) the peaks resolution is better, thus a better separation was attained. The gradient eluted peaks occurred between 10 and 30 % of B. The elution time used in (A) and (B), using the previously mentioned percentages, was not enough for efficient separation. Therefore for the following experiments, the elution profile (C) was used.

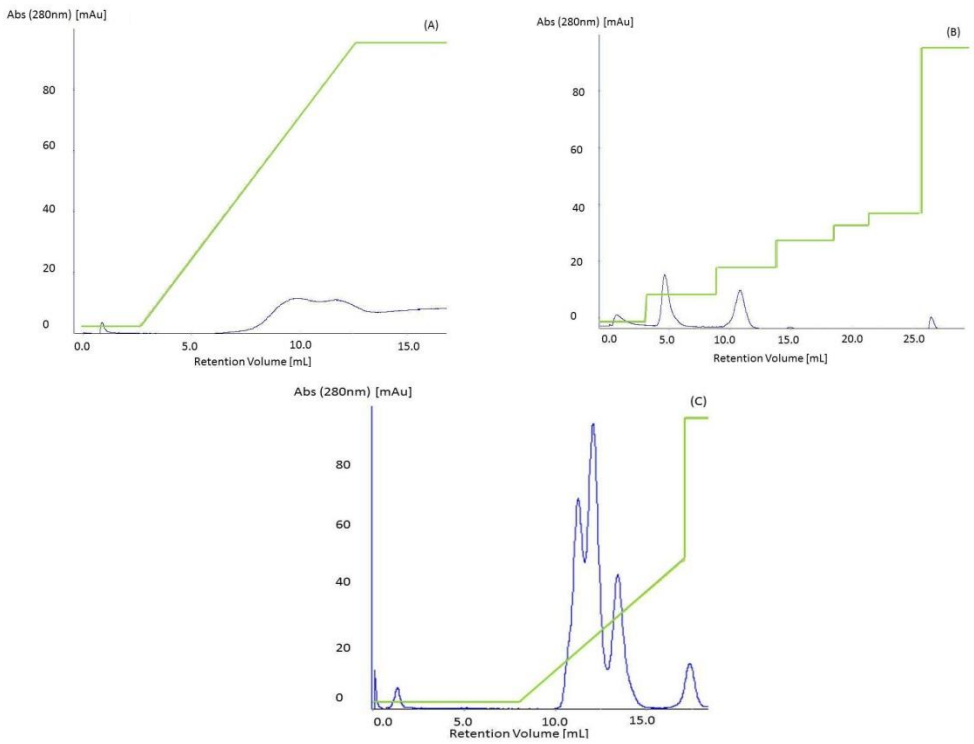

Figure 3.1.1- Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW column. Different elution gradients (A), (B) and (C)of buffer B were used. Blue line: absorption, green line: elution profile.

Figure 3.1.2 shows typical chromatographic profiles of a reaction mix of PEGylated lysozyme separated on TSKgel SP-5PW. Different molar ratios of mPEG:lysozyme, 5:1 and 25:1, were used in the production of PEGylated lysozyme and are shown in Figure 3.1.2 (A) and 3.1.2 (B), respectively.

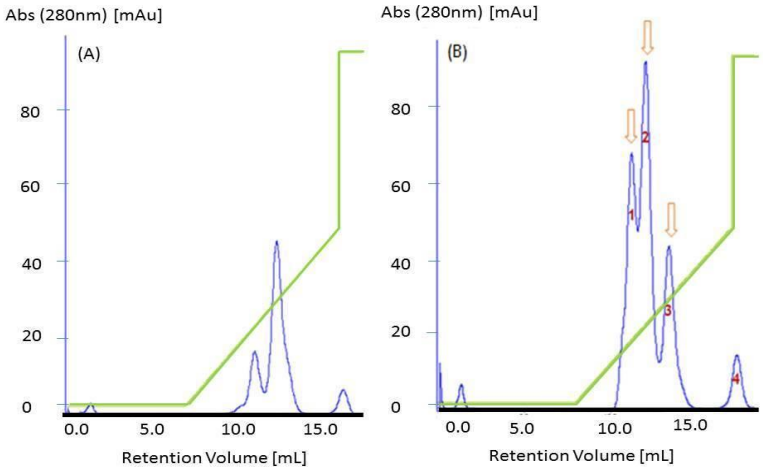

Figure 3.1.2- Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW column. Gradient elution to 1M NaCl in buffer A. Blue line: absorption, green line: elution profile. Prodution of PEGylated Lysozyme with molar ratios of mPEG: protein (A) 5:1, (B) 25:1. The peaks indicated by arrows were identified via MALDI-TOF analysis.

From what can be seen, the PEGylation reaction seems to be more efficient when used in the ratio of 25:1. More peaks were observed which may indicate the presence of more PEGylation species when compared to the ratio of 5:1. So, for the following experiments only the ratio 25:1 was used. Also, different reaction times were tested (18 h, 24 h, 48 h), however, there were no visible differences in the chromatographic separation (chromatograms shown in figure 3.1.3).

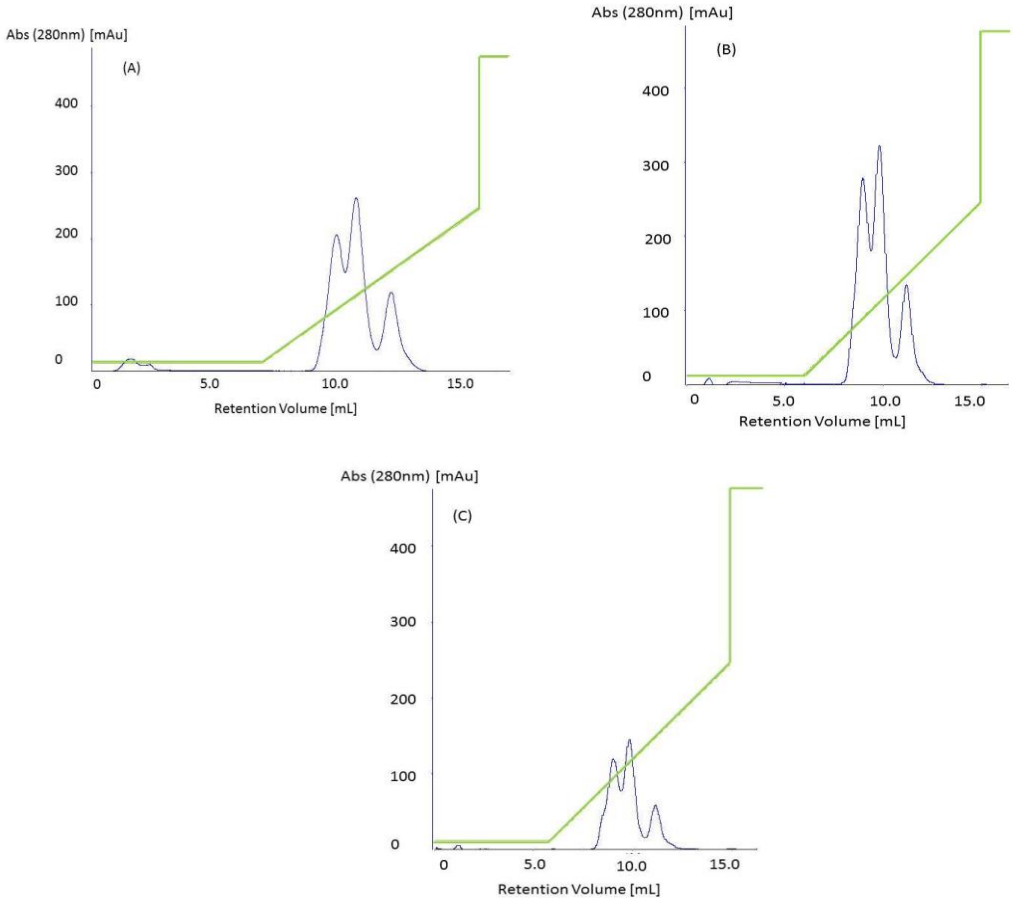

Figure 3.1.3- Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW column. Gradient elution to 1M NaCl in buffer A. Blue line: absorption, green line: elution profile. Different reaction time (A)18 h, (B)24 h and (C)48 h were used.

The chromatographic separation originated four peaks (Figure 3.1.2 (B)), which were collected and identified, as shown below. The fourth peak in the reaction mixture chromatogram (approximately 16 mL, eluted at 100% of buffer B) is related to unmodified lysozyme, this being verified by comparison with pure lysozyme (chromatogram shown in figure 3.1.4). Also, the sample was enriched with pure lysozyme (chromatogram shown in figure 3.1.5) and we found that, compared to the chromatogram of Figure 3.1.2 (B), the magnitude of peak 4 increased significantly. As mPEG-aldehyde is non-UV active, unreacted PEG could not be directly evaluated (Maiser *et al.*, 2014).

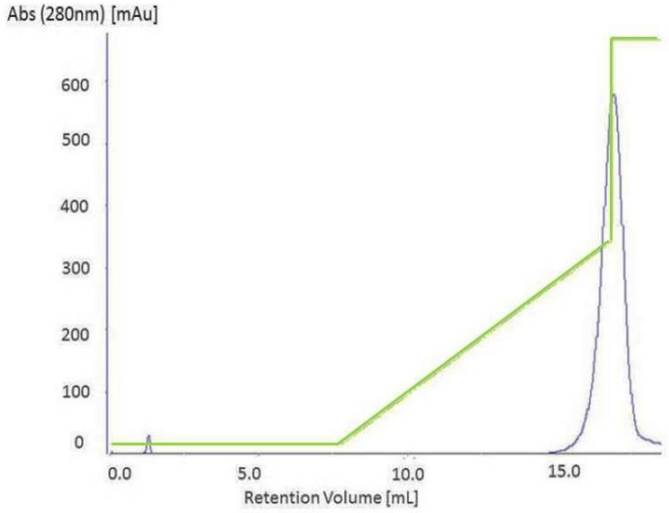

Figure 3.1.4- Purification of lysozyme on an analytical TSKgel SP-5PW column. Gradient elution to 1 M NaCl in buffer A. Blue line: absorption, green line: elution profile.

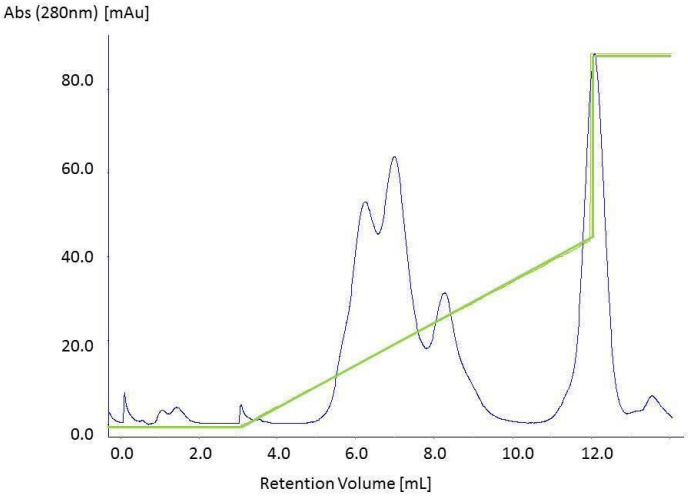

Figure 3.1.5- Purification of sample enriched with pure lysozyme on an analytical TSKgel SP-5PW column. Gradient elution to 0.5 M NaCl in buffer A. Blue line: absorption, green line: elution profile.

The gradient eluted peaks (the ones indicated by arrows in Fig. 3.1.2 (B)) were collected and analysed by MALDI-TOF. Due to the small amount obtained from peak 4, previously identified as lysozyme, it was not possible to analyse it by MALDI-TOF. This may be indicative of an efficient PEGylation of lysozyme.

MALDI-TOF has the ability to ionize high-molecular-weight thermolabile molecules, with high accuracy and sensitivity. It is a promising method for the identification of biomolecules in

complex samples, including peptides, proteins, oligosaccharides and oligonucleotides (Gevaert and Vandekerckhove, 2000). The spectra represented in figure 3.1.6, 3.1.7 and 3.1.8 correspond the peak 1, 2 and 3 respectively.

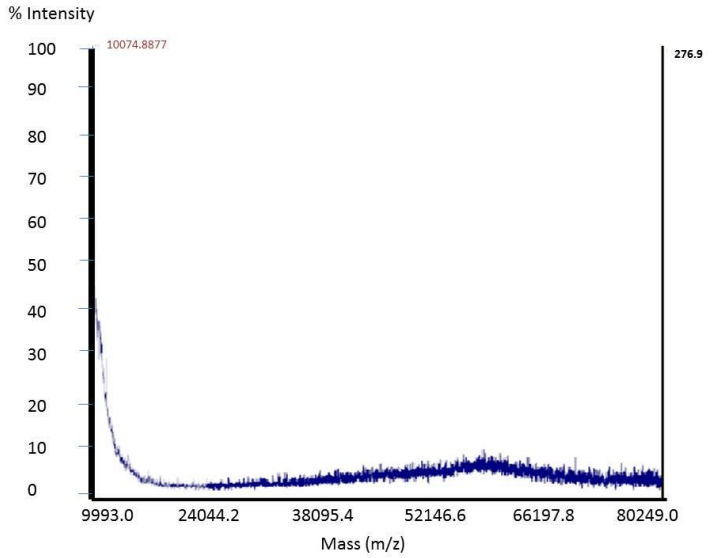

Figure 3.1.6- MALDI mass spectrum of peak 1.

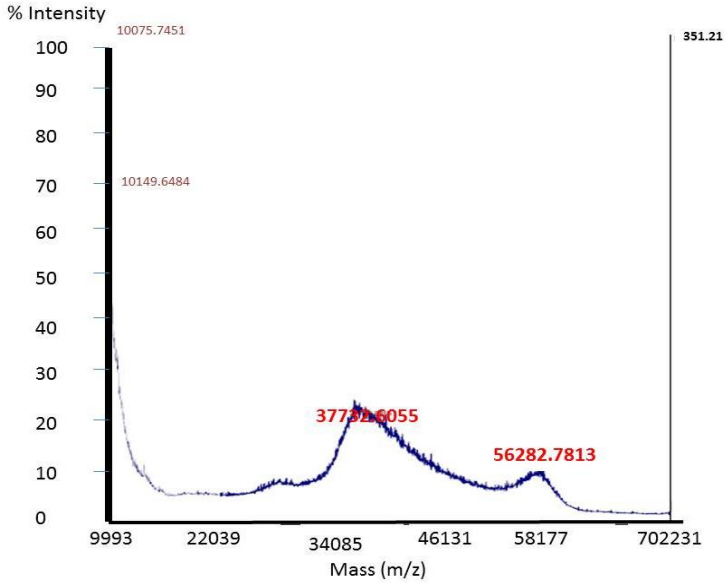

Figure 3.1.7- MALDI mass spectrum of peak 2.

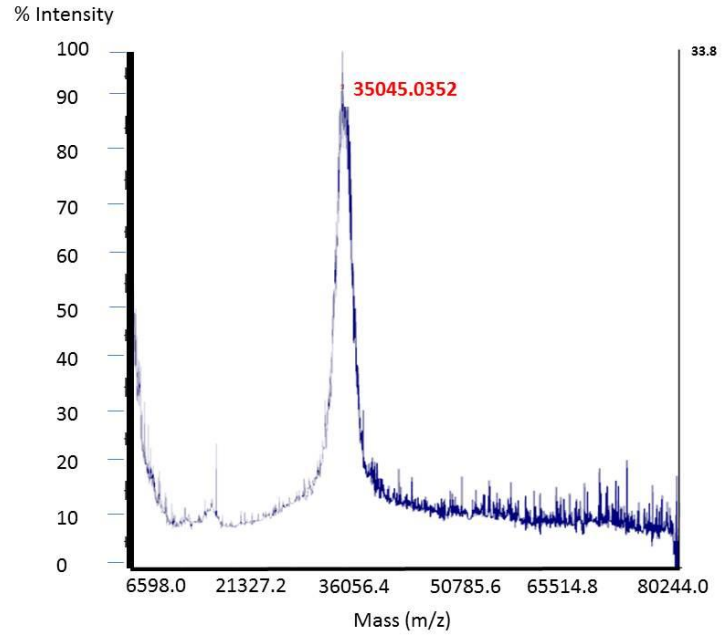

Figure 3.1.8- MALDI mass spectrum of peak 3.

As mentioned before, lysozyme with M_W of approx. 14.4 kDa was PEGylated randomly at its lysine residues with mPEG-ALD with an average molecular weight of 20 kDa. Only three of the residues: 33, 97 and 116 can be PEGgylated. The lysozyme is first PEGylated to mono-PEGylated lysozyme (Mw of approx. 35 kDa) which can then react to di-PEGylated lysozyme (Mw of approx. 55 kDa). The latter can be reacted to tri-PEGylated lysozyme (Mw of approx. 75 kDa) (Blaschke *et al.*, 2011). To identify the binding site were PEGylation occur, peptide mapping in combination with mass spectrometric analysis can be applied *(*Maiser *et al.*, 2012). However, the amount of sample obtained was not enough to perform MALDI-TOF/TOF analyses. Thus, it was only possible the identification of PEGylation degree by MALDI-TOF analyses.

A peak of low intensity, lower than 10% with a molecular mass of approximately 55 kDa is observed in figure 3.1.6. So, peak 1 may correspond to di-PEGylated Lysozyme.

As noted in figure 3.1.7, there are two peaks with a mass of approximately 35 and 55 kDa, consisting in the molecular weight of mono- and di-PEGylated species respectively. The intensity of mono-PEGylated lysozyme is higher compared with di-PEGylated lysozyme. The small amount of di-PEGylated protein may be explained by a lack of efficiency in the chromatographic separation of peak 1 and 2 (figure 3.1.2 (B)).

Peak 3 with around 35 kDa is mono-PEGylated lysozyme (figure 3.1.8). The intensity of this peak is high, being consistent with the high resolution of the peak, as seen in Figure 3.1.2.

Mueller and co-workers (Moosmann *et al.*, 2010) purified PEGylated proteins using cation exchange chromatography and showed that TSKgel SP-5PW is capable of detect different isoforms of mono-PEGylated lysozyme. So, peaks 2 and 3 may correspond to the different isoforms mono-PEGylated lysozyme, 1-mono PEG and 3-mono PEG respectively.

According to Moosmann *et al.*, multi-PEGylated species generally elute before di- and mono-PEGylated variants, followed by the native unPEGylated protein (Moosmann *et al.*, 2010). Since the PEG:protein ratio correlates with retention time, it was thought that the PEG was weakening the interaction of the protein with the resin. As the unPEGylated molecule has an additional negative charge due to the ionization of the SH group on cysteine, it would be expected to elute later. In our work it was also demonstrated that PEGylated lysozyme was eluting before native lysozyme. This effect can be explained via the masking effect of PEG as found by Seely and Richey (Seely and Richey, 2001).

3.2. Microcalorimetry, to investigate the mechanism of protein adsorption on HIC

Understanding the mechanism of protein adsorption on HIC media is still a challenging task (Haimer *et al.*, 2007). Microcalorimetry can help us in this mission. As described in Section 1.3.2, FMC enables the measurement of the energy changes associated with the sorption processes and the calculation of the amount of compound adsorbed or desorbed (Diaz *et al.*, 2005).

Protein adsorption can be exothermic or endothermic. Exothermic adsorption is associated with attractive forces between the surface and the adsorbed proteins, between the adsorbed proteins, or both. Sources of endothermic adsorption, depending on the process conditions, can be: water and ions release from the surface of the adsorbent and protein, repulsive interactions between adsorbed molecules, repulsive interactions between protein surface hydrophobic groups and adsorbent surface hydrophilic moieties, and repulsive interactions between like charge groups on the protein surface and on the adsorbent surface. Additionally, protein conformational changes and protein reorientation on the surface are other potential sources for an endothermic heat signal (Katiyar *et al.*, 2010; Silva *et al.*, 2014).

This work was performed in order to understand the behaviour of PEGylated and pure Lysozyme onto a hydrophobic resin and to check the effect of differents salts (ammonium sulphate and sodium chloride). $(NH_4)_2SO_4$ is classified as a kosmotrope, whereas NaCl is a chaotrope. Regarding the hydratation conduct, $(NH_4)_2SO_4$ has a salting-out effect, making lysozyme molecules being separate from solutions or adsorbing on the solid surfaces of resins. In the other hand, the salting-in effect of NaCl dissolves lysozyme (Chen *et al.*, 2013).

3.2.1. HIC adsorption behaviour of pure and PEGylated lysozymes

As already described in the section 2.3.4, the experiments were performed at pH 6 in phosphate buffer 25 mM in presence of 4M NaCl or 1 M $(NH_4)_2SO_4$, using the 30 µL loop in a constant flow rate of 1.5 mL/h. Peak de-convolution was performed using asymmetric Gaussian peaks. The integral heat of adsorption recorded by CALDOS was calculated from the area of the de-convoluted peaks. Lysozyme adsorption thermograms, for the systems studied here, are shown in figure 3.2.1.1. In figures 3.2.1.2 and 3.2.1.3 are depicted the adsorption thermograms of the mono- and di-PEGylated lysozyme species. The associated enthalpy changes determined from the deconvoluted thermograms are summarized in Table 3.2.1.1. As already stated in the Introduction section, common sources of endothermic heats are: protein's and adsorbent's surface water and ion release; bound and unbound molecules repulsion; adsorbed protein reorientation and alteration of conformation. As for exotherms, they can be caused by the release of energy that occurs during adsorption.

Table 3.2.1.1- Heat of adsorption for pure and PEGylated lysozyme adsorption onto Toyopearl Butyl-600M using the 30 µL loop.

Sample	Mobile phase	Surface Concentration (mg/mL gel)	Exothermic peaks (kJ/mg)		Endothermic peaks (kJ/mg)		Net heat of adsorption (kJ/mg)
			ΔH^{I}	ΔH^{II}	ΔH^{III}	ΔH^{IV}	ΔH^{Total}
Lysozyme	$(NH_4)_2SO_4$	0,87	-41,64	0,00	31,14	0,00	-10,50
	NaCl	1,13	-35,59	0,00	37,10	0,00	1,51
Mono-PEGylated Lysozyme	$(NH_4)_2SO_4$	0,74	-12,79	-61,07	77,58	60,00	63,73
	NaCl	0,55	-178,10	0,00	450,93	0,00	272,83
Di-PEGylated lysozyme	$(NH_4)_2SO_4$	0,58	-7,43	0,00	148,27	0,00	140,84
	NaCl	1,44	-29,15	0,00	20,50	0,00	-8,65

In the case of pure lysozyme, at both salt conditions an initial exothermic peak was observed, followed by an endotherm (figure 3.2.1.1). The heats started at 800-900 s, where only occurred a release of energy. The endotherm began slightly after. In the presence of ammonium sulphate, when the protein plug is within the cell, the net heat of adsorption is exothermic, indicating an enthalpically adsorption process. The endotherm has a maximum at the moment the injection pulse leaves the cell, possibly indicating reorientation of adsorbed

lysozyme. As it can be seen in Table 3.2.1.1, the net heat resulting from the adsorbed protein is only slightly exothermic (-10 kJ/mg), confirming the enthalpically driven process in the presence of the kosmotropic salt. This has been already observed when weaker hydrophobic interactions are presents (Dias-Cabral *et al.*, 2002). In the experiments with NaCl, even though the heat profile evolution is similar, big differences can be seen in the moments where events took place. It is clearly observed in Figure 3.2.1.1 (B) that practically almost every energetic event happens before the protein plug leaves the system. The observed endothermic heat maybe related with dehydration process. Kosmotropic salt ions have higher polarity and bind water strongly, which induces the exclusion of water from the protein and ligand surface, in contrast, chaotropic salts have less polarity and bind water loosely, which induces inclusion of water on the protein and ligand surfaces (Xia *et al.*, 2004; Chen *et al.*, 2003), being necessary for adsorption a greater energy consumption to expose hydrophobic areas in the support and biomolecule, which leads to the appearance of the endothermic peak before protein plug comes out of the column. Concerning the exothermic observed heat, its magnitude is bigger in presence of NaCl. As chaotropic salts are expected to surround protein's charges, this will reduced overall protein charge and consequently the repulsion between adsorbed proteins will be also reduced, resulting in an increase of heat released. The overall energy is slightly endothermic, indicating that entropy, resulting from water release, is the driving force during adsorption.

In the mono-PEGylated lysozyme experiments, distinct events can be observed for the different salt conditions used. When the mobile phase is comprised with phosphate buffer 25 mM in presence of 1.0 M $(NH_4)_2SO_4$, two exothermic peaks and two endothermic peaks are observed. The heat signal began around 800 s after the beginning of the experiment with an exotherm. Immediately after the first exotherm ended, another exothermic peak is seen, as well as the first endotherm. The last endothermic peak appears afterwards. In the case where the mobile phase is comprised with phosphate buffer 25 mM in presence of 4.0 M NaCl, only two peaks are observed. One is exothermic and the other peak is endothermic. Both began around 800 s after the beginning of the experiment and ended at the moment the protein plug left the column. Werner *et al.* (Werner *et al.*, 2012) in an ITC study with PEGylated lysozyme stated that endothermic adsorption was due to the release of water from the hydration shell of the protein and the resin. Therefore, the enthalpy of adsorption increases with PEGylation degree since there is a higher water release. From what can be seen from Table 3.2.1.1, both experiments show an endothermic adsorption net heat, concluding that both processes were entropically driven and water molecules release has a great importance in conducting adsorption in hydrophobic interaction chromatography, as expected. The fact that the adsorption net heat is almost six times greater in the presence of NaCl that it is in the presence of ammonium sulphate, is related, as already referred, to the fact that by being a chaotropic salt, NaCl has a greater influence in protein adsorption due to the associated

generated entropy. In addition, their profile is very different from each other, as already stated. Also, by being a kosmotropic salt, ammonium sulphate does not mask lysozyme charges that could result in protein repulsion (can be the cause for the second endothermic peak) which may promote reorientation and further adsorption (second exothermic peak). On the other hand, in the presence of sodium chloride, since protein charges are shield by the salt, reorientation seems not to be a major contribution.

Lastly, the enthalpy profiles for di-PEGylated lysozyme, represented in Figure 3.2.1.3, are very distinct for both salt conditions used. Both experiments began with an exothermic peak, followed by an endotherm. In the presence of $(NH_4)_2SO_4$, the exotherm is very short in time and magnitude and the overall net heat is greatly positive, consequence of the very intense endothermic contributions, proving that under these conditions the process is conducted by entropy, consistent with HIC, as referred in the last paragraph. On the other hand, in the presence of NaCl, the adsorption net heat is mildly exothermic due to the greater contribution of adsorption heat. This can be the result from the greater effective hydrophobic area (two PEG chains) available for adsorption and repulsion between absorbed molecules redution, since NaCl has a shielding effect on the protein charge. In this case, again, there are practically no associated heats when the protein plug leaves the cell, helping supporting the hypothesis that PEG chains bind to the matrix ligands in a very effective way, preventing posterior reorientation.

As already discussed, one of the differences between the PEGylated and non-PEGylated lysozyme experiments is what happens inside the cell when the protein plug exits. It is seen that only in the experiment with native lysozyme in the presence of ammonium sulfate there is still thermic events happening when the protein leaves the cell. In this moment an endotherm is shown, being a indication of molecular reorientation to a more stable position. On the contrary, every thermal event occurs when the PEGylated lysozyme is still in the column. These results may indicate that PEG bounds to the adsorbent strongly enough in the primary adsorption not allowing lysozyme subsequent reorientation (Pasut and Veronese, 2007).

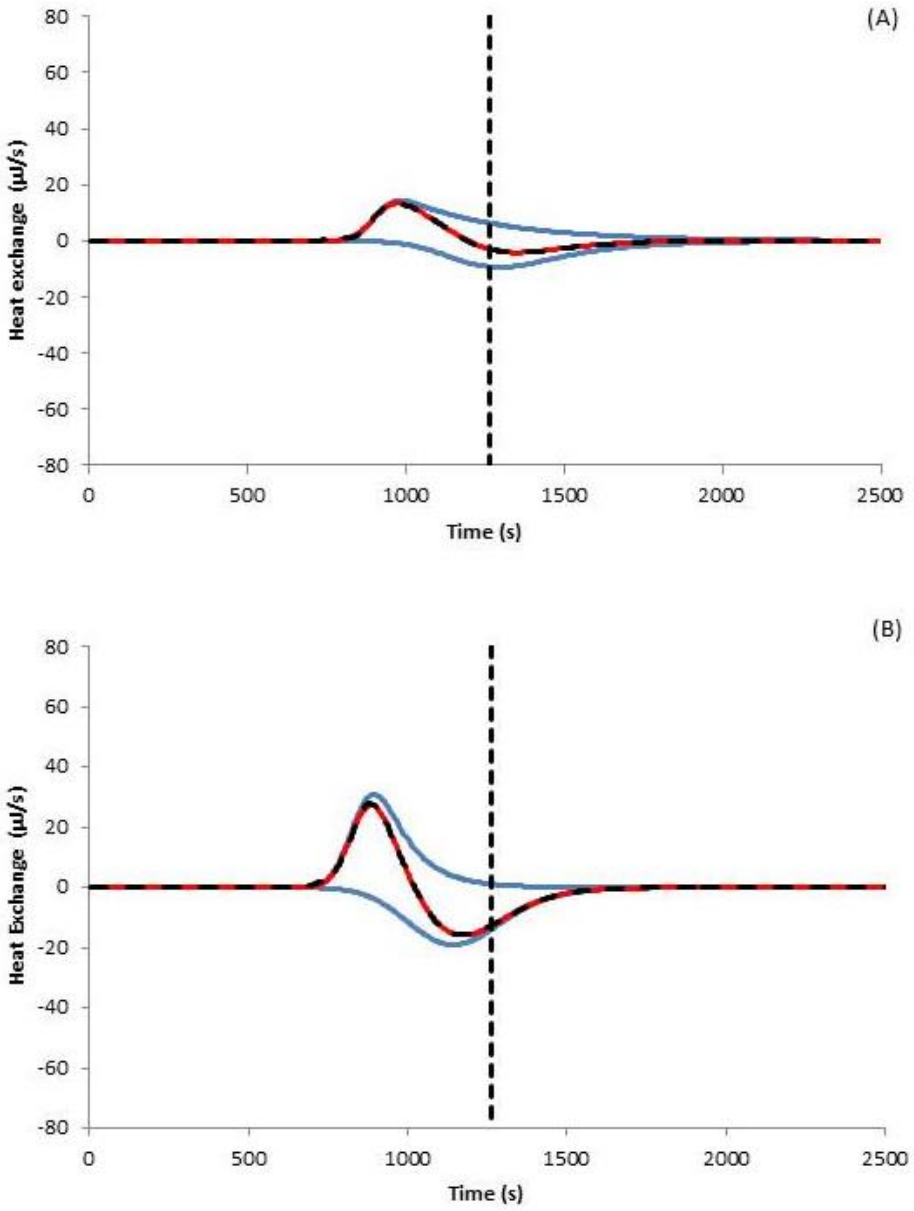

Figura 3.2.1.1- PEAKFIT de-convolution of thermograms for lysozyme adsorption onto Toyopearl Butyl-600M at pH 6. Sample loop volume: 30 µL; flow rate: 1.5 mL h^{-1}; temperature: 25 °C. Black line: experimental peak; Red line: generated peak; Blue line: de-convolutions peaks; Vertical line: time where the protein-containing plug of solution is replaced with protein-free mobile phase. Mobile phase contains (A) 1.0 M $(NH_4)_2SO_4$, and (B) 4.0 M NaCl.

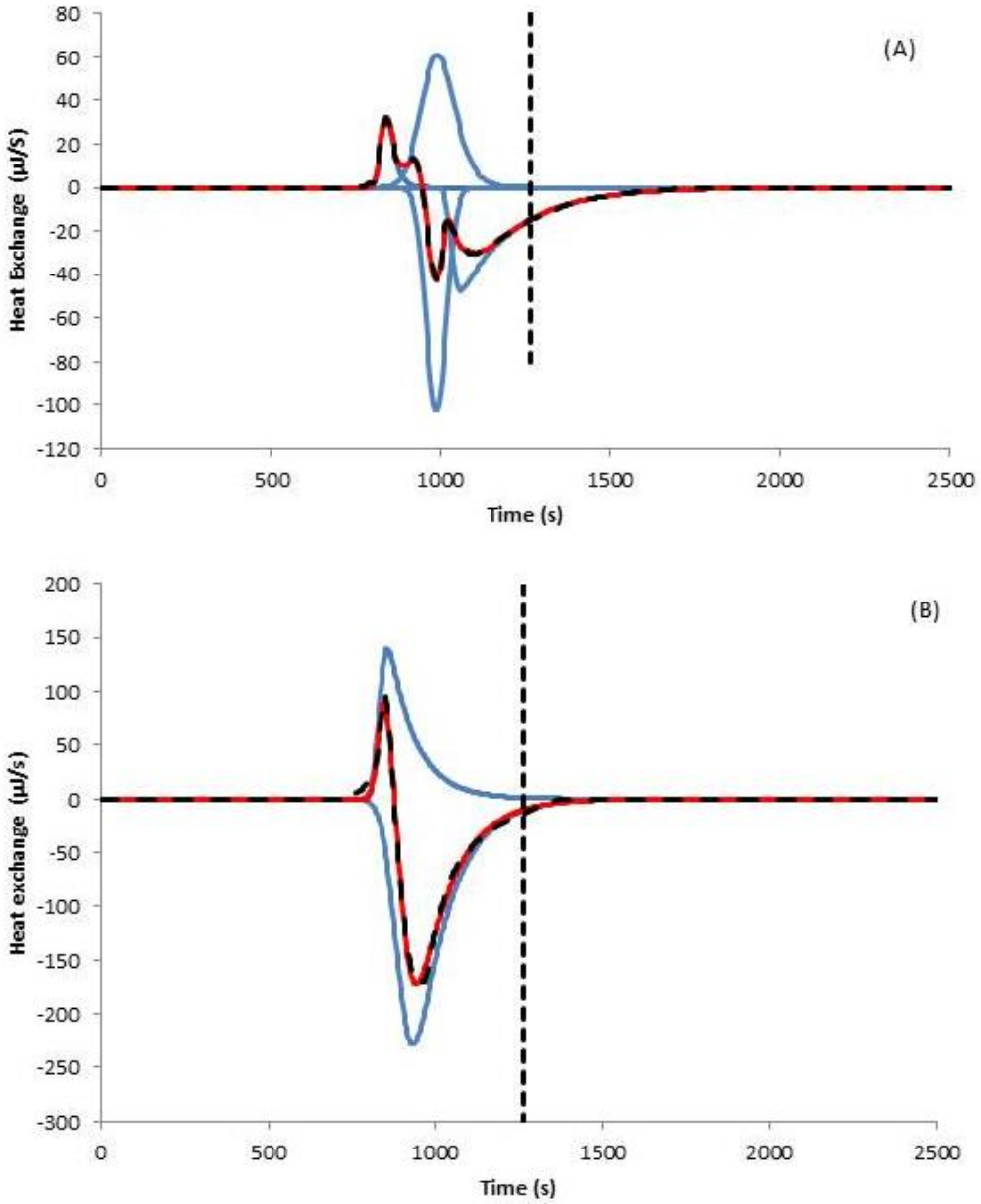

Figura 3.2.1.2- PEAKFIT de-convolution of thermograms for mono-PEGylated lysozyme adsorption onto Toyopearl Butyl-600M at pH 6. Sample loop volume: 30 µL; flow rate: 1.5 mL h^{-1}; temperature: 25 °C. Black line: experimental peak; Red line: generated peak; Blue line: de-convolutions peaks; Vertical line: time where the protein-containing plug of solution is replaced with protein-free mobile phase. Mobile phase contains (A) 1.0 M $(NH_4)_2SO_4$, and (B) 4.0 M NaCl.

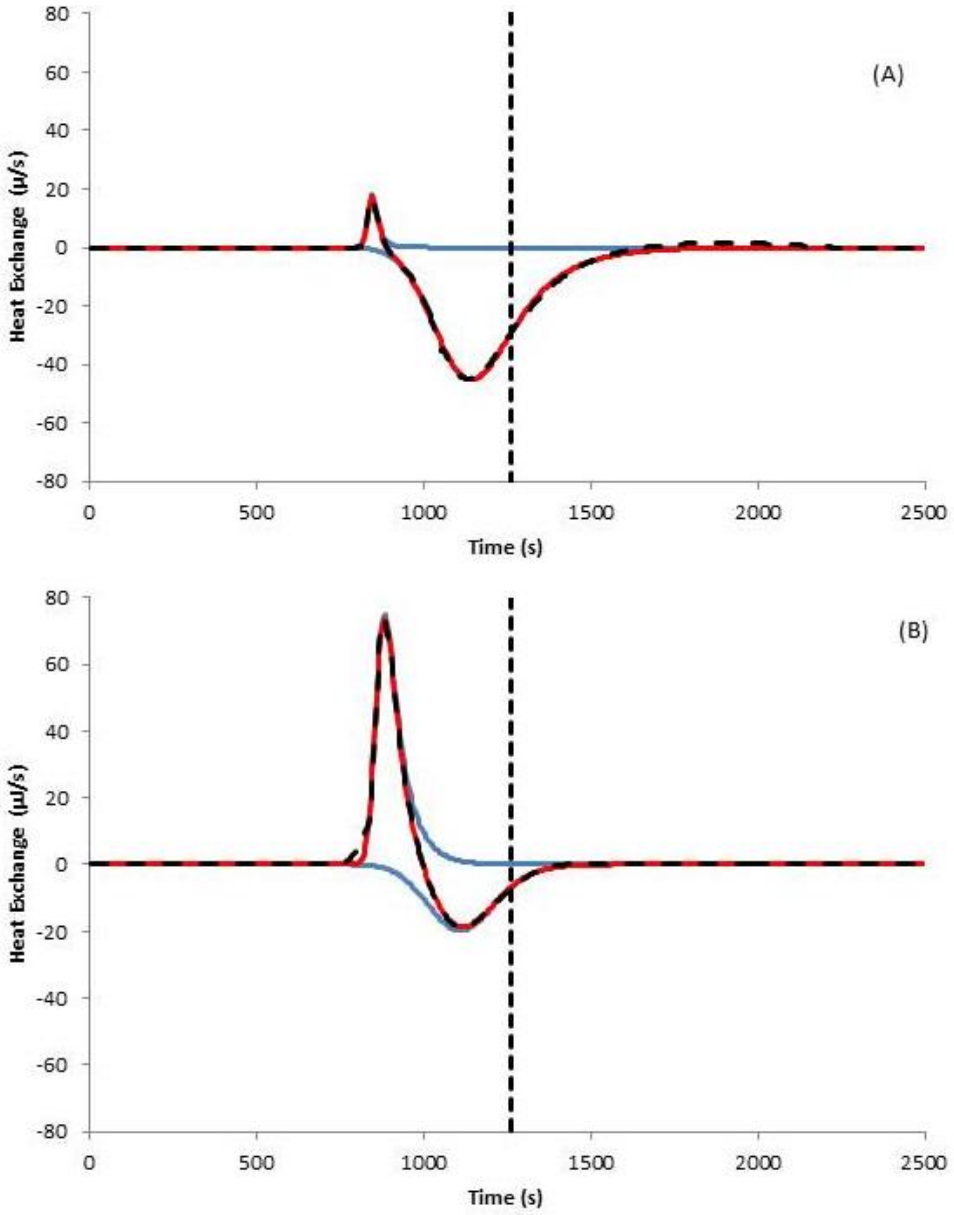

Figura 3.2.1.3- PEAKFIT de-convolution of thermograms for di-PEGylated lysozyme adsorption onto Toyopearl Butyl-600M at pH 6. Sample loop volume: 30 µL; flow rate: 1.5 mL h^{-1}; temperature: 25 °C. Black line: experimental peak; Red line: generated peak; Blue line: de-convolutions peaks; Vertical line: time where the protein-containing plug of solution is replaced with protein-free mobile phase. Mobile phase contains (A) 1.0 M $(NH_4)_2SO_4$, and (B) 4.0 M NaCl.

Chapter 4 – Conclusions and future work

Several chromatographic techniques have been used to resolve mixtures resulting from PEGylation reactions such as size exclusion chromatography, and ion exchange chromatography. Little work has been done with hydrophobic interaction chromatography, consequently the mechanisms of protein adsorption are not completely understood (Moosmann *et al.*, 2010).

In this study the adsorption mechanism of native and PEGylated lysozymes was studied on a hydrophobic resin (Toyopearl Butyl-600M) as function of salt type (ammonium sulfate and sodium chloride). To this, flow microcalorimetry was used as a central technique. With the exception of thermogram of mono-PEGylated lysozyme (mobile phase containing ammonium sulfate) where are obtained two endothermic peaks and two exothermic peaks, all other thermograms are composed of a first exothermic peak followed by an endothermic peak, which resulted from overlapped peaks. The presence of different peaks suggests the existence of different events occurring through the adsorption process.

The calorimetric analysis of native lysozyme in the presence of ammonium sulfate, an anti-chaotropic salt showed that the heat resulting from the adsorbed protein was slightly exothermic, indicating that the process is conducted enthalpically. In the experiments with sodium chloride solution, the total energy was slightly endothermic, showing that entropy is the driving force during adsorption. Werner *et al.* (Werner *et al.*, 2012) in an ITC study with PEGylated lysozyme stated that endothermic adsorption was due to the release of water from the hydration shell of the protein and the resin. In experiments with mono-PEGylated lysozyme, it appears an endothermic adsorption net heat, concluding that both processes were entropically driven and water molecules release has a great importance in conducting adsorption in hydrophobic interaction chromatography, as expected. In the case of di-PEGylated lysozyme, differences in overall heat process are more pronounced, being this endothermic in the presence of an anti-chaotropic and is exothermic in the presence of a chaotropic salt. These differences result from the greater PEGylated biomolecule hydrophobic area and reduced repulsion between absorbed biomolecules in presence of sodium chloride. Furthermore, it was found that in experiments with native lysozyme occurs molecular reorientation after adsorption. On the other hand, when the lysozyme is PEGylated, this doesn't happen. These results make one conclude that PEG bounds to the adsorbent strongly enough to not allow lysozyme reorientation (Pasut and Veronese, 2007) and secondary adorption. All these results confirm that flow microcalorimetry is a useful technique

illustrating the underlying mechanism associated with protein adsorption and elucidating the role of non-specific effects in the establishment of the adsorptive process.

Future work will be focus on static binding capacity studies to supplement the heat measurements performed in this work. Besides of mPEG-ALD with an average molecular weight 20 kDa, it will be also evaluated the adsorption behaviour of PEG 5 kDa, 10 kDa and, 30 kDa on a Toyopearl Butil-600M. In order to study the mechanisms that can lead to adsorption of unmodified and PEGylated lysozyme to the hydrophobic resin, the analysis of chromatographic retention data will be also essential.

References

Basu, A., Yang, K., Wang, M., 2006. Structure-function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. *Bioconjugate Chem*. 17(3), 618-630.

Bellot, J. C., Condoret, J. S., 1993. Review Modelling of Liquid Chromatography Equilibria. *Process Biochem*. *28*, 365–376.

Benkerroum, N., 2008. Antimicrobial activity of lysozyme with special relevance to milk. *Afr. J. Biotechnol*. *7*(25), 4856–4867.

Berek, D., 2010. Size exclusion chromatography- A blessing and a curse of science and technology of synthetic polymers. *Journal of Separation Science*. *33*, 315–335.

Blaschke, T., Varon, J., Werner, A., Hasse, H., 2011. Microcalorimetric study of the adsorption of PEGylated lysozyme on a strong cation exchange resin. *Journal of Chromatography A*. *1218*, 4720–4726.

Blaschke, T., Werner, A., Hasse, H., 2013. Microcalorimetric study of the adsorption of native and mono-PEGylated bovine serum albumin on anion-exchangers. *Journal of Chromatography A*. *1277*, 58-68.

Bonomo, R. C. F., Minim, L. A., Coimbra, J. S. R., Fontan, R. C. I., Silva, Silva, L. H. M., Minim, V. P. R., 2006. Hydrophobic interaction adsorption of whey proteins: Effect of temperature and salt concentration and thermodynamic analysis. *Journal of Chromatography B*. 844, 6-14.

Bouchemal, K., 2008. New challenges for pharmaceutical formulations and drug delivery systems characterization using isothermal titration calorimetry. *Drug Discovery Today*. *13* (21-22), 960–972.

Bowen, W. R., Pan, L.-C., 1997. Ion Exchange of Bovine Serum Albumin at a Natural Organic Anion Exchanger: Thermodynamics and Energetics. *Journal of Colloid and Interface Science*. *189*, 328–336.

Callewaert, L., Michiels, C. W., 2010. Lysozymes in the animal kingdom. *Journal of Biosciences*. *35*(1), 127–160.

Carta, G., Jungbauer, A., 2010. Protein Chromatography: Process Development and Scale Up. *Wiley-VCH Verlag GmbH & Co. KGaA*.

Cegielska-radziejewska, R., Leśnierowski, G., and Kijowski, J., 2008. Properties application of egg white lysozyme and its modified preparations – a review. *Polish journal of food and nutrition sciences*. *58*(1), 5–10.

Chen, W.-Y., Huang, H.-M., Lin, C.-C., Lin, F.-Y., Chan, Y.-C., 2003. Effect of temperature on hydrophobic interaction between proteins and hydrophobic adsorbents: studies by isothermal titration calorimetry and the van't Hoff equation. *Langmuir*. 19, 9395-9403.

Chen, W.-Y., Hsu, M.-Y., Tsai, C.-W., Chang, Y., Ruaan, R.-C., Kao, W.-H., Huang, E.-W., Chuan, H.,-Y., 2013. Kosmotrope-like Hydration Behaviour of Polyethylene Glycol from Microcalorimetry and Binding Isotherm Measurements. *Langmuir*. 29, 4259-4265.

Chung, W. K., Hou, Y., Freed, A., Holstein, M., Makhatadze, G. I., Cramer, S. M., 2009. Investigation of protein binding affinity and preferred orientations in ion exchange systems using a homologous protein library. *Biotechnology and Bioengineering*. 102(3), 869–81.

Cisneros-Ruiz, M., Mayolo-Deloisa, K., Przybycien, T. M., Rito-Palomares, M., 2009. Separation of PEGylated from unmodified ribonuclease A using sepharose media. *Separation and Purification Technology*. 65(1), 105–109.

Dias-Cabral, A. C., Pinto, N. G., Queiroz, J. A., 2002. Studies on hydrophobic interaction adsorption of bovine serum albumin on polypropylene glycol-sepharose under overload conditions. *Separation science and technology*. 37(7), 1505-1520.

Dias-Cabral, A. C., Queiroz, J. A., Pinto, N. G., 2003. Effect of salts and temperature on the adsorption of bovine serum albumin on polypropylene glycol-Sepharose under linear and overloaded chromatographic conditions. *Journal of Chromatography A*. 1018(2), 137–153.

Diaz, L., Liauw, C. M., Edge, M., Allen, N. S., McMahon, A., Rhodes, N., 2005. Investigation of factors affecting the adsorption of functional molecules onto gel silicas: 1. Flow microcalorimetry and infrared spectroscopy. *Journal of Colloid and Interface Science*. 287, 379-387.

Diogo, M. M., Queiroz, J. A., Monteiro, G. A., Ferreira, G. N. M., Martins, S. A. M., Prazeres, D. M. F., 2000. Purification of a cystic fibrosis plasmid vector for gene therapy using hydrophobic interaction chromatography. *Biotechnol. Bioeng*. 68, 576.

Esquibel-King, M. A., Dias-Cabral, A. C., Queiroz, J. A., Pinto, N. G., 1999. Study of hydrophobic interaction adsorption of bovine serum albumin under overload conditions using flow microcalorimetry. *Journal of chromatography A*. 865, 11-122.

Fee, C. J., Van Alstine, J. M., 2006. PEG-proteins: Reaction engineering and separation issues. *Chemical Engineering Science*. 61(3), 924–939.

Freyer, M. W., Lewis, E. A., 2008. Isothermal titration calorimetry: experimental design, data analysis, and probing macromolecule/ligand binding and kinetic interactions. *Methods in Cell Biology*. 84(07), 79–113.

GE Healthcare, 2006. *Hydrophobic Interaction and Reversed Phase Chromatography*. *Amersham Bioscience*.

Green , A. A. , Hughes , W. L., 1955. Methods in Enzymology. *Academic Press Inc*. 1, 67-90.

Hage, D. S., 1999. Affinity chromatography: a review of clinical applications. *Clinical Chemistry*. 45(5), 593–615.

Haidacher, D., Vailaya, A., Horváth, C., 1996. Temperature effects in hydrophobic interaction chromatography. *Proceedings of the National Academy of Sciences of the United States of America*. 93(6), 2290–5.

Haimer, E., Tscheliessnig, A., Hahn, R., Jungbauer, A., 2007. Hydrophobic interaction chromatography of proteins IV kinetics of protein spreading. J*ournal of chromatography A*. 1139, 84-94.

Harris, J. M., Chess, R. B., 2003. Effect of pegylation on pharmaceuticals. *Nature Reviews, Drug Discovery*. 2(3), 214–21.

Hedhammar, M., Karlström, A. E., and Hober, S., 2006. Chromatographic methods for protein purification. *Royal Institute of Technology*.

Heftmann, E., 2004. Chromatography 6th edition: Fundamentals and applications of chromatography and related differential migration methods, part A Fundamentals and techniques. *Elsevier B.V*.

Hong, P., Koza, S., Bouvier, E. S. P., 2012. Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and their Aggregates. *Journal of Liquid Chromatography and Related Technologies*. 35(20), 2923–2950.

Huang, H., Lin, F., Chen, W., Ruaan, R., 2000. Isothermal Titration Microcalorimetric Studies of the Effect of Temperature on Hydrophobic Interaction between Proteins and Hydrophobic Adsorbents. *Journal of Colloid and Interface Science*. 229(2), 600–606.

Jevsevar, S., Kunstelj, M., Porekar, V. G. 2010. PEGylation of therapeutic proteins. *Biotechnology Journal*. 5(1), 113–28.

Katiyar, A., Thiel, S. W., Guliants, V. V, Pinto, N. G., 2010. Investigation of the mechanism of protein adsorption on ordered mesoporous silica using flow microcalorimetry. *Journal of Chromatography A*. 1217(10), 1583–8.

Kim, J., Desch, R. J., Thiel, S. W., Guliants, V. V, Pinto, N. G., 2011. Energetics of lysozyme adsorption on mesostructured cellular foam silica: effect of salt concentration. *Journal of Chromatography A*. 1218(38), 6697–704.

Korfhagen, J., Dias-Cabral, A. C., Thrash, M. E., 2010. Nonspecific Effects of Ion Exchange and Hydrophobic Interaction Adsorption Processes. *Separation Science and Technology*. 45(14), 2039–2050.

Lantenois, S., Prélot, B., Douillard, J.-M., Szczodrowski, K., Charbonnel, M.-C., 2007. Flow microcalorimetry: Experimental development and application to adsorption of heavy metal cations on silica. *Applied Surface Science*. 253(13), 5807–5813.

Li, W., Zhan, P., De Clercq, E., Lou, H., Liu, X., 2013. Current drug research on PEGylation with small molecular agents. *Progress in Polymer Science*. 38(3-4), 421–444.

Lin, F. Y., Chen, C. S., Chen, W. Y., Yamamoto, S., 2001. Microcalorimetric studies of the interaction mechanisms between proteins and Q-sepharose at pH near the isoelectric point (pI) effects of NaCl concentration, pH value, and temperature. *Journal of Chromatography A*. 912(2), 281–9.

Lund, M., Jungwirth, P., 2008. Patchy proteins, anions and the Hofmeister series. *Journal of Physics: Condensed Matter*. 20(49), 494218 (4pp.).

Mahn, A., 2008. Hydrophobic Interaction Chromatography : Fundamentals and Applications in Biomedical Engineering.*Biomedical science, engineering and technology. In Tech*.

Mayolo-Deloisa, K., González-Valdez, J., Guajardo-Flores, D., Aguilar, O., Benavides, J., Rito-Palomares, M., 2011. Current advances in the non-chromatographic fractionation and characterization of PEGylated proteins. *Journal of Chemical Technology and Biotechnology*. 86(1), 18–25.

Mayolo-Deloisa, K., Lienqueo, M. E., Andrews, B., Rito-Palomares, M., Asenjo, J. a., 2012. Hydrophobic interaction chromatography for purification of monoPEGylated RNase A. *Journal of Chromatography A*. 1242, 11–6.

Mir, M. A., 1977. Lysozyme: a brief review. *Postgraduate Medical Journal*. 53(619), 257–9.

Moosmann, A., Christel, J., Boettinger, H., Mueller, E., 2010. Analytical and preparative separation of PEGylated lysozyme for the characterization of chromatography media. *Journal of Chromatography A*. 1217(2), 209–15.

Müller, E., Josic, D., Schröder, T., Moosmann, A., 2010. Solubility and binding properties of PEGylated lysozyme derivatives with increasing molecular weight on hydrophobic-interaction chromatographic resins. *Journal of Chromatography A*. 1217(28), 4696–703.

Nfor, B. K., Hylkema, N. N., Wiedhaup, K. R., Verhaert, P. D. E. M., van der Wielen, L. A. M., Ottens, M., 2011. High-throughput protein precipitation and hydrophobic interaction chromatography: salt effects and thermodynamic interrelation. *Journal of Chromatography A*. 1218(49), 8958–73.

Pabst, T. M., Buckley, J. J., Ramasubramanyan, N., Hunter, A. K., 2007. Comparison of strong anion-exchangers for the purification of a PEGylated protein. *Journal of Chromatography A*. *1147*(2), 172–82.

Pasut, G., Guiotto, A., Veronese, F., 2004. Protein, peptide and non-peptide drug PEGylation for therapeutic application. *Expert Opinion on Therapeutic Patents*. 14(6), 859–894.

Pasut G., Veronese, F. M., 2007. Polymer-drug conjugation, recent achievements and general strategies. *Prog. Polym. Sci.* 32, 933-961.

Pasut, G., Veronese, F. M., 2012. State of the art in PEGylation: the great versatility achieved after forty years of research. *Journal of Controlled Release : Official Journal of the Controlled Release Society*. 161(2), 461–72.

Perkins, T. W., Mark, D. S., Root, T. W., Lightfoot, E. N., 1997. Protein retention in hydrophobic interaction chromatography: modeling variation with buffer ionic strengh and column hydrophobicity. *Journal of Chromatography A*. 766, 1-14.

Queiroz, J. a, Tomaz, C.T., Cabral, J.M., 2001. Hydrophobic interaction chromatography of proteins. *Journal of biotechnology*, 87(2),143–159.

Roberts, M. J., Bentley, M. D., Harris, J. M., 2002. Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews*. 54(4), 459–76.

Sato, H., 2002. Enzymatic procedure for site-specific pegylation of proteins. *Advanced Drug Delivery Reviews*. 54(4), 487–504.

Seely, J. E., Richey, C. W., 2001. Use of ion-exchange chromatography and hydrophobic interaction chromatography in the preparation and recovery of polyethylene glicol-linked protein. *J. chromatogr. A*. 908, 235-241.

Shaunak, S., Godwin, A., Choi, J.-W., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger,S., Teo, I., Zloh, M., Brocchini, S., 2006. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. *Nature Chemical Biology*. 2(6), 312–313.

Silva, G. L., Marques, F. S., Thrash, M. E., Dias-Cabral, a C., 2014. Enthalpy contributions to adsorption of highly charged lysozyme onto a cation-exchanger under linear and overloaded conditions. *Journal of Chromatography A*. 1352, 46–54.

Staby, A., Johansen, N., Wahlstrøm, H., Mollerup, I., 1998. Comparison of loading capacities of various proteins and peptides in culture medium and in pure state. *Journal of Chromatography A*. 827(2), 311–318.

Stefuca, V., Gemeiner, P., 1999. Investigation of catalytic properties of immobilized enzymes and cells by flow microcalorimetry. *Advances in Biochemical Engineering/biotechnology*. *64*, 69–99.

Thrash, M. E., Pinto, N. G., 2001. Flow microcalorimetric measurements for bovine serum albumin on reversed-phase and anion-exchange supports under overloaded conditions. *Journal of Chromatography A*. *908*(1-2), 293–9.

Thrash, M. E., Pinto, N. G., 2002. Characterization of enthalpic events in overloaded ion-exchange chromatography. *Journal of Chromatography A*. *944*(1-2), 61–8.

Thrash, M.E., Phillips, J.M., Pinto, N.G., 2004. An Analysis of the Interactions of BSA with an Anion-Exchange Surface Under Linear and Non-Linear Conditions. *Adsorpt. Int. Adsorpt. Soc.* 10, 299–307.

TOSOH Bioscience, 2007. Chromatographic Process Media Catalog.

TOSOH Bioscience, Process media products.

Ueberbacher, R., Rodler, A., Hahn, R., Jungbauer, A., 2010. Hydrophobic interaction chromatography of proteins :Thermodynamic analysis of conformational changes. *Journal of Chromatography A.* 1217, 184-190.

Veronese, F. M., 2001. Peptide and protein PEGylation: a Review of problems and solutions. *Biomaterials.* 22, 405-417.

Veronese, F. M., Pasut, G., 2005. PEGylation , successful approach to drug delivery REVIEWS. *Drug discovery today. 10*(21), 1451–1458.

Veronese, F. M., Harris, J. M., 2008. Peptide and protein PEGylation III: advances in chemistry and clinical applications. *Advanced Drug Delivery Reviews. 60*(1), 1–2.

Veronese, F. M., Mero, A., 2008. The impact of PEGylation on biological therapies. *BioDrugs : Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy. 22*(5), 315–329.

Veronese, F. M., 2009. PEGylated Protein Drugs: Basic Science and Clinical Applications. *Birkhauser Verlag.*

Werner, A., Blaschke, T., Hasse, H., 2012. Microcalorimetric study of the adsorption of PEGylated lysozyme and PEG on a mildly hydrophobic resin: influence of ammonium sulfate. *Langmuir : The ACS Journal of Surfaces and Colloids. 28*(31), 11376–11383.

Wettig, S. D., Kamel, A. O., 2013. Thermodynamic Studies of DNA-Cationic Components Interactions Using Titration Calorimetry. *J. Thermodyn. Catal.* 4(1), 1-6.

Wiseman, T., Williston, S., Brandts, J. F., Lin, L. N., 1989. Rapid measurement of binding constants and heats of binding using a new titration calorimeter. *Analytical Biochemistry. 179*(1), 131–137.

Xia, F., Nagrath, D., Cramer, S. M., 2005. Effect of pH changes on water release values in hydrophobic interaction chromatographic systems. *Journal of Chromatography A. 1079*(1-2), 229–235.