UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Development of new chromatographic support based on gellan gum for pharmaceutical biomolecules isolation

Armanda Isabel Carvalho Gonçalves

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

Orientador: Prof. Doutor Luís António Paulino Passarinha
Coorientador: Prof. Doutora Ângela Maria Almeida de Sousa

Covilhã, junho de 2013

Dedicatória

Aos meus ídolos, os meus pais

Resumo

A cromatografia é uma técnica usada para separação e/ou purificação de diferentes biomoléculas com finalidade analítica ou preparativa. Esta técnica tem sido muito aplicada na área da indústria farmacêutica, para a obtenção de proteínas e ácidos nucleicos, entre outras biomoléculas. Com o passar dos anos, a quantidade de amostras e a pureza necessária aumentaram, tornando esta técnica numa das mais usadas na indústria biotecnológica. O desenvolvimento de novas matrizes cromatográficas tem sido um tema de grande importância, a fim de encontrar uma matriz ideal que reúna características como custos associados, eficiente, estável física e quimicamente, com uma elevada eficiência de transferência de massa e reutilizável.

A gelana é um polímero polissacárido natural linear que tem sido aplicado em diversas áreas, como indústria alimentar (agente gelificante e espessante), indústria farmacêutica (entrega direcionada de fármacos), podendo também ser aplicada como um substituinte do agar. Este polímero linear em determinadas condições pode sofrer uma alteração conformacional, formando uma dupla hélice. Na presença de catiões, forma uma rede tridimensional, devido às interações ocorridas entre a gelana, os catiões e as moléculas de água em solução, dando origem a um gel. Tendo em conta estas considerações, o presente trabalho foi realizado com o intuito de formular um gel de gelana estável, a fim de adquirir um comportamento de matriz cromatográfica. A preparação dos géis foi feita de acordo com os seguintes parâmetros: concentração de gelana, concentração de catião (zinco), concentração de DMF, temperatura e tempo de reação. Uma vez que a estabilidade dos géis de gelana é afetada por todos estes parâmetros, foram preparadas várias formulações em que se variou apenas um destes parâmetros para verificar qual o seu efeito na estabilidade do gel. As formulações foram testadas de acordo com as seguintes variações: concentração de gelana (0,75% - 2%), concentração de sulfato de zinco (30 – 120 mM), concentração de DMF (0% - 30%), temperatura (temperatura ambiente – 110ºC) e tempo de reação (0.5 horas – "overnight"). Posteriormente foi aplicada uma estratégia de desenho experimental no sentido de definir as condições ideais para a preparação do gel, e consequentemente obter um gel mais estável. Assim, os melhores resultados quanto à estabilidade do gel foram obtidos quando se usou 0,75% de gelana, 48 mM de sulfato de zinco, 0 % DMF, 25ºC, e 0,5 horas.

Adicionalmente e aproveitando a natureza aniónica do polímero de gelana, foi possível explorar diferentes interações com as seguintes proteínas modelo (albumina sérica bovina, α-quimotripsina e lisozima). Na presença do tampão MÊS com pH 6,2, a albumina sérica bovina encontrava-se com carga negativa, enquanto que a α-quimotripsina e lisozima se encontravam com carga positiva, tendo em conta os respetivos pontos isoelétricos. Sendo assim, a albumina sérica bovina não interagiu com a matriz, visto que ambas têm carga

negativa. Pelo contrário, a α-quimotripsina e lisozima ligaram-se à matriz, devido à oposição de cargas. A separação das três proteínas foi conseguida através da eluição por passos, com o aumento gradual da concentração de NaCl no tampão de eluição. A albumina sérica bovina foi a primeira proteína a eluir após a aplicação da amostra, ainda com a passagem do tampão de eluição sem sal. Posteriormente aumentou-se a concentração de sal promovendo-se a eluição da α-quimotripsina . Por fim, para concentrações de sal mais elevadas, e a lisozima que promoveu uma interação mais forte com a matriz de gelana acabou por eluir também. A fim de melhor caracterizar esta nova matriz, foi determinada também a capacidade dinâmica de ligação utilizando uma estratégia de saturação da coluna com uma solução de lisozima 0,05 mg/mL. Os valores obtidos para a capacidade dinâmica de ligação da gelana a 10% e a 50% foram 3,9 mg/ml e 17,4 mg/ml, respetivamente. Comparando com outras matrizes cromatográficas, os valores de capacidade dinâmica de ligação da matriz de gelana estão dentro do esperado. Estes estudos iniciais permitiram concluir que a gelana interagiu com as proteínas, considerando a diferença de cargas, e que a sua eluição também foi possível com o aumento de sal, o que revela uma provável potencialidade como matriz cromatográfica de troca catiónica. Para poder afirmar e provar que o polímero gelana pode constituir uma matriz cromatográfica, devem ser feitos mais ensaios. Nomeadamente testar a purificação de amostras mais complexas e determinar mais parâmetros (como por exemplo a capacidade iónica e o tipo e tamanho de poros) que permitam a otimização da matriz. No futuro, poderemos vir a ter uma matriz versátil com aplicação em diversos domínios científicos.

Palavras-chave

Cromatografia; Matriz; Polímero de gelana; Proteínas; Troca iónica

Abstract

Higher separation efficiency and resolution have been of great interest in chromatography and have become increasingly important in recent years mainly driven by the challenges of either more complex samples or high sample quantity. Therefore, for the development of new chromatographic matrices is increasingly important to improve the purification efficiency and to decrease the use of resources. Gellan gum is a polysaccharide polymer with natural anionic nature and ability to form thermoreversible gels, seeming to have potential to be used as a chromatographic matrix. In the presence of cations, the gellan polymer suffers conformational transition accompanied by the formation of a three dimensional network, forming a gel. In this work, it was intended to prepare of a stable gellan gum gel to be used as a chromatographic matrix. In order to increase the stability of the gellan gels, different experimental conditions were tested. Experimental design was used to obtain optimal conditions for the gel stability. Due to negative charge of these gels, it was possible to study the interactions established with three model proteins (bovine seric albumin (BSA), α-chymotrypsin and lysozyme). Gellan gum was able to interact with two of these proteins, being able to elute them with an increase in the ionic strength. In this assays a MES buffer with pH 6.2 was utilized. This pH conferred negative charge to BSA and positive charge to α-chymotrypsin and lysozyme, due to their isoelectric points. Assays of dynamic binding capacity were performed to find more characteristics of this new matrix and comparing with commercial resins. The values of dynamic binding capacity of the gellan gum to 10% and 50% breakthrough were 3,9 mg/ml and 17,4 mg/ml, respectively. These values were similar to commercial resins. These results showed that gellan gum might be an innovative and promising chromatographic matrix due to its versatility to interact with different biomolecules and its gelling ability. Thus, the gellan gum gel could have potential to be applied in different scientific domains (purification of complexes cellular extracts or nucleic acids).

Keywords

Chromatography; Gellan gum; Ion exchange; Matrix; Proteins

Contents

List of Figures

List of Tables

List of Acronyms

AC	Affinity chromatography
ANN	Artificial neural network
ANX	Diethylaminopropyl
BSA	Bovine serum albumin
CM	Carboxymethyl
CVs	Column volumes
DEAE	Diethylaminoethyl
DMF	N,N-Dimethylformamide
FDA	Food and Drug Administration
HIC	Hydrophobic Interaction Chromatography
IEC	Ion Exchange Chromatography
IMAC	Immobilized Metal Ion Affinity Chromatography
MES	4-Morpholineethanesulfonic acid
NaCl	Sodium chloride
$NiSO_4$	Nickel sulfate
PAGE	Polyacrylamide Gel Electrophoresis
PSA	Ammonium persulfate
Q	Quaternary ammonium
RPC	Reversed Phase Chromatography
S	Methyl sulfonate
SDS	Sodium dodecyl sulfate
SEC	Size Exclusion Chromatography
SP	Sulfopropyl
TDP	Thymine-5-diphosphate
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine-5-diphosphate
USA	United States of America
$ZnSO_4$	Zinc sulfate

Chapter I - Introduction

1. Chromatography

Protein purification has been performed for more than 200 years. Until the beginning of the 20th century, the only available separation technologies were filtration, precipitation, and crystallization methods. In 1906 the botanist Mikhail Tswett introduced the term chromatography, describing his work on separation of plant pigments on a column of calcium carbonate [1, 2]. In a few years, the purification of other biomolecules, such as proteins, nucleic acids, antibodies, was possible [3, 4].

Nowadays, most purification schemes involve some form of chromatography. As a result, chromatography has become an indispensable tool in the separation and purification of biological substances in every laboratory, due to its simplicity, robustness, versatility, and high reproducibility [5, 6].

The aim of a purification process is not only removal of unwanted contaminants, but also to obtain and maintain quality, stability and biological activity of biomolecules. The purity required depends on the science area in which the biomolecule will be applied. If the application consists on pharmaceuticals, the biomolecule must have a high purity degree, in contrast to biomolecules applied in laundry detergents where the purity degree is inferior [7, 8, 9].

Chromatography has become a preferential technique for separation, due to its high resolving power, the existence of several chromatographic methods with different selectivity and its applicability to an extensive spectrum of compounds [10, 11].

1.1 Chromatographic process

Chromatography is a method used to separate components of a sample, taking into account the physical and chemical properties of each biomolecule [10]. Charge, molecular size, hydrophobicity and polarity determine the type of interaction between the solute in a mobile phase with a stationary phase [7].

Solutes can stay in the mobile phase or be distributed in the stationary phase by promoting specific interactions. Stationary phase is packed into a vertical column and should be insoluble in the buffer. Mobile phase is pumped through the column and it can be liquid or gaseous [2].

Separation of the different sample solutes can occur when they interact with different intensities on the support, running the column with different times. This differential separation occurs due to continuous addition of mobile phase and, which is known as elution [10, 12]. Differential elution of several biomolecules causes a sequence of separated peaks. These peaks reflect the concentration of biomolecules versus time or volume of eluent at the column exit, which is typically represented in a chromatogram [2].

The chromatographic process consists in five steps: equilibrium phase, loading of sample, washing and removal of unbound material, elution of the biomolecule and regeneration/cleaning matrix [12]. Equilibrium phase involves the adjustment of the mobile and stationary phase to optimal binding conditions of solutes. The sample loading corresponds to injection of a specific quantity protein solution in the column, depending of the sample origin and if the process has an analytical or preparative objective. In washing step, unbound material components that remained in the mobile phase are removed, because did not interact with the stationary phase [12, 13]. The elution of retained species corresponds to desorption of desired compound by stepwise or continuous change of mobile phase composition. Elution buffer decreases interactions between the matrix and target biomolecules [2, 12].

The target biomolecule that strongly adsorbed to the stationary phase elute slower than the weakly bound biomolecules, and the application of significant alterations in the mobile phase is necessary [14]. Alterations on ionic strength (type and salt concentration), pH and competitive agent addition are the most common means of eluting adsorbed proteins by linear gradient or stepwise gradient [5, 8, 15].

In linear gradient elution, the mobile phase composition is continuous changing for conditions that favor the dissociation of the protein and thus, the solute interaction with the support is variable. This elution form improves the separation and it is normally used when the sample is unknown. The eluent composition is changed step by step in stepwise gradient and various substances can be eluted with this strategy [15]. Stepwise elution is frequently favored for routine, large-scale purifications and additionally the buffer consumption is reduced [1, 16]. Alternatively, the composition of the eluent is selected to give weak or no existent interactions between sample components and chromatographic matrix, and these conditions are kept unchanged during binding and elution. The target biomolecule passes through the column slower or faster than impurities, resulting in different elution positions. This type of elution is known by isocratic elution where in composition of the mobile phase and the solute interaction with the support are constant [1, 16]. The gradient elution allows the separation of components with a wider range of properties when compared with isocratic elution [1].

The regeneration and cleaning matrix step is important, because it permits to reuse the matrix for new chromatographic assays [1]. In this step, sticky impurities such as endotoxins

and lipids are removed to maintain the native matrix characteristics such as selectivity and support life-time [9, 12]. A dirty medium may also induce decreased binding capacity and increased pressure [1]. Cleaning procedure depends on matrix type, but normally it is made with low or high-salt concentrated solutions, highly acid or basic conditions, organic solvents or some detergents. This process also avoids microbial contamination during storage [13].

The purification of the biomolecule of interest may happen by two different processes. The biomolecule itself can be separated from contaminants by binding to the stationary phase, followed by selective elution; or by binding impurities, allowing the target biomolecule to pass through the column without being retained (designated as negative chromatography) [1].

1.2 Preparative versus analytical chromatography

The product amount, purity degree and biomolecule stability are important requirements for choosing the type of chromatography [12].

Chromatography can be used for preparative or analytical scale. Liquid chromatography (mobile phase is a liquid) is the most used for preparative scale. Gas chromatography (mobile phase is a gas) is an indispensable tool to the analytical level [17–19].

In the last years, chromatographic stationary phase materials based on new concepts where developed to increase speed at both analytical and preparative scale [20].

Preparative liquid chromatography has become the primary tool for the purification of proteins from complex biological mixtures in biotechnological processes. In preparative chromatography, the aim is to separate very similar compounds, requiring high column efficiencies [16, 18]. Preparative separations require the introduction of larger amounts of sample onto the chromatographic column. Sample dissolution in the same solvents as used for elution is ideal to minimize peak broadening and distortion, because the higher the solubility, the higher the efficiency of the preparative productivities [21].

Preparative chromatography is the most tool used in the biotechnological industry, therefore it is used on a large scale, producing sometimes kg or even tons of the target biomolecule [20]. For this reason, large diameter columns and gels very porous to increase the binding capacity are necessary to use. Diffusion pathways for the proteins are relatively long, therefore low flow velocities are required to improve the resolution [22]. Preparative chromatography is becoming a well-established separation and purification method including chiral separations, extraction of recombinant proteins from fermentation broths and purification of basic compounds [17].

Whether the amount of purified compound isolated is going to be used of sale as a reagent or a pharmaceutical, among them the need to produce as concentrated a fraction as possible, to collect and transfer without impurities, and to do the separation as quickly and cheaply as

possible. As it is a large-scale process, the buffer should be cheap, because it will be used in large quantities [1, 17]. Separation costs account for 50-80% of total production costs and chromatography is often the most expensive unit process in a separation. Due to this fact it is important to develop more cost efficient chromatographic systems, to become the purification technique more economically viable and reproductive for to large-scale-up process [9].

Preparative chromatography is a powerful separation technique, but it is often associated with significant dilution of the product. Other problem is the gradient, for the typical large-diameter preparative columns linear gradients are not a good choice. Thus, step gradients are more popular in preparative elution chromatography or displacement elution [2].

Separation power of preparative chromatography attracts the attention of those interested in producing pure chemicals in large amounts for a variety of purposes. Once produced, the identity, purity, potency, and safety of biopharmaceutical compounds must be demonstrated to the regulatory agencies before they can be used in humans, avoiding unwanted side effects. This aim may require the use of simple detectors (such as UV or FID) or coupling with the most complex detectors (such as multiple MS or high field strength NMR). From the moment required signals have been acquired for a detector, the chemical can be discarded [9, 24].

Analytical chromatography permits to identify and quantify the components of mixtures either simple or complex. In analytical chromatography, a small sample is applied to the column and maximum resolution is sought, by manipulating the characteristics of the different phases of the system [12, 17]. Preparative column loads more protein in three or four preparative cycles than an analytical column loads in two or three hundred cycles [9]. In analytical chromatography high flow velocities and cycle times of a few seconds are reported [24].

The goal of analytical chromatography remains to be the rapid determination of the structure of the component, through the direct acquisition of the proper information, and the calculation of its concentration, through calibration of the detector signal [17]. In this type of chromatography it is required a high selectivity and sensitivity, because the purification conditions may create very similar populations of proteins with structural variants (such as deamidated, amino acid residues oxidized and cleavage fragments of a single polypeptide) and their analysis becomes more difficult [24, 25]. Lately, development of complex instruments and the coupling two or several columns, have been applied to solve analytical or preparative separation problems [17].

1.3 Chromatographic equipment

Protein purification is always a race against time and there is a need to accelerate the procedure, because during the isolation step proteins are exposed to changes in environment, enzymatic degradation by proteases, or even time itself, leading to protein losses and reduction of biological activity. The separation process is fast to increase the yield of the target protein and helping to improve the cost effectiveness of the production process is essential [2, 22].

The combination of hardware, chemicals, operation mode, sample volume, purity, amount of purified protein required, presence of toxic components, number of samples to be processed, are determinant to choose of the purification format [1]. The principal purification format is based on chromatography in closed columns (chromatography system or an independent pump) or in open columns (gravity flow columns or spin columns) used in manual purification. A chromatographic system should be used when reproducible results are important and when the manual purification becomes more slow and inefficient [1].

The purification equipment has evolved into hardware and software that impart a high level of control over the process. These automated systems not only decrease labor costs, but perhaps more importantly contribute to the reproducibility of operation and recording [2]. Automatic systems provide more control than manual purification because it permits to control the flow rate, monitor the progress of the purification and allows automatic collection of narrow peaks as well as to make controlled gradients. These systems can perform simple step-gradient elution as well as high-resolution separations using accurately controlled linear-gradient elution. Although proteins may be separated using a variety of matrices, the basic components of a chromatographic system are similar [1, 15].

Usually the typical components of a chromatography system are pump, injection system, column, detector, recorder and collector, (Figure 1). The pump allows a constant flow during the process and variable pressure. Injection system is responsible for the injection of the sample into the column. The packaging is made of the stationary phase in the column where the separation occurs. Depending on the process in which it is employed, it can have different lengths, degree of chemical resistance and mechanical strength [2].

The detector allows the measurement of UV absorbance, fluorescence, conductivity, radioactivity or optical density of the output eluent. The recorder allows to record the chromatograms on paper or digitally. Finally the collector enables automatic collection of output eluent fractions according to the recorder [2, 14, 15]. This type of chromatographic systems designed for analysis contain narrow tubing in order to give optimum performance with high resolution columns [1].

Equipment and materials of construction should be nonreactive with mobile phase. System components should be able to pass a rigorous cleaning and validation test. In order to keep the equipment and consequently the chromatographic process in a controlled or validated state, regular preventive maintenance and calibration procedures must be made [2].

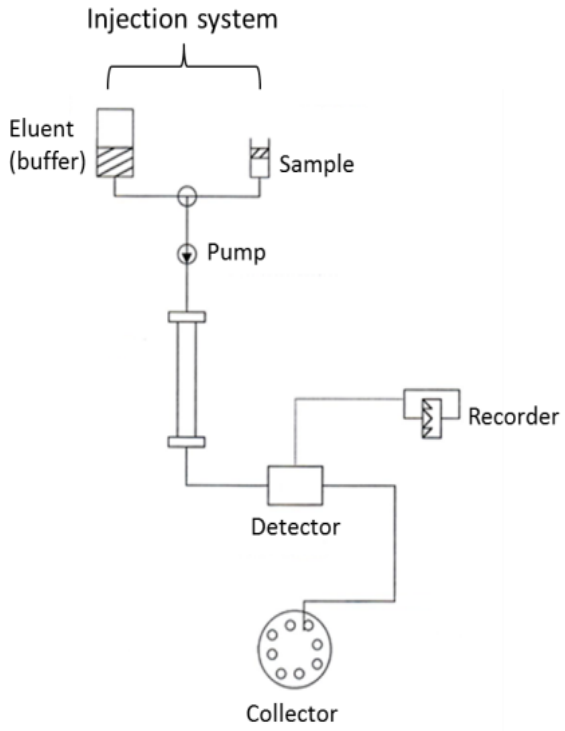

Figure 1 - Schematic representation of the low pressure chromatographic equipment adapted from [1].

1.4 Chromatographic methods

Depending on characteristics of target biomolecules several chromatographic methods can be used, which in turn explore different interactions between the support and these molecules [6]. Therefore, the interaction established with the target biomolecule is normally determined by the chemical composition of the chromatographic resins, being the objective to retain the biomolecule and to elute the contaminants [26, 27].

Basically, there are two mechanisms used for chromatographic separation of proteins: adsorption (separation proteins occurs according to some property that provides interaction to certain matrices) and molecular filter chromatography (retardation from proteins occurs without this property) [2, 5]. On the other hand, based on properties of the target biomolecule such as size, charge, hydrophobicity, polarity or affinity, different chromatographic methods can be applied in purification.

1.4.1 Size Exclusion Chromatography (SEC)

This type of chromatography is sometimes also referred as gel filtration, molecular sieve chromatography or gel-permeation chromatography [8]. SEC matrices consist on a variety of beads with a size pores similar to the target proteins. Thus, the pore size of the gel can be adjusted to delete or retain all molecules above a certain size [8, 28].

This technique is simple to use, because separation occurs according to molecular weight and conformation, and it is achieved by the differential exclusion or inclusion within porous particles [29]. The largest proteins cannot penetrate in the channels beads because the pores are too small, so they flow quickly around the external of the beads and elute out first. The smallest proteins are able to penetrate into the pores in the beads and thus get isolated temporarily after a while, they elute out. The gel beads have a range of pore size, so that intermediate sized proteins can spend some time inside in the beads, but not as much as the smallest proteins. Therefore, proteins are eluted in order of decreasing size and since proteins differ in size, gel filtration can be used to purify a target protein [2, 28, 30].

Gels may be formed from natural polymers (such as agarose or dextran) or synthetic polymers (such as polyacrylamide) [31]. A crosslinking process is applied to these polymers to form a three-dimensional network and the degree of crosslinking will define the pore size. Nowadays, many gels are commercially available in a broad range of porosities [8, 32].

Since molecules are not adsorbed but only retarded, proteins are isocratically eluted, which means a single buffer is used throughout the separation process. However, some proteins may exhibit ionic or hydrophobic interactions with the matrix resulting in slower elution or retention in the column [5]. In these cases, buffer conditions can be varied to suit the sample type, increase purification or analysis and maintain biological activity of proteins. Indeed, factors such as polarity, pH and ionic strength of mobile phase may have influence on their elution behavior [14, 30].

The main limitations of gel filtration are the low matrix capacity that allows only small sample volumes, viscosity effects and largest dilution of the sample. So, maximum resolution can be obtained with sample volumes of 0.5% to 2% of the total column volume, however, up to 5% may give acceptable separation [32, 33]. Concentrations above 70 mg/mL should be avoided, because viscosity effects may cause severe band broadening and consequently, reduces the resolution. To avoid these effects and increase the resolution, several parameters can be manipulated such as increase retention time, column length, decrease the flow rate and use beads of different sizes [1, 31]. When lower resolution is obtained, a pair of proteins with a molecular mass difference of 500 Da cannot be distinguished. However, identify the presence of aggregates, and investigating protein folding can be employed [28].

This technique is ideal for final polishing steps in purification when sample volumes have been reduced, for desalination, buffer exchange and it also allows to determinate molecular mass of proteins [5, 32].

1.4.2 Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interactions are involved in the biological systems, namely they are responsible by structure stabilization, antibody-antigen reactions, enzyme-substrate reactions, protein folding and self-association of phospholipids and other lipids to form the bilayer of the cellular membrane [7].

HIC allows the biomolecule separation, under relatively soft conditions, according to differences in their hydrophobicity [7, 33]. The degree of hydrophobicity of a protein depends on the amount and position of hydrophobic amino acids. Isoleucine, valine, leucine and phenylalanine are examples of hydrophobic amino acids and they have side chains without active groups for formation of hydrogen-bonds with water [33]. Therefore, the separation occurs due to hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the protein surface [7, 34]. These interactions are reversible and caused by Van del Waals forces, the most important hydrophobic interaction [35].

The adsorption of biomolecules to the matrix increases with high salt concentration in the mobile phase and the elution occurs by decreasing the salt concentration of the eluent [7, 34]. When proteins with nonpolar side chains are dissolved in water, the water molecules present an extremely orderly form. In this process there is a decrease in entropy, energetically unfavorable, consequently it does not occur spontaneously. In the presence of salt, there is an increase in entropy, due to displacement of the ordered water molecules around the hydrophobic groups and the interaction occurs. This process is energetically favorable, consequently occurs spontaneously. All this interactions are mainly determined by the change in entropy [2, 7].

Anions and cations have influence in hydrophobic interactions and they can be sorted according Hofmeister (lyotropic) series. Starting with anions that highly favor the interaction to those that will reduce hydrophobic forces are: PO_4^{3-}, $SO_4^{2-,}$ CH_3COO^-, Cl^-, Br^-, NO_3^-,ClO_4^-, I^- and SCN^-. The cations are: NH_4^+, Rb^+, K^+, Na^+, Li^+, Mg^{2+}, Ca^{2+} and Ba^{2+}. The ions at the beginning of this series are called cosmotropes or antichaotropic and they promote hydrophobic interactions. The ions at the final of this series are called chaotropic, because they decrease the strength of hydrophobic interactions [2, 7]. The salt increment can affect the surface tension of water, resulting on an increase in the strength of interaction between proteins and the matrix. But specific interactions between the protein and the salt may change the protein structure and the protein hydration and counteract these effects [7, 8].

The elution is usually performed by continuous decreasing of salt gradient. Sometimes, step-wise elution is preferred in large scale preparative applications since it is technically more simple and reproducible than gradient elution [36]. Proteins elute based on their different hydrophobicities, according to the increasing hydrophobicity [7, 14]. From theoretical calculations, ammonium sulfate is the best salt for the protein retention and it is applied in high-ionic-strength solution, but the used salt concentration must be kept below to the concentration that precipitates any protein in the sample [28]. In some cases the binding is too strong, therefore it is needed to add a decrease of the solvent polarity (such as ethylene glycol), detergents, organic solvents or chaotropic agents (such as urea, guanidine hydrochloride) [1].

Generally, the increase of temperature enhances protein retention and the decrease of temperature generally promotes the protein elution. The pH of buffers has a decisive influence on the protein adsorption. An increase in the pH value up to 9-10 decreases the hydrophobic interaction, due to the increased hydrophilicity promoted by the change in the charge of the protein. All these factors require a special attention because they can lead to protein denaturation [8, 28].

In general, HIC can be used for capture, intermediate to other techniques or polishing steps in a purification protocol [34]. This is a powerful bioseparation technique, since it allows the protein separation that differ in one amino acid residue, separating a native protein from incorrectly folded forms and the amount of recovered biomolecule is high. Therefore, at laboratorial and industrial scales, the biomolecule purification such as serum proteins, nuclear proteins, recombinant proteins, membrane proteins, enzymes and hormones is extremely used [35, 37].

1.4.3 Reversed Phase Chromatography (RPC)

Reversed Phase Chromatography is very similar to HIC, because both are based upon interactions between hydrophobic surfaces of biomolecules and the hydrophobic chromatographic matrix. However, the surface of a RPC medium is usually more hydrophobic than a HIC medium and the eluents are also different [34].

A typical biological sample contains a complex mixture of molecules. Some of these molecules can be sufficiently hydrophobic to bind strongly in the hydrophobic matrix, mainly proteins, peptides and oligonucleotides [35]. The sample is applied under conditions that favor binding, typically using an aqueous solution and a low concentration (3-5%) of organic solvent. Sometimes, an ion-pairing agent, such as trifluoroacetic acid (TFA), to enhance the hydrophobic interactions is used [8, 34].

As the binding between the biomolecule and the matrix is very strong, the application of organic solvents to elute the biomolecules is necessary [30, 39]. The organic solvent

decreases the polarity, causing elution. A large variety of organic solvents can be used, but the two most widely used are acetonitrile and methanol. The protein retention decreases according to following series of solvent modifiers: methanol, ethanol, acetonitrile and isopropanol. The use of isopropanol is limited due to its high viscosity that causes a decrease of column efficiency and an increase of pressure. Consequently, acetonitrile becomes the more used organic solvent. Other reason to use this organic solvent is the fact it promote much lower background absorbance at low wavelengths. This characteristic is important since the column elution is normally monitored by UV detectors [1, 8, 30].

However, the use of organic solvents has also disadvantages. They are explosive and flammable by nature, have an intense odor and its recycling has to be proper [28]. Normally, the addition of an organic solvent to the eluent leads to the protein denaturation. This phenomenon is a consequence of the disruption of the hydrophobic interactions between nonpolar side chains in the protein and disruption of the hydrogen bindings, destroying their three-dimensional structure. Thus, RPC is not recommended for protein purification if the recovery of activity and refolding to tertiary structure are required, but it is possible to use for to determination of protein primary structure [1, 34, 39]. In contrast, for proteins with molecular weight lower than 30 KDa, denaturation effects are often minimal or rapidly reversible, and they can be isolate in a biologically active form [8].

The elution is usually done by decreasing the polarity of the mobile phase and the separations frequently use elution gradients in order to minimize the run time. However, if there is a large difference in hydrophobicity between the separating proteins a step elution can be used [30]. Samples are eluted in order of increasing hydrophobicity or in order of decreasing polarity [34]. The nature of the stationary phase most commonly used in RPC consists in porous silica beads with modified groups and synthetic organic polymers, such as beaded polystyrene [34, 38].

Due to the high resolving power, RPC is an extremely useful tool for final polishing of oligonucleotides and peptides. This method is also important for analytical separations requiring high selectivity and for the separation, purification and analysis of polypeptides and small proteolytic fragments such as peptide mapping [5, 34]. RPC has been applied in large-scale purification of recombinant proteins and synthetic peptides (insulin and growth hormone), which were obtained with a high chemical purity and biological activity [28].

1.4.4 Affinity Chromatography (AC)

Affinity Chromatography can include all types of interactions that occur in adsorption chromatographic techniques. However, interactions between biomolecules that interact in natural binding sites are much more significant [37]. The solute retention in this method is based on the same types of specific and reversible interactions established in biological

systems such as antibodies-antigens, hormone-receptor, enzyme-inhibitor or inhibitor-drug or other compounds with serum proteins [40, 41].

Ligand-protein interactions are often based on electrostatic interactions, hydrophobic and hydrogen bonds [2]. AC separates proteins on the basis of a specific and reversible interaction between the target protein and a specific ligand covalently attached to the chromatographic matrix [34, 41]. AC ligands can be of biological or non-biological origin such as metal ion complexes and synthetic dyes [37]. The sample of interest is applied in the affinity matrix under conditions that favor the specific binding to the ligand and unbounded biomolecules are washed away, staying only the target protein retained [38].

The choice of elution conditions depends on the nature and strength of the ligand-protein interaction. The elution is undertaken using a competitive ligand or by changing pH, ionic strength or polarity the buffer [1, 41]. Competitive ligand can bind either to the ligand or to the target molecule. The competitor binds to the matrix-bound ligand, by having a higher affinity or being in a higher concentration, and thereafter the competitor replaces the target molecule that is then eluted. On the other hand, if the target molecule forms a stronger binding with the free ligand, it will desorb from the matrix-bound ligand and elute together with the free ligand [8]. When the elution is due to pH change, a change in the state of ionization of ligand groups occurs and the interaction with target molecule weakens. If the interaction between ligand and biomolecule is constituted predominantly by electrostatic interactions, an increase on the ionic strength of the buffer is necessary for elution of the bounded proteins. Finally, in some cases it is necessary to reduce the polarity or include a chaotropic salt in the buffer. This elution type is typical when the binding is dominated by strong hydrophobic interactions. However, this elution method can lead to protein denaturation [5, 8, 41].

AC is ideal for a capture or intermediate step, because it offers high selectivity, resolution and usually high capacity for the protein of interest. So, this technique has been used in pharmaceutical science and biotechnology for purification of enzymes, immunoglobulins, glycoconjugates, nucleotides and cell fragments [5, 38]. Some advantages of this method are the reutilization the same ligand preparation for multiple experiments, and the relatively short periods of time required [37]. The nature of the chromatographic support is usually constituted by agarose, dextran, cellulose, silica, polystyrene and polyacrylamide [40, 41].

1.4.4.1 Immobilized metal ion affinity chromatography (IMAC)

IMAC is a specific type of affinity chromatography. This technique is based on the interaction between electron donor species present on the surface of biomolecules and metal ions immobilized via a chelating ligand [1, 42]. Generally, the most used ions are $Fe3^{+}$, $Co2^{+}$, $Ni2^{+}$, $Cu2^{+}$, Zn^{2+}, Al^{3+} and Ca^{2+} due to their acidic character. These ions after being chelated form

reversible bonds with certain amino acid residues such as imidazole of histidine, thiol of cysteine and indole of tryptophan [42, 43].

However, many proteins do not have these electron donors. Nonetheless, due to recombinant DNA technology, it is possible to incorporate tails (tag) into proteins, which do not naturally contain electron donor species, making this separation process possible [42, 43]. Usually, the elution of bound proteins is performed using a competitive ligand, the imidazole [1, 42].

1.4.5 Ion exchange chromatography (IEC)

IEC separates biomolecules according to differences in their surface charge. The set of all charged side chains will give the protein surface charge [28]. The separation is based on the reversible interaction between protein surface charges and oppositely charged groups on the matrix [28]. Proteins vary considerably in their charge properties, depending on ionizable amino acid residues on their structures; proteins can have both positive and negative charges. Charged groups within a molecule possess different pKa values depending on the pH buffer [44, 45].

The protein isoelectric point corresponds to the pH at which the net charge is zero, and under this condition the protein will not interact with a charged medium. When the buffer pH is higher than the isoelectric point, the protein acquires negative net charge and will bind to a positively charged medium. However, if the buffer pH is lower than of the protein point isoelectric, the protein acquires positive net charge and will interact to a negatively charged medium [33, 45–47]. Therefore, if the matrix is negatively charged it is being applied cation exchange chromatography, whereas, when the matrix has a positive charge it corresponds to anion exchange chromatography [45].

Proteins with the same charge as the resin will pass through the column without adsorbing while proteins with the opposite charge will bind. Normally, in anion exchange chromatography, pH values above the isoelectric point of the protein of interest are used. On the contrary, cation exchange chromatography is carried out with pH values below the protein isoelectric point. Nonetheless, pH values to be used are limited by the pH range in which the protein is stable and optimal pH is the one that confers the largest charge difference as possible between the target protein and contaminants [5, 48].

Target protein elution occurs due to the change of the buffer pH or by increasing the ionic strength. When the ionic strength is increased, salt ions compete with bound biomolecules for charged groups on the matrix surface and they begin to elute. When the buffer pH is changed, the net charge of proteins is equal to the matrix charge and there is no longer interaction between both molecules. The elution normally takes place under mild conditions, so that the protein can maintain its native conformation during the chromatographic process [28, 44, 49].

IEC is a methodology that provides high resolution, high sample loading capacity and ability to separate molecular species that have only minor differences in their charge properties, for example two proteins differing by one charged amino acid [48, 49]. Nevertheless, this method has a incompatibility with mass spectrometry, especially in case of ionization mode, because the ionization of proteins and peptides are severely perturbed by ions [41]. This type of chromatography is adequate for capture, intermediate purification or polishing steps and it is used to microscale analysis or in large scale production of proteins [46]. Table 1 summarizes the main characteristics of the chromatographic methods referred above.

Table 1 - Characteristics and applications of several chromatographic methods, adapted from [1].

Method	Protein property	Typical characteristics		Purification phase			Sample start Conditions	Sample end conditions
		Resolution	Capacity	Capture	Intermediate	Polishing		
AC	Specific ligand recognition (biospecific or nonbiospecific)	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
IMAC	Metal binding	+++	++	+++	++	+	For purifying histidinetagged proteins using Ni Sepharose columns: 20-40 mM imidazole; pH > 7; 500 mM NaCl; no chelators Other proteins: low concentration of imidazole	High concentration of imidazole, pH > 7, 500 mM NaCl
GF	Size	++	+	+		+++	Most conditions acceptable, limited sample volume	Buffer exchange possible, diluted sample
IEX	Charge	+++	+++	+++	+++	+++	Low ionic strength. pH depends on protein and IEX type	High ionic strength or pH changed
HIC	Hydrophobicity	+++	++	++	+++	+++	High ionic strength, addition of salt required	Low ionic strength
RPC		+++	++		+	++	Ion-pair reagents and organic modifiers may be required	Organic solvents (risk for loss of biological activity)

1.5 Chromatographic applications

Chromatography is probably the most powerful technique to achieve high levels of biomolecule purification. Peptides, native proteins and recombinant proteins produced by bacteria, fungi, yeasts, animal cells, insect cells and plants, as well as nucleic acids (DNA and RNA) have been purified by different chromatographic methods which reflect the use of various chromatographic matrices [20, 42]. The ideal chromatographic technique for separating different proteins from a mixture depends on the most relevant properties and nature of proteins. The application of different chromatographic types within the same process is possible [1].

Nowadays, chromatography is established in the biotechnologic industry as a productive or an analytical tool. Thus, the protein purification is now performed in scales from micrograms and milligrams in research laboratories or kilograms and tonnes in industrial settings [9]. Requirements of a large-scale purification protocol are principally determined by the nature and quality of the desired final product and its intended use. Therefore, proteins for therapeutic use need to be extremely pure to minimize the risk of unwanted side effects or immunogenic response. Distinctly, materials to be used in industrial processes (for example laundry detergents) do not need always to be absolutely pure. Therefore, parameters such as purity, biological activity, necessary amount, costs and time frame for the work should be considered before this process implement [9].

According to Passarinha and coworkers, an example of protein purification through HIC is the recombinant human soluble catechol-*O*-methyltransferase purification [47]. This protein is involved in the biotransformation and detoxification of many endogenous and xenobiotic compounds [47]. Sousa and coworkers applied affinity chromatography as other purification methodology of the plasmid DNA for the development of gene therapy and DNA vaccine [4].

2. Matrices

2.1 Matrices and ligands used in chromatography

Over the years, it is increasingly relevant to increase the resolution, selectivity, efficiency and speed of the chromatographic process, because samples complexity and quantity have also increased. So, complex samples separation and to maintain satisfactory recovery yields is necessary to development new chromatographic supports [6, 23].

An ideal chromatographic support for successful application in chromatography should possess the following properties: solid, macroporous, inert, uniform, hydrophilic, nontoxic, incompressible, cheap, simple to use, chemical, physically stable and insoluble in the solvent. Furthermore, other characteristic should be considered such as to present high binding

capacity and mass transfer, keep good flow properties throughout the process, exhibit low nonspecific adsorption, promote stable immobilization of the ligand which should be reusable in various chromatographic runs and allow regeneration with extreme conditions [40, 51–53].

2.1.1 Chemical nature of the chromatographic supports

Normally, the chemical nature of proteins determines surface properties of the chromatographic medium, while the physical properties are determined by its size. Constitutive materials of the matrix can be classified as natural polymer, synthetic polymer, inorganic material and composite material [6, 54].

Natural polymers include agarose [52], cellulose [53], nitrocellulose [54] and dextran [55]. They are highly hydrophilic due to the high amount of hydroxyl groups in the polymer chain. In some cases, natural polymers have poor mass transfer properties and limited stabilities at high flow rates, because a high pressure causes the compression of the chromatographic medium. To counteract this effect and increase the stability, the materials have to be crosslinked with ion-exchange or hydrophobic groups [50].

Polyacrylamide derivatives, polymethacrylate and polystyrene are synthetic polymers. Due to the hydrophobic character of these polymers, their surface area should be coated with hydrophilic molecules in order to make possible a reversible interaction with target biomolecule [6, 54].

Hydroxyapatite, silica and glass are examples of inorganic materials used in chromatography. Unlike hydroxyapatite, the glass does not present good selectivity but has excellent flow and mass transfer properties due to its rigid and porous structure. In the case of silica, it is necessary inactivate the residual Si-OH groups, because they can interact with biomolecules. For this case, silica can be coated with natural or synthetic polymers also resulting in a good selectivity [55, 56].

One cryogel polymerized into a porous skeleton of methacrylate, silica or zirconium is considered a composite material and it can be operated at high speeds without losing its binding capacity. This material allows the direct capture of proteins from the supernatant of cell cultures, without additional clarification steps [57, 58].

2.1.2 Physical nature of the chromatographic supports

Packed particles can be non-porous or porous. Nonporous particles allow quick separation and analysis with high efficiency, because the mass transfer resistance and intraparticulate diffusion effects are eliminated. The absence of internal pore structure allows good recovery, avoiding biomolecules conformational changes, but the column loading capacity is relatively low due to small surface area of these particles [6,59]. Porous particles appeared to overcome some limitations of nonporous particles. These particles increase surface area and

consequently increase binding capacity. The pore size should be at least five times larger than the average size of the target biomolecule for its easy access. Porous particles allow faster mass transfer and higher flow rates by maintaining an efficient capture [53, 60].

Monoliths are considered the fourth generation of the chromatographic stationary phases, because they overcome some limitations of conventional columns [6]. The material can be polymerized into rigid disk and cylindrical or conical tubes as a solid block interlaced with branched channels, which avoid air bubble problems. The solute transport to their surfaces is done only by convection, in contrast to conventional supports that operate by diffusion. Therefore, this methodology allows fast separations with high mass transfer at low pressure [48, 61, 62]. Typically, monoliths applied for preparative separation are composed of polyacrylamide or polymethacrylate and for analytical separation are composed of polyacrylamide, polymethacrylate or polystyrene. One advantage of the monolithic column is the fact that the same column can be used on both small and industrial scales [6].

Membrane technology is often used to concentrate samples, to separate large molecules from small ones, in desalting processes and sometimes to remove cell debris. However, membrane absorbers arise as potential chromatography supports increasing resolution, efficiency and consequently productivity. In industrial applications they present a limitation, because they have higher costs [6].

Cryogels are also a type of chromatography support. They are prepared at low temperatures, forming ice crystals. The shape and size of the pores depend on the freezing process of cryopolymerization. Indeed, cryogels with macropores have the ability to process crude solutions that contain non-clarified cell or entire cell suspensions [6,62].

2.1.3 Role of ligand immobilization on chromatographic supports

Ligands play a very significant role in the success of the purification protocol, because they confer specific interactions to explore. The binding of adequate ligands allows high specificity for the target biomolecule and an efficient and directed purification [6,9].

The ligand immobilization procedure consists on activating the matrix to make it reactive with the ligand functional group. Then, the ligand is covalently coupled to the matrix through a chemical reaction. Finally, to ensure that all interactions are responsible by the ligand and the sample, unreacted groups are blocked by a large excess of a suitable low molecular weight substance such as ethanolamine [2]. When small ligands are immobilized, a spacer arm between the ligand and the matrix should be introduced to increase the ligand availability and reduce the steric hindrance [36].

Normally, several ligands such as nucleotides, lectins, protein A and protein G or non-biological such as dyes, metals and amino acids can be classified in biological ligands [9].

The ideal ligand would be a synthetic and stable molecule, safe, nontoxic, compatible with the solvents used during the procedure and inexpensive. Some dyes have been used in this way, but a great inconvenient has been a lack of specificity. However, protein ligands usually provide higher selectivity but are not ideal for production, since they are expensive, they can be irreversibly denatured and they must be pharmaceutically pure. Ligands can bind a single or a very small number of proteins with similar chemical characteristics and it should be compatible with the solvents used during the process [36–38].

2.2 Typical matrices and ligands in ion exchange chromatography

In general, ion exchangers are more densely substituted than other adsorbents used in protein chromatography and its capacity for protein binding is very high. Chromatographic matrices for ion exchange are made from porous or non-porous particles and they should trail several characteristics [44, 63] referred in the section 1.2. Matrices commonly used in ion exchange chromatography are summarized in Table 2.

The functional groups substituted into a chromatographic matrix determine the medium charge and they will also influence the separation. For example, the following functional groups Quaternary ammonium (Q), Diethylaminoethyl (DEAE) and Diethylaminopropyl (ANX) are anion exchangers. On the other hand, the functional groups Sulfopropyl (SP), Methyl sulfonate (S) and Carboxymethyl (CM) are cation exchangers. Ion exchangers are classified as weak (Q, DEAE, CM) or strong (Q, SP, S). This classification refers to the pKa values of their charged groups and does not reflect anything about the interaction strength. Strong ion exchangers are not affected by change of pH medium. On the contrary, weak ion exchangers have a limited pH range for their use [9,28,64].

Table 2 - Ion exchange chromatographic matrices, adapted from [41,42].

Stationary phase	Base matrix	Mean particle size (µm)
SP Sepharose fast flow	Cross-linked agarose	90
Sepharose XL	Agarose 6%, dextran chains coupled to agarose	90
Sepharose	Big beads agarose 6%	200
Capto S	Highly cross-linked agarose with flexible dextran surface extender	90
UNOsphere rapid S	Polyacrylamide network	80
S-HyperD M	Ceramic shell filled with polyacryalamide soft gel	80
Fractogel EMD SO3 – M	Polymethacrylate with Polyacrylamide surface extender	65
POROS HS	Polystyrene- divinylbenzene with through pores	50
Source 30 S	Polystyrene- divinylbenzene monobeads with hydrophilic coating	30
SOURCE 15	Polystyrene with divinyl benzene	15
Toyopearl SP-650 M	Polymethacrylate	65
GigaCap S	Polymethacrylate with flexible polymeric surface extender	65
MiniBeads	Polystyrene with divinyl benzene	3
MonoBeads	Polystyrene with divinyl benzene	10

2.3 Dynamic Binding Capacity

Over the last 30 years there have been significant improvements in the expressed levels of recombinant proteins, thus quantities increasing than µg/mL for mg/mL. The assessment of column binding capacity is a significant component for evaluate the efficiency of a purification processe [63, 65].

Dynamic binding capacity predicts the target protein amount that will bind to the matrix under adequate flow conditions, representing the adsorption capacity of the column for the target protein. The breakthrough curve can be also used to determine how much of the column capacity has been used, how much biomolecule has been wasted during the adsorption phase and the processing time or applied volume [66,67]. Dynamic binding capacity can be determined by different methods [69].

The binding capacity of the support is directly proportional to its surface area and consequently is affected by the amount of immobilized ligand. The larger is the surface area, the greater is the amount of immobilized ligands. On the other hand, surface area increase with decreasing porous size. All these parameters are important for evaluating the application of a new matrix in purification processes [6,9]. Dynamic binding capacity can forecast the mass transfer limitations value that may occur when the flow rate is increased [67].

3. Gellan gum

3.1 Physical and chemical properties of gellan gum

Gellan gum is a polysaccharide produced extracellularly by a non-pathogenic bacterial strain, *Sphingomonas paucimobilis* of the *Pseudomona elodea species* [66, 67]. The gellan chemical structure consists on a straight-chain constituted of repeating units of a tetrasaccharide (1,3-ß-D-glucose; 1,4-ß-D-glucuronic acid; 1,4 ß-D-glucose and 1,4-α-L-rhamnose). The composition of this polysaccharide is approximately glucose 60%, rhamnose 20% and glucuronicacid 20% [68, 69].

Native polymer contains L-glyceryl group at C2 and acetyl group at C6 on glucose residues. In presence of these groups, high acyl gellan is obtained (Fig. 2) [66,70]. Immediately after the production by fermentation, native gellan gum acquires glyceryl and acetyl groups, but when exposed to strong alkali treatment at high temperatures they become hydrolyzed, and the low acyl gellan is obtained (Fig. 3). Commercially, the gellan gum is available in these two forms [68, 71].

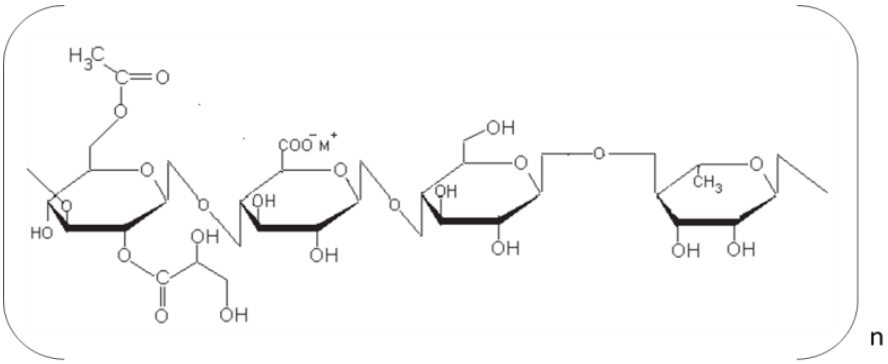

Figure 2 - Representation of the repeating unit of chemical structure of native gellan gum, adapted from [76].

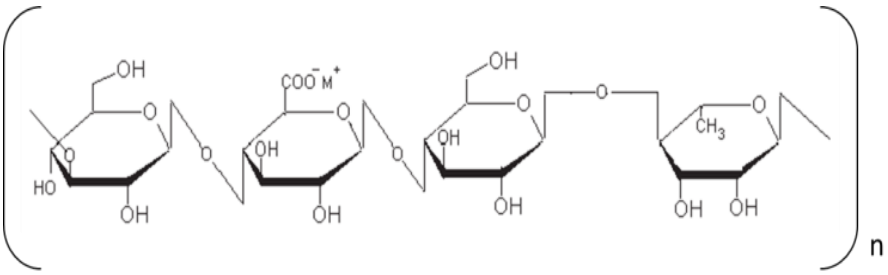

Figure 3 - Representation of the repeating unit of chemical structure of deacetylated gellan gum, adapted from [76].

Gellan gum is an anionic natural polymer with high molecular weight (500 kDa) and in solution at high temperature it acquires a random coiled structure. However, gellan gun conformation is changed to a double helix by decreasing temperature [77]. When dissolved in water and in presence of ions, gellan gum forms three dimensional double-helix networks. Low acyl gellan

gum forms a strong, firm and brittle gels. On the contrary, high acyl gellan gum produces soft and elastic gels [66,74].

Glycoside molecules have all their connections carbon 1 to carbon 4, with flat structure and similar to a loop. However, gellan gum has three of four glycosidic linkages, according other glycoside molecules, but it has one glycosidic linkages localized in carbon 1 and carbon 3 [79]. Gel rheological properties and melting point vary depending on the high acyl gellan to low acyl gellan proportion, gel concentration and temperature, as well as the type and concentration of cations added [72,76].

Previously, gellan gum had been reported by its S-60 and PS-60. Due to its unique properties, the gellan gum has been found to be applicable to a wide range of applications in the food, pharmaceutical and chemical industries [76].

3.2 Fermentative production of gellan gum

For the industrial production of gellan gum, the bacterium *Sphingomonas paucimobilis* ATCC 31461 is used. *Sphingomonas* is a group of gram-negative, chemoheterotrophic and strictly aerobic. These microorganisms are rod form and typically produce yellow-pigmented colonies [66, 77].

The growth media suitable for the production differs according to each polysaccharide nature. Accordingly, several factors can affect the gellan gum production such as media components, pH, agitation rate and temperature, in order to increase production yields [76]. The media used in the gellan gum production can be simple media, only containing carbon source, nitrogen source and inorganic salts or can be more complex with nutrient supplements, for example vitamins and amino acids [76]. Carbon source is the most important component of the media and the carbon amount varies between 2–4 % by mass. Carbohydrates such as glucose, fructose, maltose, sucrose and mannitol can be used either alone or in combination as carbon source [72,78].

According to Nampoothiri and coworkers (2003) and Bajaj and coworkers (2006), the soluble starch is the best carbon source for gellan gum production, when compared with glucose, lactose, maltose and sucrose [79, 80]. Following carbon source, nitrogen is the second more important medium component for gellan gum production. However, copious secretion of exopolysaccharide is usually most noticeable with an abundant carbon source and minimal nitrogen [85]. The choice of the nitrogen source has strong effect on broth characteristics. Several studies were made to find the best nitrogen source [85–87]. Among the organic and inorganic nitrogen sources used, tryptone supported the maximum gellan gum production according Nampoothiri and collaborators [84].

The pH variation is a very important parameter in gellan production, because it significantly influences both cell growth and product formation. The pH value usually recommended for gellan production ranges from 6.5 to 7. Values out of this range reduce the cell growth, and consequently the gellan production [79, 80, 84].

According to Dreveton and coworkers (1994), an agitation of 250 rpm is adequate for the mixing of gellan gum broth. Lower values than 250 rpm showed that agitation was insufficient for homogenous conditions and the broth exhibited gelling characteristics. High stirring rates (600 to 800 rpm) result in the formation of stagnant zone and the medium becomes heterogeneous [89]. On the contrary, the bacterium growth increases with vigorous agitation and high aeration rate. Oxygen is vital for gellan synthesis. Although gellan formation occurs mainly parallel with cell growth, the increase of cell number does not always reflect high gellan production. For example, a study made by Giavasis and coworkers showed that the maximum growth happened at high agitation rates, 1000 rpm, however a great gellan gum concentration was also obtained at 500 rpm [86, 87]. The gellan gum formation is also affected by the temperature manipulation, reaching its peak at 20ºC , remaining quite high at 25ºC , and significantly decreasing above 30ºC [92].

The molecular mass average and intrinsic viscosity of gellan gum depend on media constitution, however, they increase with the most homogeneous conditions [85, 89]. To ensure high yield of gellan gum fermentation, some production parameters, as well as an efficient recovery and purification should be optimized. The recovery process consists on heating the culture broth to 90–95ºC in order to kill the cells and reduce the viscosity. Following, the polysaccharide is separated from the cells by filtration or centrifugation. Native gellan gum is obtained after alcohol precipitation and lyophilization. If acyl groups are removed from the native gellan gum, the final product will have a low acyl gellan gum [72,83].

3.2.1 Biosynthetic pathway of gellan gum

In spite of recent advances in knowledge of the gellan biosynthetic pathway, yet there are poorly understood steps and its knowledge is crucial for the eventual success. Many researchers have investigated the route of repeating tetrasaccharide units and gellan synthesis, and found out that a role of enzymes is involved. Genetic engineering has a fundamental role in this process [72, 81, 90].

Gellan biosynthesis pathway can be divided in three levels: synthesis of sugar-activated precursors (Fig. 4); repeat unit assembly and gellan to the inner membrane; and finally polymerization and export of the polymer through the outer membrane (Fig. 5) [80]. Gellan synthesis requires activated precursors before the repeating unit be assembled. These gellan precursors were found to be nucleotide phosphate sugars. They are UDP-glucose, TDP-

rhamnose and UDP-glucuronic acid. The Figure 4 represents a possible formation pathway of the nucleotide sugar precursors [81,91].

After having the precursors, synthesis of the repeat unit by sequential transfer of the precursors for a lipid carrier occurs. UDP-glucose is the first precursor to bind the lipid carrier, followed by UDP-glucuronic acid, second UDP-glucose molecule, TDP-rhamnose and finally glyceryl and acetyl groups. The last step corresponds to polymerization and exportation of the polymer through the outer membrane for cell surface [76, 91].

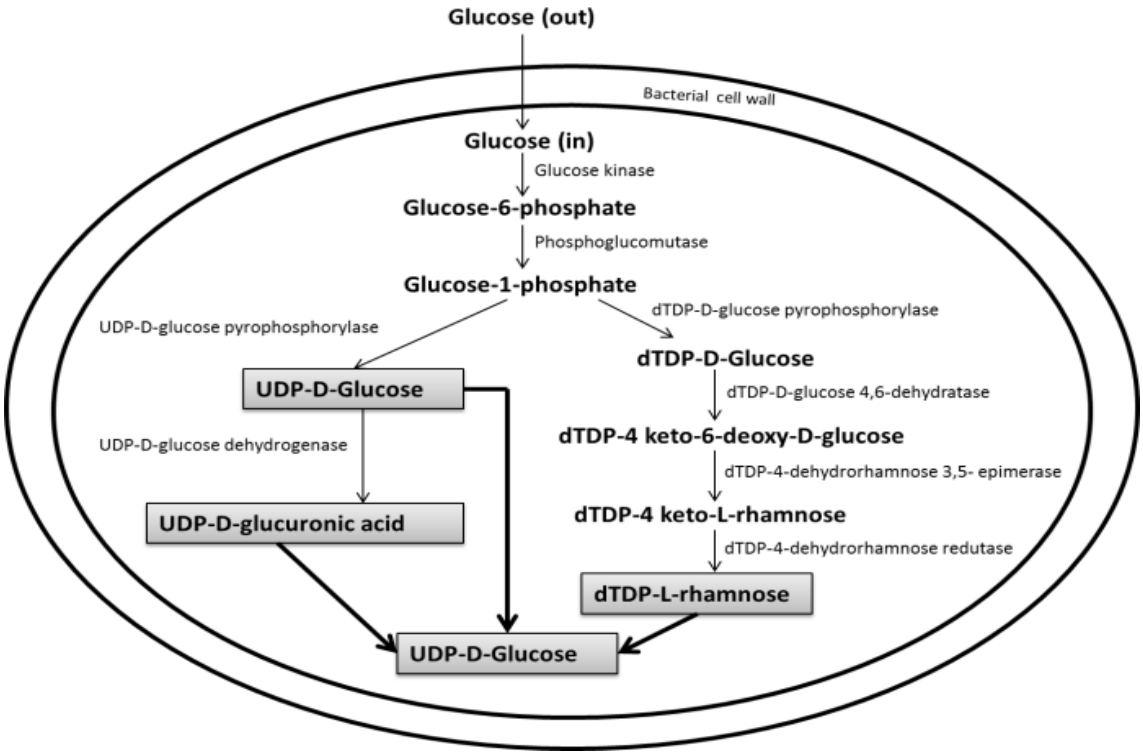

Figure 4 - Schematic representation of the postulated pathway leading to the nucleotide sugar precursors, adapted from [95].

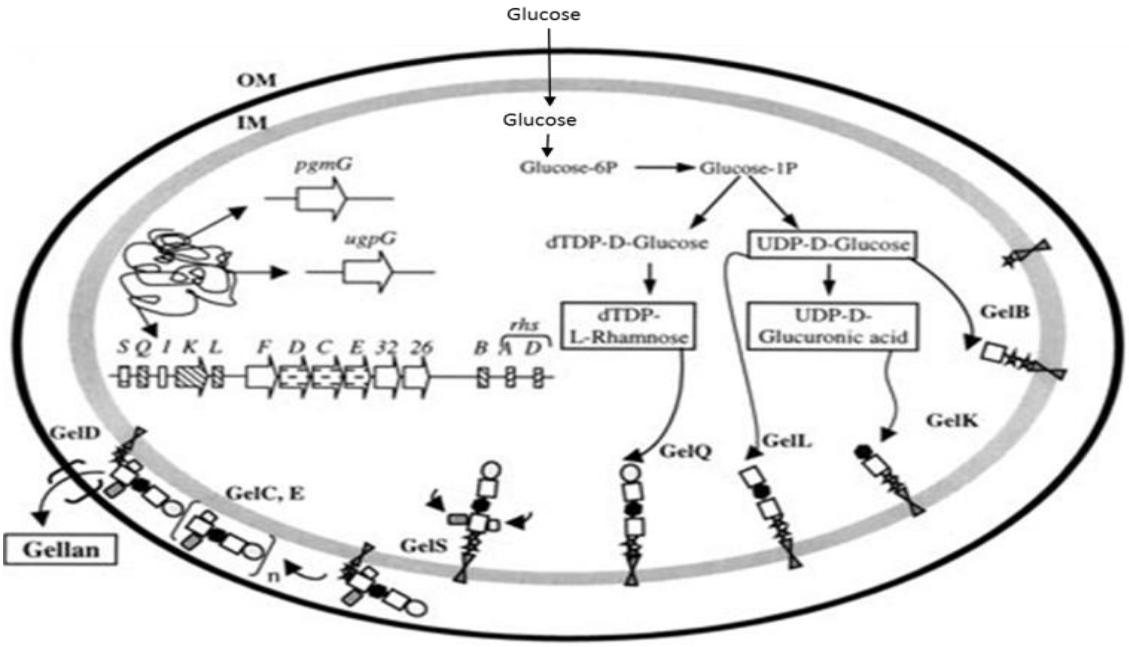

Figure 5 - Schematic representation of the polymerization and export of the gellan gum through the outer membrane for cell surface adapted from [95]. Abbreviations: GelB, priming glucosyltransferase; GelK, glucuronosyltransferase; GelL and GelQ, putative glycosyltranferases; GelS, putative translocase; Gel D, C and E, proteins putatively involved in polysaccharide chain length regulation and secretion; OM, outer membrane; IM, inner membrane; ⋈ , lipid carrier; ★, phosphate; □, glucose; ⬢, glucoronic acid; ○, rhamnose; ▭, glycerate; ▮, acetate.

3.3 Gellan gum applications

Toxicological studies concluded that gellan gum is relatively nontoxic. Therefore, in 1992 gellan gum was approved by US FDA for use as a food additive. Currently, C.P. Kelco produced gellan gum in Japan and USA [76]. Commercially, gellan gun is available with four different trade names: Kelcogel® (thickener and gelling agent) and Gelrite®, Phytagel® and Gel-Gro® (solidifying agent, a substitute for agar) [72, 92].

Gellan gum has many advantages such as an exceptional thermal and acid stability, adjustable gel elasticity, rigidity, high transparency, and good flavor release [68, 76, 93]. Due to these characteristics, gellan has gained importance in the food or pharmaceutical industries, cosmetic and personal care products, gel electrophoresis, immobilization of cells and enzymes and bioremediation [76].

Gellan gum has a high potential in various food products. Its application is not only in foods which require a highly gelled structure, but it can be used in systems to provide body and mouth-feel rather than gelatin. Sometimes, a combination with other hydrocolloids to obtain optimal product texture and stability is needed [72, 75].

In confectionery and bakery products, gellan gum provides structure and texture to the products and it can prevent moisture fluctuations in sugary foods, icings and toppings. The

amount used in these cases is only one fifth of the commonly used agar [98]. Gellan gum provides dessert gels with mouth-feel characteristics similar to those of gelatin, but gellan gum is preferred due to its high clarity. Pectin can be replaced by gellan gum in jams [76]. This polysaccharide gives excellent sheen and has good spread ability. In pie fillings and puddings, gellan gum can be used as a structuring agent to partly replace starches. Negatively charged hydrocolloids like gellan interact with the positively charged milk proteins. Thus, the gellan can also be used in cheese, ice cream and yogurts production it increases the total yield and reduces solids loss, since gellan gum provides an environment which does not melt during pasteurization. Foods retain their characteristic shape under the processing conditions [72, 76]. According to Bajaj and coworkers, gellan gum can reduce oil uptake during frying due to its hydrophilic character [99].

Regarding pharmaceutical industry applications, gellan gum has an important role in controlled drug release. Studies made by Wataru and collaborators (2003) showed sustained delivery of paracetamol by gellan gum and sodium alginate formulation was similar to that of a commercially available suspension containing an identical dose of paracetamol [96]. Use of gellan gum for controlled bioavailability of ophthalmic formulations has been showed by Sanzgiri (1993). They concluded that gellan-based ophthalmic solutions have longer residence time in tear fluid than saline solution [66, 96]. Biopolymers of gellan with calcium are promising as potential physiological alternatives for vitreous substitution, it being considered best proprieties than silicone oil that is a common vitreous substitute [101].

Gellan Gum has been recently proposed for tissue engineering applications, due to highly tunable mechanical and degradation properties. For example, three-dimensional encapsulation of fibroblast cells in gellan networks demonstrated in vitro biocompatibility, because the amount of cell survival was high [98, 99]. Oliveira and coworkers demonstrated that gellan gum hydrogels adequately support the growth and deposition of human articular chondrocytes *in vitro* and *in vivo* [104].

This natural polymer is an added-value for solid culture media in microorganisms and plants growth. Gellan polymer withstands to several autoclave cycles and is also resistant to a variety of enzymes. So, gellan gum is also considered an economical alternative to agar as a gelling agent [105]. Gellan is a polymer cheap, so it is considered a good alternative as a replacement for agarose. However, this gel must include a second polymer (such as hydroxymethylcellulose or polyethylene oxide) to reduce electroosmosis [76]. Some studies concluded that gellan gels provide a mechanically stable matrix for the immobilization of bacterium. Taking this ability in consideration, delivery of probiotic cultures to human gastrointestinal tract may be useful. The wastewater treatment and removal of gasoline hydrocarbons by encapsulation of cultures in gellan gum is also possible [94, 102, 103]. In cosmetic and personal care applications, gellan gum has a role as gelling agent of the lotions, creams, and toothpastes [80].

3.4 Gellan gum gels

Gellan gum gelation is dependent of the polymer concentration, temperature as well as type and concentration of the ions [70]. As previously mentioned, gellan gum acquires a random coiled structure at high temperatures and with decreasing temperature of the solution gellan changes its conformation for double helix. Therefore, gelation process involves aggregation of the double helical segments, complexation with cations and bonding with water through hydrogen bridges forming a three-dimensional network. Addition of monovalent or divalent cations during the cooling markedly increases the number of salt bridges between double helical segments [67, 72, 104].

The gel strength is affected by acetyl content in gellan gum. The native gellan has acetyl and glyceryl groups. Gels formed with high acyl gellan are soft and elastic, because acetyl and glyceryl groups prevent close association between gellan polymer chains, due to their size. With decreasing of the acylation levels, gellan gels become more firm and brittle [109].

Gellan gum does not form gel in odeling water, but the addition of the ions influences gel strength and brittleness. Divalent cations are more important than monovalent cations, because in presence of the divalent cations occur a direct "cross-link" decreasing electrostatic repulsion between helices. The opposite happens with monovalent cations which form indirect "cross-linking" of the gellan double helix. Therefore, divalent cations form ordered structures, which become extremely thermostable with progressive addition of cations [75, 104]. Divalent cations efficiently fortify the gel matrix at lower concentrations more than monovalent cations [110]. The best monovalent cation is the hydrogen, followed by cesium, potassium, sodium, lithium and finally tetramethylammonium. In case of divalent cations, there is difference between transition metals and alkaline-earth metals. Therefore, transition metals are more efficient in gellan gum gelation and the most efficient is lead, followed copper and zinc. With regard to the alkaline-earth metals, magnesium, calcium, strontium and barium contribute equally to the gel formation [75, 104].

Sometimes, an electrostatic repulsion between the helices can avoid the gel formation. However, by reducing the solution pH, this repulsion decrease and formation of gels with higher thermal stability occurs. This problem happens because there is a modification on charge carboxyl groups. So, these groups cease to have negatively charged, getting neutrally charged [79].

The gel formulation is favored at a pH range of 3.5 to 8 and 90°C. In some cases that monovalent cations are used, increasing pH leads to an increase in melting temperature. Sometimes, the modification of the melting temperature can be advantageous in order to reduce the amount of thickeners and stabilizers needed [76].

Parameters as gelling temperature, clarity, and texture properties of gellan gels are influenced by addition of hydrophilic ingredients, such as sucrose and fructose. According to Tang and collaborators (2001), gelling temperatures increase on the addition of sucrose, whereas addition of fructose up to 35% (w/v) had no effect. Thus, gel clarity increased with these two molecules. Relatively to texture properties, the addition of the sucrose is only advantageous when cation concentrations are low [111].

The mixture with other food hydrocolloids (for example sodium alginate, gelatin, carrageenan and xanthan) influence textural properties of gellan gum gels. Various studies concluded that the incorporation of moderate concentrations of gellan in sodium alginate gels increased the strength, but did not change their elasticity. The results suggested that hardness was dependent on the concentration of gellan gum in the mixture with gelatin. On the other hand, the brittleness, springiness and cohesiveness were dependents on the ions concentrations. These studies also concluded that the mixture with carrageenan or xanthan increased the gel elasticity [72, 105, 108].

Chapter II – Objectives

This master thesis has as main aim the development of a new chromatographic matrix taking advantage of the fact that gellan gum has negative charge and provides possible interactions with the proteins loaded. Thus, this work intended to:

- Formulate different gels with different conditions;
- Optimize the preparation conditions in order to increase stability of gellan gum gels;
- Explore gellan gum gel as a matrix on ion exchange chromatography;
- Study model proteins to verify if they interact with the matrix;
- Determine dynamic binding capacity to characterize the gellan gum as a chromatography matrix.

Chapter III – Materials and Methods

1. Materials

All the solutions were prepared using deionized water with ultra-pure grade, purified with a Milli-Q system from Millipore/Waters (Billerica, MA, USA). Gellan gum (Gelrite ®), 4-Morpholineethanesulfonic acid (MES), zinc sulfate ($ZnSO_4$), nickel sulfate ($NiSO_4$), *N,N*-Dimethylformamide (DMF), lysozyme, BSA (Bovine serum albumin), α-chymotrypsin, Sodium chloride (NaCl), methacrylic anhydride, sodium carbonate and bromophenol blue were acquired from Sigma-Aldrich (St. Louis, MO). Acrylamide 30% / Bis-acrylamide solution was obtained from BioRad (Hercules, CA). Tris (hydroxymethyl)aminomethane and glycine were bought from Fisher Scientific (Epson, United Kingdom). Sodium dodecyl sulfate (SDS) and glycerol were acquired from Himedia (Mumbai, India). Ammonium persulfate (PSA) was obtained from Eurobio (Courtaboeuf, France). N,N,N',N'-tetramethylethylenediamine (TEMED) and ß-mercaptoethanol were purchased from Merck (Darmstadt, Germany). NZYTech colour protein marker II was bought from NZYTech (Lisbon, Portugal). The employed buffers were filtered through a 0.20 µm pore size membrane (Schleicher Schuell, Dassel, Germany) and ultrasonically degassed. In this work, low acyl gellan was used.

2. Formulation of Gellan gum gels with different conditions

In order to increase the stability of gellan gels, several experimental conditions were tested. Gels formulations involved various parameters: gellan concentration (%(w/v)), counter ion concentration, DMF (%(v/v)) temperature and reaction time. The gel preparation consisted in dissolving the counter ion (zinc) in water, with constant magnetic stirring at room temperature. Then, DMF was added and the solution was progressively heated. Followed, gellan gum was added to the mixture being kept with a constant magnetic stirring of 300 rpm for a determined reaction time. Finally, the obtained suspension was left at room temperature in order to cool it down. After this process, the gel was packed in an Econo-Pac column (BioRad, Hercules, CA) and equilibrated with 10 mM MES buffer pH 6.2.

Gellan gel formulations involved different concentrations of gellan gum (from 0.75% to 2%), zinc sulfate (from 30 to 120 mM), DMF (from 0% to 30%), temperature (from temperature ambient to 110 ° C) and reaction time (from 0.5 hours – overnight).

3. Gellan gum gel stability assays

After packaging of an Econo-Pac column with the gellan gum formulation, stability assays were made. These assays consisted on registering the time that 15 mL of 10 mM MES buffer pH 6.2, took to pass through each gel formulation before losing its integrity. Consequently, this strategy allowed registering the number of column volumes (CVs) that each gel formulation remained without being affected by the loss of stability.

4. Optimization of gellan gum gel formulation conditions

After testing various gel formulations, experimental design was applied to define ideal experimental conditions that allowed a more stable gel.

4.1 Experimental Design

A feed-forward artificial neural network (ANN) was applied to predict the number of column volumes as function of the concentration of gellan, $ZnSO_4$, DMF, temperature and time.

The ANN models were implemented in MATLABTM using the Neural Network Toolbox. The ANN structure included an input layer with five neurons (one for each input variables), an output layer with one neuron (number of column volumes) and one hidden layer with four neurons (5/1/4), resulting in 29 model parameters. The transfer function of the input and output layers was linear function 'purelin' and of the hidden layer was log-sigmoid function 'logsig'.

5. Gellan gum gel formulation with methacrylic anhydride

The gellan gum gels with methacrylic anhydride were prepared according to followed conditions: 1.25% (w/v) gellan, 50 mM zinc sulfate, 2.5% (v/v) methacrylic anhydride, 90ºC and 0.5 hours. Preparation of these gels occurred according to the process described in 3.2. However, instead of adding DMF, it was added methacrylic anhydride.

5.1 Gellan gum gel formulation with methacrylic anhydride and nickel as contour ion.

Gellan gum gels with nickel as contour ion were prepared according to followed conditions: 1.25% (w/v) gellan, 50 mM nickel sulfate, 2.5% (v/v) methacrylic anhydride, 90ºC and 0.5 hours. Preparation of these gels occurred according to the process described in 3.2. However, DMF and zinc sulfate were substituted for methacrylic anhydride and nickel sulfate, respectively. Following, the column was equilibrated with 10 mM MES buffer pH 6.2. After, in order to study stability of the gels in presence of NaCl molecules, it passed 750 mM NaCl 10 mM MES buffer pH 6.2.

6. Ion Exchange Chromatography

All chromatographic assays were executed using a workbench column (Econo-Pac column) at room temperature.

The matrices used in these experiments were prepared according these conditions: 0.75% (w/v) gellan, 48 mM zinc sulfate, 0.5 hours at 25 ° C and 1.25% (w/v) gellan, 50 mM zinc sulfate, 0.5 hours at 90 ° C.
The samples used in these tests were three model proteins (BSA, lysozyme and α-chymotrypsin).

Fractions of 1 mL were collected in each chromatographic step that was maintained for three column volumes. Then, the absorbance of the collected fractions was monitored at 280 nm in a Pharmacia Biotech Ultraspec 3000 spectrophotometer. Finally, the fractions corresponding to the chromatographic peaks were concentrated and desalted in Vivaspin® 6 concentrators (Sartorius Stedim Biotech, Goettingen, Germany). The samples were preserved at 4ºC for further analysis.

6.1 Chromatographic assays with isolated or combined model proteins

In assays made with isolated proteins (BSA; α-chymotrypsin; lysozyme) or with combined proteins (mixture of BSA + α-chymotrypsin; BSA + lysozyme), 500 µL of each sample was injected into the matrix with a concentration of 10 mg/mL. These assays consisted in two chromatographic steps. First, the column was equilibrated and the sample was loaded to the gellan matrix with 10 mM MES buffer pH 6.2. The second step corresponded to the elution of bound biomolecules. The elution was made with the same buffer, but with addiction of 50 mM $ZnSO_4$ and 750 mM NaCl to maintain the gel stability and to increase the ionic strength, respectively.

6.2 Chromatographic assay of combined model proteins (α-chymotrypsin + lysozyme)

These experiments were made to determine the NaCl concentration that allowed the separation of α-chymotrypsin and lysozyme. Thus, different NaCl concentrations (from 50 to 350 mM) were added to the elution buffer. First, the column was equilibrated with 50 mM $ZnSO_4$ in 10 mM MES buffer pH 6.2 and a determined concentration of the NaCl. For the protein elution was utilized 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. The injected sample contained 10 mg/ml of the model protein mixture (α-chymotrypsin and lysozyme).

6.3 Chromatographic assay of combined model proteins (BSA + α-chymotrypsin + lysozyme)

This chromatographic assay consisted in three steps. First, the column was equilibrated with 25 mM $ZnSO_4$ in 10 mM MES buffer pH 6.2 and the protein mixture was loaded. After elution of unbound material, a stepwise gradient at 60 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES pH 6.2 was applied. Finally, the more retained protein was eluted at 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM MES pH 6.2. The amount of injected sample in this assay was 1 mL of the BSA (2mg/ml), α-chymotrypsin (5mg/ml) and lysozyme (5mg/ml).

6.4 Chromatographic assay of combined model proteins (BSA + α-chymotrypsin + lysozyme) and pH variation

This chromatographic assay was made in order to study pH buffer effect in the protein elution. This chromatographic assay consisted in three steps. First, the column was equilibrated with 25 mM $ZnSO_4$ in 10 mM MES buffer pH 6.2 and the protein mixture was loaded. For elution of unbound material, 50 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10 was applied. Finally, the more retained protein was eluted at 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10. The amount of injected sample in this assay was 1 mL of the BSA (2mg/ml), α-chymotrypsin (5mg/ml) and lysozyme (5mg/ml).

7. Electrophoretic analysis

Reducing Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [113] and as previously described [114].

Samples resultants from the chromatographic peaks were treated by adding 10 µL of a reduction buffer (500 mM Tris-HCl (pH 6.8), 10% (w/v) SDS, 0.02% bromophenol blue (w/v), 0.2% glycerol (v/v), 0.02% β-mercaptoethanol (v/v)) to the 30 µL of concentrated samples. Samples were denatured at 100 ºC during 5 min. Then, an electrophoretic assay on 5% stacking and 15% resolving gels, in the running buffer containing Tris (25 mM), glycine (192 mM), SDS (0.1% w/v) at 150 V for 90 min was executed. Finally, the gel was stained by Comassie brilliant blue.

8. Dynamic Binding Capacity

During the dynamic binding capacity studies, the prepared gellan gel formulation was the same as for chromatographic assays. The matrix was packed into a column C 10/10 (GE Healthcare, Portugal) to a total volume of 500 µL. This column was connected to an Akta Purifier system to determine the dynamic binding capacity.

The column was equilibrated with 25 mM $ZnSO_4$ in 10 mM MES buffer pH 6.2. Thereafter, the column was overloaded with 0.5 mg/mL of lysozyme in 25 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2, at a flow rate of 1 mL/min.

Determination of dynamic binding capacity was carried out by recording breakthrough curves and calculating the amount of bound lysozyme per mL support at 10% and 50% of breakthrough curve. Dynamic binding capacity values were then obtained by subtracting the value obtained under non-binding conditions. Afterwards, the elution of the bound lysozyme was achieved by increasing the ionic strength to 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2.

Chapter IV – Results and Discussion

1. Gellan gum gel stability assays

The zinc ion was chosen for the gellan matrix formulations, because divalent cations are considered more effective in the gelation of gellan gum [115]. Taking into account that the zinc is a divalent ion and a transition metal, this ion is considered the most adequate for the formation of gellan gels [43].

To assess the stability of the gellan gum gels, various parameters were studied such as gellan concentration (%(w/v)), counter ion concentration, DMF concentration (%(v/v)), temperature and reaction time. To study these parameters, various formulations were prepared, by manipulating only one parameter and keeping the other constant. This procedure was undertaken in order to find the result that permitted a higher number of CVs. The gel is more stable the greater the number of CVs that it allows, without losing their integrity. The best result obtained for the gellan concentration study was 1.25% (w/v) gellan, within the interval of 0.75% to 2% (w/v) as shown in table 3.

Table 3 – Number of column volumes obtained by varying the gellan concentration for the gellan gum gel formulation.

Constant conditions	Formulations	Variable condition: Gellan (%m/v)	Number of column volumes
90 mM Zinc sulfate 0% DMF 90ºC 300 RPM 0.5 Hours	1	0,75	18
	2	1.25	51
	3	2	16

In the case of formulations wherein the variable parameter was the concentration of counter-ion, zinc, the range studied was 30 to 120 mM. The formulation that allowed a greater number of CVs was the one which contained 50 mM of zinc sulfate, as shown in Table 4.

Table 4 – Number of column volumes obtained by varying the zinc sulfate concentration for the gellan gum gel formulation.

Constant conditions	Formulations	Variable condition: $[ZnSO_4]$ (mM)	Number of column volumes
0% DMF 1.25% Gellan 90ºC 300 RPM 0.5 Hours	1	30	25
	2	40	22
	3	50	45
	4	60	28
	5	70	28
	6	80	22
	7	90	28
	8	100	31
	9	110	28
	10	120	19

When the variable parameter was the reaction time, four conditions were tested: 0.5, 2, 4 hours and overnight. This study showed that the formulation which allowed a greater number of CVs took the reaction time of 0.5 hours, as shown in table 5.

Table 5 – Number of column volumes obtained by varying the reaction time for the gellan gum gel formulation.

Constant conditions	Formulations	Variable condition: Time (hours)	Number of column volumes
90 mM Zinc sulfate 0% DMF 1.25% Gellan 90ºC 300 RPM	1	overnight	7
	2	4	10
	3	2	16
	4	0.5	28

In the case of formulations wherein the variable parameter was the temperature, the range studied was room temperature to 110 ºC. The best result was obtained when the reaction occurred at 90 ºC, as shown in table 6.

Table 6 – Number of column volumes obtained by varying the temperature time for the gellan gum gel formulation.

Constant conditions	Formulations	Variable condition: Temperature (°C)	Number of column volumes
90 mM Zinc sulfate 0% DMF 1.25% Gellan 300 RPM 0.5 Hours	1	Room temperature	22
	2	50	21
	3	60	24
	4	70	34
	5	80	31
	6	90	51
	7	100	22
	8	110	16

Finally, the DMF concentration from 0% to 30% was also evaluated. The best results were obtained when it was used 10% DMF or when DMF was absent, as shown in table 7. Since the results were satisfactory at both 0% and 10% DMF concentration, it was chosen do not use DMF in the gel formulation due to its toxicity.

Table 7 – Number of column volumes obtained by varying the DMF concentration for the gellan gum gel formulation.

Constant conditions	Formulations	Variable condition: DMF (%v/v)	Number of column volumes
90 mM Zinc sulfate 1.25% Gellan 90ºC 300 RPM 0.5 Hours	1	0	51
	2	5	28
	3	10	51
	4	20	36
	5	30	13

In summary, the best conditions for the formulation of gellan gum gels found through stability assays are 1.25% (w/v) gellan, 50 mM zinc sulfate, 2.5% (v/v) methacrylate, 90ºC and 0.5 hours. Following these results, experimental design was applied in order to relate all parameters and obtain the optimum formulation conditions for a stable gel and allowing the greatest number of CVs.

2. Optimization of gellan gum gel formulation conditions

An ANN model aiming the optimization of experimental conditions of a gellan gel with zinc sulfate was developed by the structure described in the section 3.4.1. The ANN model was trained using the experiments defined in Tables 1-5.

The ANN model was developed using a stepwise process until the maximum number of columns volumes was achieved by applying the optimal conditions. In the present case, the final ANN model was reached after two iterative steps. The maximum number of column volumes achieved under optimal conditions (48 mM $ZnSO_4$, 0 % DMF, 25ºC, 0.75% gellan and

0.5 hours) proposed by the ANN model in the first optimization step was 369. Then, the predictive capacity of the model was validated experimentally and the obtained number of column volumes obtained was 324. In order to fine-tune the model predictive capacity under the optimal conditions, a new iterative step was carried out. In this second iteration, the model proposed the same optimal conditions but the predicted number of column volumes was adjusted to 328 which are really close to the experimental result obtained in the previous iteration. Therefore, the iterative optimization was stopped and the final ANN model was identified.

Figure 6 shows the linear regression of predicted over experimental number of column volumes for the train, validation and test dataset with its slope, intercept value and correlation coefficient, R^2. The ANN model is almost unbiased because the slope and R^2 for all dataset were close to 1 (1.001 and 0.991, respectively).

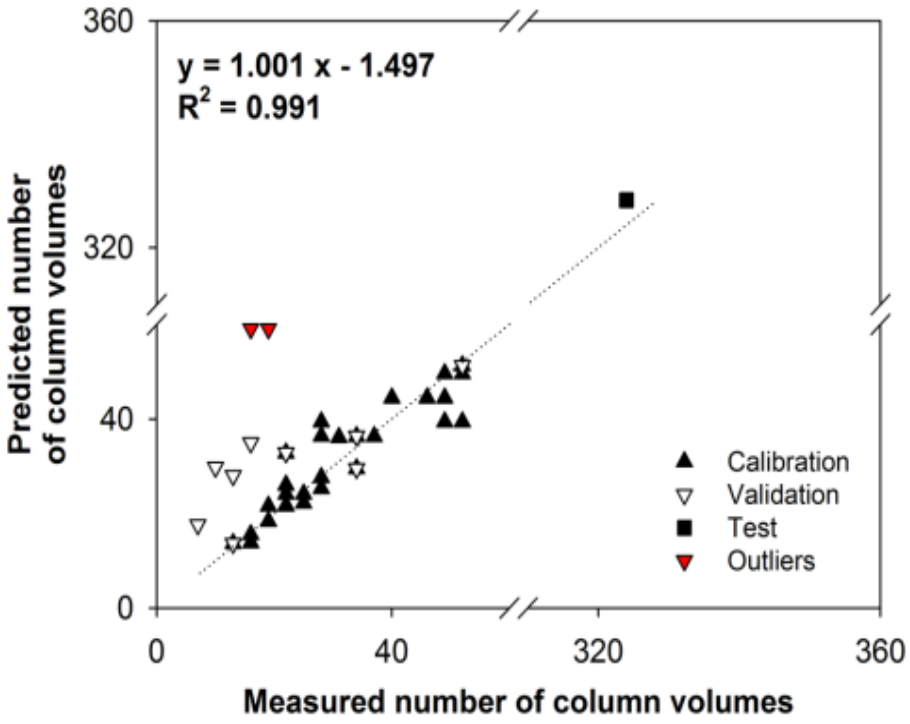

Figure 6 - Representation of the linear regression of predicted over experimental number of column volumes.

Figure 7 show the contour plot obtained from the ANN model for the three combinations of the conditions (gellan with $ZnSO_4$, gellan with temperature and gellan with time). The modeling results showed that the input variables have a significant impact on the number of column volumes. In comparison to the experiments used for the model training, an increasing of more than six-fold was achieved by applying a model based optimization.

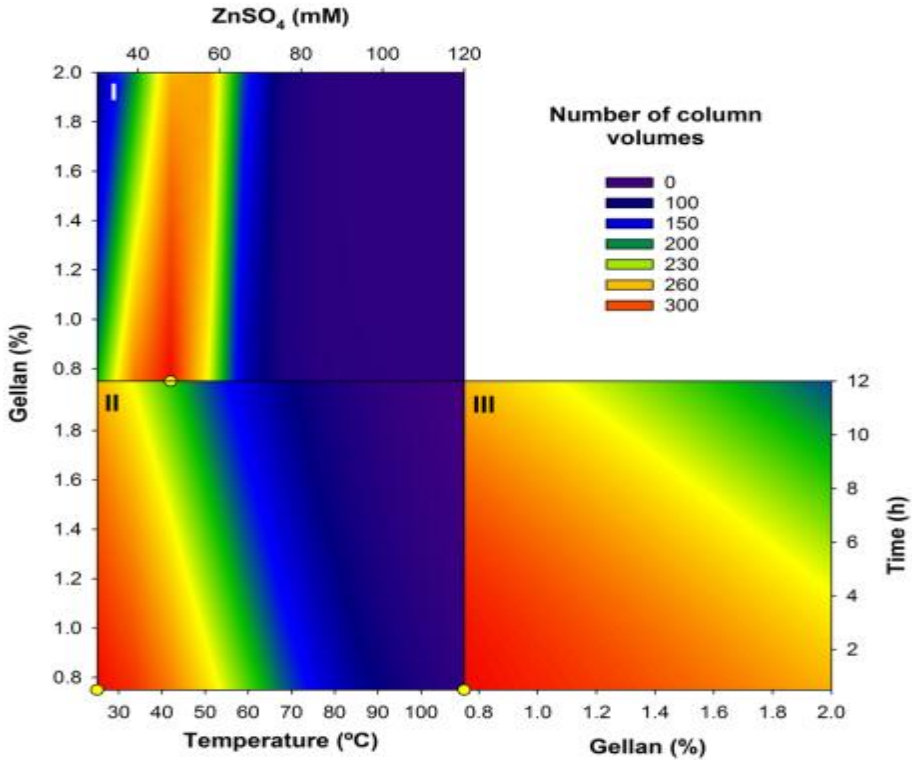

Figure 7 - Representation of the three combinations of the gel preparation conditions (gellan with $ZnSO_4$, gellan with temperature and gellan with time).

In spite of $ZnSO_4$ concentration and reaction time are similar in two forms of gels preparation (according stability assays conditions or according experimental design conditions), gellan concentration and temperature differences are accentuated. Therefore, it was concluded that small differences in gels preparation affected considerably gels stability, and consequently the number of CVs.

3. Gellan gum gel formulation with methacrylic anhydride

After founding the ideal conditions for the formulation of gellan gels through stability assays, the gellan gel was prepared and equilibrated with 10 mM MES buffer pH 6.2. Following some CVs, the buffer was changed to 750 mM NaCl in 10 mM MES buffer pH 6.2. During the passage of this buffer, the stability of the gel decreased. Probably, NaCl ions have replaced divalent cations in the gel, making three-dimensional structure was lost, become an impermeable gel. Therefore, methacrylic anhydride was used as additional parameter for the formulation of these gels to increase gels stability. This reagent concentration 2.5% (v/v) was added in place of DMF. Following the gel preparation, its stability was tested with 10 mM MES buffer pH 6.2. By registering the number of CVs, it was observed that this formulation was more stable because it allowed 153 CVs, without losing stability. Thus, the methacrylic anhydride reagent increased the stability of the gels.

As shown in figure 8, gellan gum has acyl groups on its structure that can be functionalized with the reactive methacrylate groups. Gellan gum with methacrylate groups become a hydrophobic molecule, repelling water molecules [103]. Therefore, a three-dimensional network formation occurred due to aggregation of the double helical segments and complexation with cations. The links with water ceased to be included in three-dimensional network formation. So, this reaction allowed a more stable gel formulation.

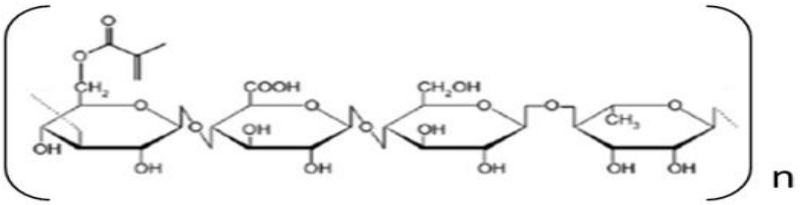

Figure 8 - Representation of the repeating unit of chemical structure of gellan gum methacrylate adapted from [103].

3.1 Gellan gum gel formulation with methacrylate and nickel as counter ion

This assay was made with nickel, because it is also a divalent ion and a transition metal. As referred previously, they are more efficient cations for gellan gum gelation. Despite these characteristics, this study was performed with nickel sulfate because the nickel ion confers blue color to the chromatographic matrix, by allowing the visual observation if the matrix instability is due to the competitive elution of divalent cations by the presence of NaCl in the elution buffer.

The gellan gel was prepared with the nickel ion and it was packed into workbench column and equilibrated with 10 mM MES buffer pH 6.2. Following some CVs, the buffer was changed to 750 mM NaCl in 10 mM MES buffer pH 6.2. During the passage of this buffer, the gel gradually lost its color and stability due to the divalent cation elution. The gel stability was diminishing, because cations are fundamentals in three-dimensional network formation and they were replaced by the NaCl ions.

However, gellan gum gel formulation with methacrylic anhydride reagent should not be used in chromatographic assays, because it has a residual absorbance at 280 nm throughout the purification assay. Given that proteins have also absorbance at this wavelength, the presence of methacrylic anhydride interferes in the absorbance reading of recovered fractions, camouflaging the peak identification.

To counteract the loss of divalent ions from the gellan matrix and in order to maintain the gel stability, a solution was found by adding a residual cation amount to the NaCl buffer.

Consequently, with this procedure there was a compensation cations lost, allowing the performance of purification assays.

4. Ion Exchange Chromatography

Ion exchange chromatography has been widely used in the purification of several biomolecules with proper characteristics that can be useful in the development of therapeutic strategies. In this way, gellan gum has an important role in chemical and pharmaceutical areas [46, 112].

Due to its gelling ability, gellan gum allowed the preparation of a gel which can be used as chromatography matrix. To prove that gellan has a chromatographic matrix-like behavior, chromatographic assays with three model proteins (BSA, lysozyme and α-chymotrypsin) were made. Two different matrices were prepared according to the conditions: 1.25% (w/v) gellan, 50 mM zinc sulfate, 0.5 hours at 90 ºC; and according to experimental design conditions: 0.75% (w/v) gellan, 48 mM zinc sulfate, 0.5 hours at 25 ºC.

Conditions referred above can define the gellan gel stability but both preparations allowed the same chromatographic behavior in model protein separation. Interactions with proteins and their separation occurred in an equal form, when used stability assays conditions or experimental design conditions. Taking these considerations into account, for chromatographic assays is indifferent to use one or other preparation. Nonetheless, in gel stability it was necessary taking into account these differences, because they influence the results. With a slight variation in these parameters we can obtain a gel more or less stable. As chromatographic results are the same independently of the gellan preparation, results shown below are referent to assays made with the preparation of experimental design conditions.

4.1 Chromatographic assays with isolated model proteins

Chromatographic assays with isolated model proteins were fundamental, because they served as an initial matrix characterization. Accordingly, with these basic assays, it is possible to predict whether the chromatographic matrix interacts with some protein and what kind of conditions are necessary to promote its elution.

These assays for each protein were constituted by two steps. First, the column was equilibrated with 10 mM MES buffer pH 6.2 and then the sample was loaded to the matrix under the same buffer. After elution the unbound material, a second step was imposed by changing of the elution buffer to 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. The zinc sulfate addition was necessary in this buffer, in order to maintain the gel stability in NaCl presence, as described above.

Figure 9 corresponds to BSA chromatographic profile and the result showed that this protein did not bind to the matrix, it being immediately eluted in the flowthrough. This behavior was

the expected, given that the isoelectric point of this protein is 5.4 [30]. Taking into account that the pH used in these assays was higher (6.2) than the BSA isoelectric point, this protein was negatively charged. Consequently, BSA did not interact with the matrix, because the gellan polymer also presents a negative character.

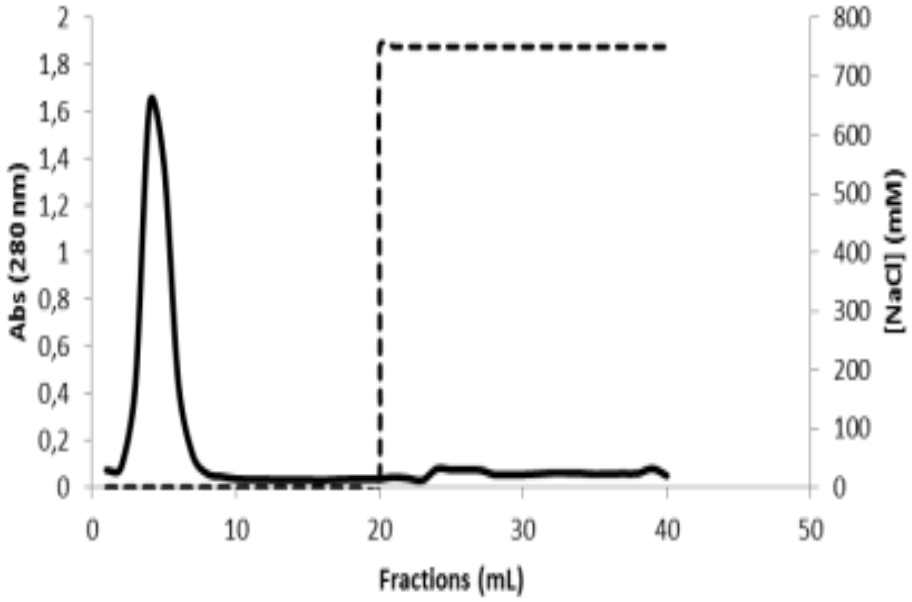

Figure 9 - Chromatographic profile obtained for BSA. The first step was performed with 10 mM MES buffer pH 6.2 and the second step was performed with 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Continuous line and dashed line represent the absorbance at 280 nm of the fractions and salt concentration, respectively.

In Figure 10 is presented the chromatographic profile for α-chymotrypsin. The result obtained under chromatographic conditions described above indicated that α-chymotrypsin remained bound to matrix with the equilibrium conditions. Given that α-chymotrypsin isoelectric point is 8.6 [31], this protein is positively charged at 10 mM MES buffer pH 6.2. As the matrix was negatively charged, the retention of α-chymotrypsin is expected. Nonetheless, the elution of this protein occurred when a second step with addition of 750 mM NaCl and 50 mM $ZnSO_4$ in MES buffer pH 6.2 was performed. The presence of NaCl in the buffer indicated that the interaction of α-chymotrypsin with the matrix was weakened by competition, resulting in its elution.

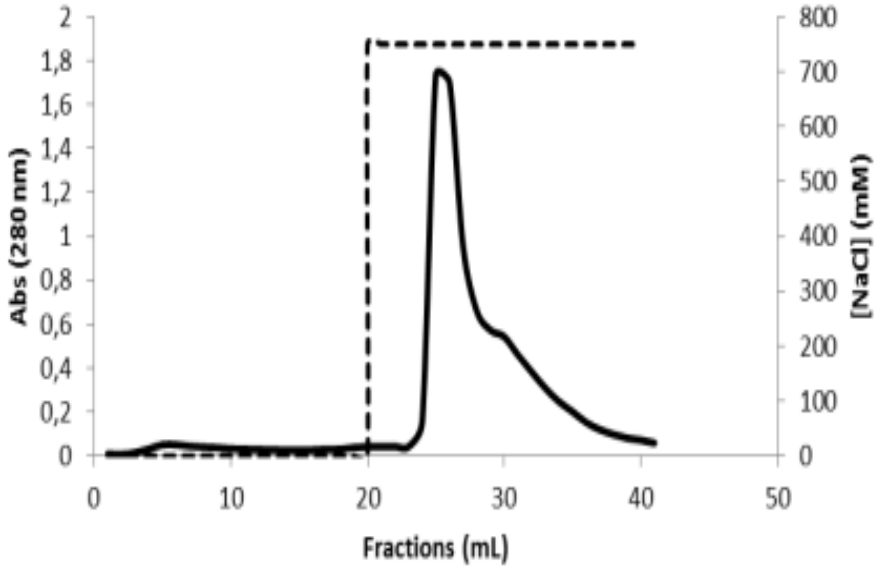

Figure 10 - Chromatographic profile obtained for α-chymotrypsin. The first step was performed with 10 mM MES buffer pH 6.2 and the second step was performed with 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Continuous line and dashed line represent the absorbance at 280 nm of the fractions and salt concentration, respectively.

The same behavior was observed with lysozyme study under the same elution conditions (Figure 11). The isoelectric point of this protein is 11 [32], which conferred positive charge to the lysozyme at 10 mM MES buffer pH 6.2. As the matrix is negatively charged, the retention of lysozyme is expected. However, the elution of this protein occurred when a second step with addition of 750 mM NaCl in 50 mM $ZnSO_4$ in MES buffer was performed. The elution occurred due to the weakening of interactions between the protein and matrix, caused by NaCl competition effect.

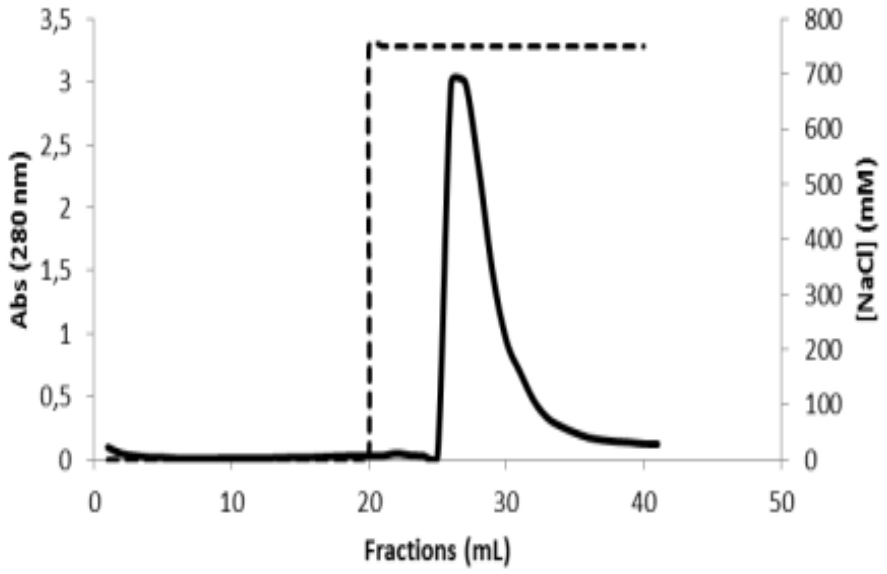

Figure 11 - Chromatographic profile obtained for lysozyme. The first step was performed with 10 mM MES buffer pH 6.2 and the second step was performed with 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Continuous line and dashed line represent the absorbance at 280 nm of the fractions and salt concentration, respectively.

Overall, the chromatographic behavior observed for α-chymotrypsin and lysozyme was the same, because both proteins were positively charged and separation assays were performed in same conditions. However, to determine the interaction force of both proteins to the matrix, a screening of several NaCl concentrations should be performed in order to find a concentration in which one protein is eluted and the other not.

4.2 Chromatographic assays with combined model proteins (BSA + α-chymotrypsin; BSA + lysozyme)

According to data obtained in previous results, the separation of two mixed proteins, which revealed different chromatographic behaviors, could be a promising strategy. For the separation of BSA + α-chymotrypsin and BSA + lysozyme it was applied the previously described elution strategy for the isolated proteins, because under these equilibrium conditions BSA should be eluted.

Figure 12 shows the resulting chromatogram for the separation of combined BSA + α-chymotrypsin. The column was equilibrated with 10 mM MES buffer pH 6.2 and the sample was loaded with the same buffer. After elution the unbound material, a second step was

imposed by changing of the elution buffer to 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2 and a second peak was also obtained.

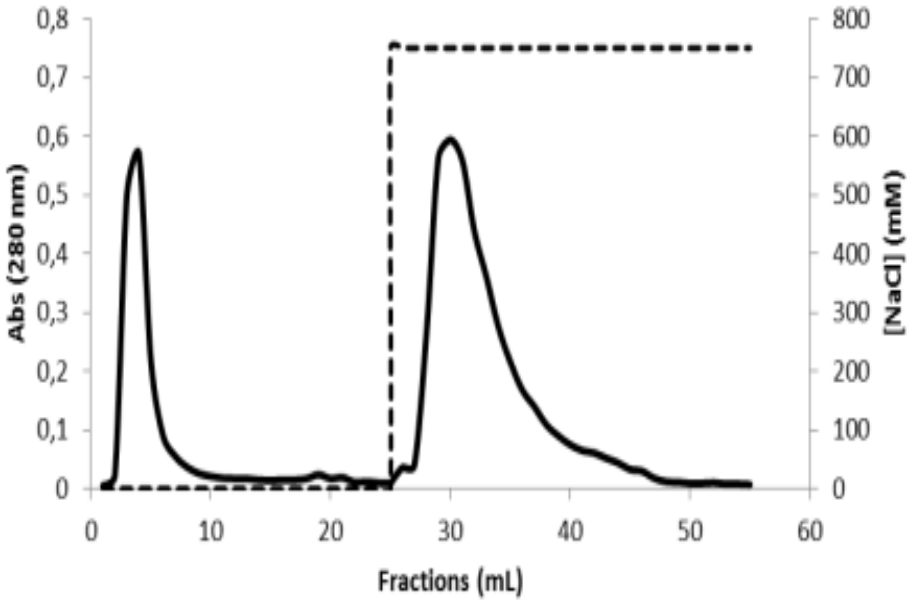

Figure 12 - Chromatographic profile obtained for BSA + α-chymotrypsin. The first step was performed with 10 mM MES buffer pH 6.2 and the second step was performed with 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Continuous line and dashed line represent the absorbance at 280 nm of the fractions and salt concentration, respectively.

Figure 13 shows SDS-PAGE electrophoresis analysis of peak fractions collected in the chromatographic assay of combined model proteins BSA + α-chymotrypsin. Lane III is according to lane I (BSA standard), so it concluded that line III corresponds to BSA. The bands appeared around 66 KDa [117], according its molecular weight, 66 KDa. Lane IV corresponds to α-chymotrypsin, because it was in accordance with line II, which correspond to its standard. α-Chymotrypsin molecular weight is 24.8 kDa [118] and electrophoresis bands appeared around this value. A good separation of the BSA and α-chymotrypsin is clearly evident.

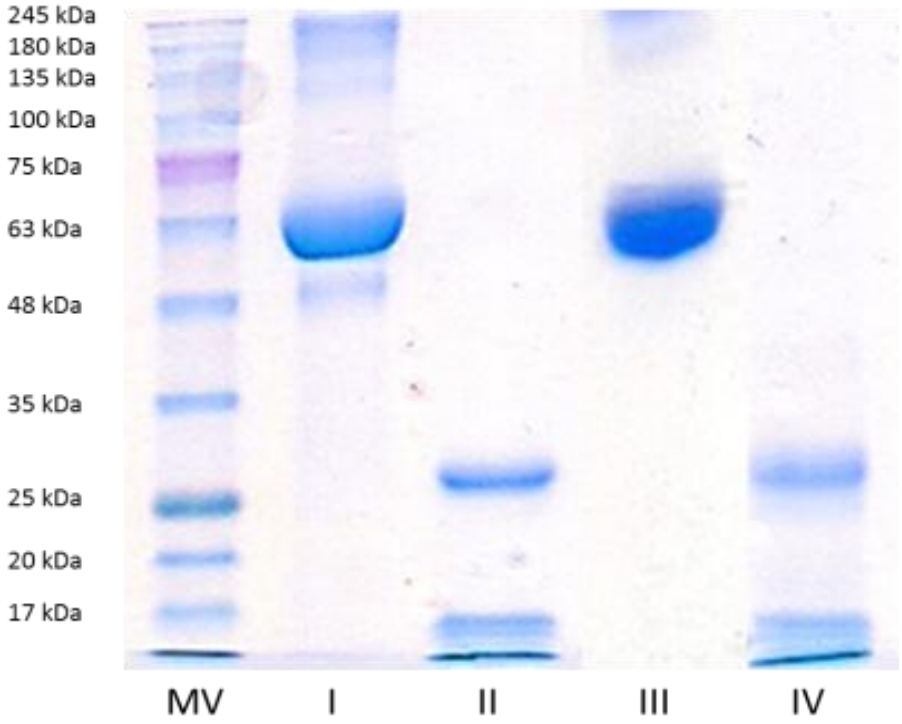

Figure 13 – SDS-PAGE electrophoresis analysis of peak fractions collected in the chromatographic assay. MW – Molecular weight standards; Lane I – BSA standard; Lane II – α-chymotrypsin standard; Lane III – First peak from combined BSA + α-chymotrypsin assay; Lane IV – Second peak from combined BSA + α-chymotrypsin.

By agarose gel electrophoresis analysis (Figure 13) of both chromatographic peaks represented in Figure 12, it was confirmed that BSA was immediately eluted in the flowthrough as it was expected, because this protein has the same net charge than the matrix, and α-chymotrypsin was eluted in the second peak. Unlike BSA, α-chymotrypsin was positively charged due to its isoelectric point be lower than the buffer pH (6.2) which favored the interaction with the matrix (negatively charged). The elution of α-chymotrypsin by increasing the NaCl concentration indicated that the ionic interaction was weakened by competition.

Figure 14 presents the chromatographic profile of the assay with combined model proteins BSA + lysozyme. As above mentioned for the previous assay, the column was equilibrated and the sample was loaded to the gellan matrix with 10 mM MES buffer. After elution the unbound material, a second step was imposed by changing of the elution buffer to 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2 and a second peak was also obtained.

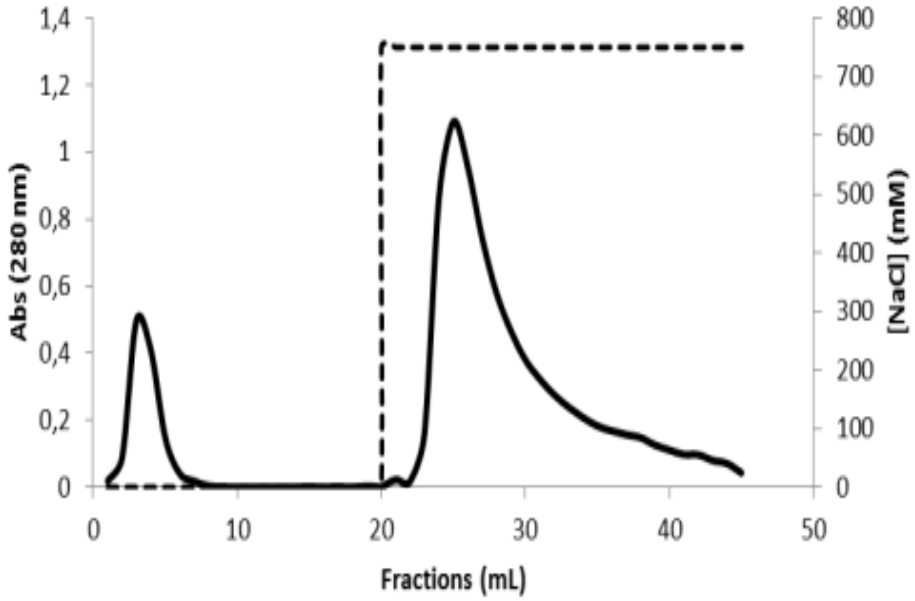

Figure 14 - Chromatografic profile obtained for BSA + lysozyme. The first step was performed with 10 mM MES buffer pH 6.2 and the second step was performed with 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Continuous line and dashed line represent the absorbance at 280 nm of the fractions and salt concentration, respectively.

Figure 15 shows SDS-PAGE electrophoresis analysis of peaks obtained in the chromatographic assay with combined model proteins BSA + lysozyme. Lane III corresponds to BSA, because it is in accordance with its standard, lane I that appeared at 66 KDa. Lane IV corresponds to lysosome, because its molecular weight is 13.900 KDa [119] and the band is in agreement with the molecular weight marker. When compared with the lysozyme standard, lane II, bands are in the same zone of migration. These results indicate that a good separation occurred between BSA and lysozyme.

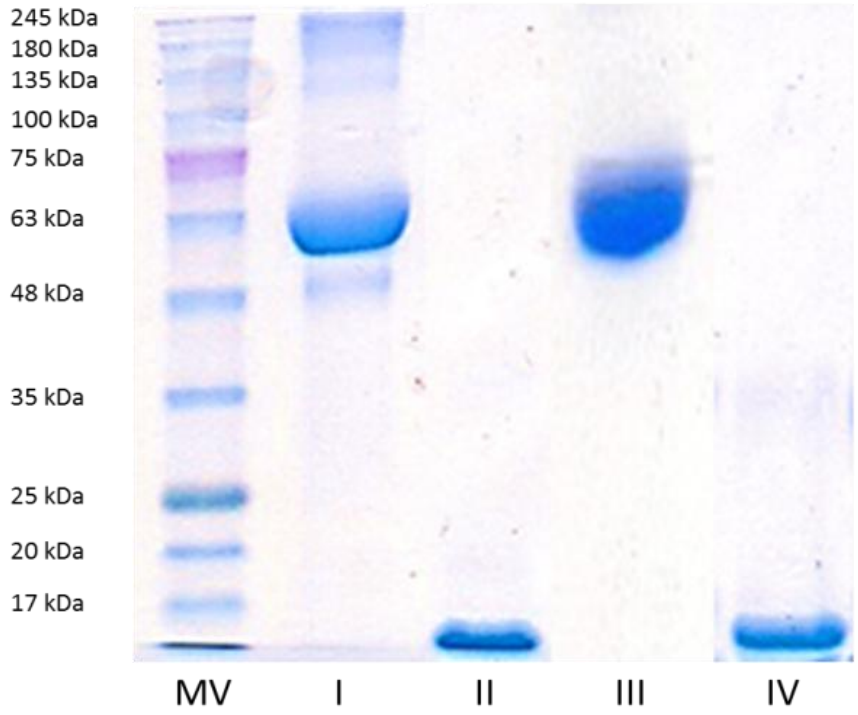

Figure 15 - SDS-PAGE electrophoresis analysis of peak fractions collected in the chromatographic assay. MW – Molecular weight standards; Lane I – BSA standard; Lane II – lysozyme standard; Lane III – First peak from combined BSA + lysozyme assay; Lane IV – Second peak from combined BSA + lysozyme.

By agarose gel electrophoresis analysis (Figure 15) of both chromatographic peaks represented in Figure 14, it was confirmed that BSA was immediately eluted in the flowthrough, because this protein has the same net charge than the matrix, there is no possible interaction. Taking into account that lysozyme was positively charged due to the pH buffer used in this assay, lysozyme interacted with the matrix. The interaction happened, because lysozyme and gellan polymer had opposite charges. However, the elution of lysozyme occurred when a second step with addition of 750 mM NaCl and 50 mM $ZnSO_4$ in MES buffer was performed, indicating that the ionic interaction was weakened by competition.

4.3 Chromatographic assay with combined model proteins (α-chymotrypsin + lysozyme)

As can be seen by previous assays, α-chymotrypsin and lysozyme eluted whenever 750 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2 passed into matrix. Therefore, to separate these two proteins other elution conditions which allowed their separation should be studied. Given that both proteins are retained when the column is equilibrated with 10mM MES buffer pH 6.2, a possible strategy to separate them could be the study of a small amount of NaCl in the equilibrium buffer in order to obtain a condition where one of these proteins do not bind to the matrix.

In these assays were tested a range of 50 – 350 mM NaCl concentrations in 50 $ZnSO_4$ and 10 mM MES buffer pH 6.2 for the first step. The best separation between α-chymotrypsin and lysozyme was obtained when 60 mM NaCl was applied in the equilibrium buffer (Figure 16). At these conditions, α-chymotrypsin was eluted and lysozyme was linked to the gellan matrix. Lysozyme elution only happened when the buffer changed to 750 mM NaCl in 50 $ZnSO_4$ and 10 mM MES buffer pH 6.2. As α-chymotrypsin elution occurred with lower NaCl concentration, it was concluded that the interactions of this protein with matrix are weaker than the interactions of lysozyme with matrix. Consequently, lysozyme elution needed a greater amount of NaCl.

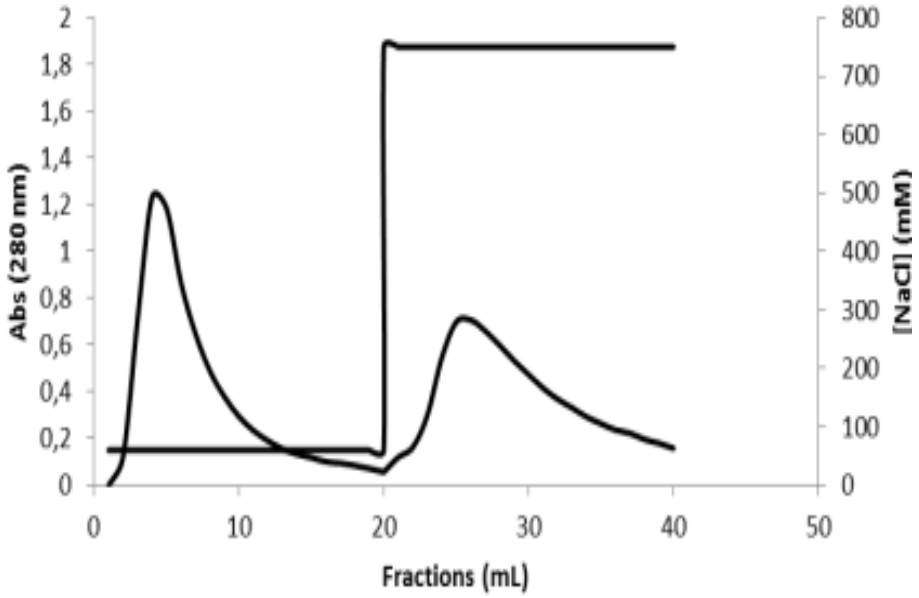

Figure 16 - Chromatographic profile obtained for α-chymotrypsin + lysozyme. The first step was performed with 60 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2 and the second step was performed with 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Continuous line and dashed line represent the absorbance at 280 nm of the fractions and salt concentration, respectively.

This test was performed to give some additional information of the behavior of lysozyme and α-chymotrypsin proteins that can be useful when a mix of three proteins (BSA + lysozyme + α-chymotrypsin) were used.

4.4 Chromatographic assay with combined model proteins (BSA + α-chymotrypsin + lysozyme)

After studying three model proteins and its combination two by two, it becomes interesting the combination study of three proteins in simultaneous. This assay was a little different from the previous, because it was constituted by three steps with a different composition buffer for each step. Thus, the gellan column was firstly equilibrated with 25 mM $ZnSO_4$ and 10 mM

MES buffer pH 6.2 and the sample of combined model proteins (BSA + α-chymotrypsin + lysozyme) was loaded under the same buffer. After elution the unbound material, a second step was performed with 60 mM NaCl in 50 $ZnSO_4$ and 10 mM MES buffer pH 6.2 and a second peak was obtained. Finally, the third peak was eluted when the elution buffer was changes to 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Figure 17 shows the chromatographic profile resultant of combined model proteins (BSA + α-chymotrypsin + lysozyme) and it is evident that different interactions occurred with the matrix.

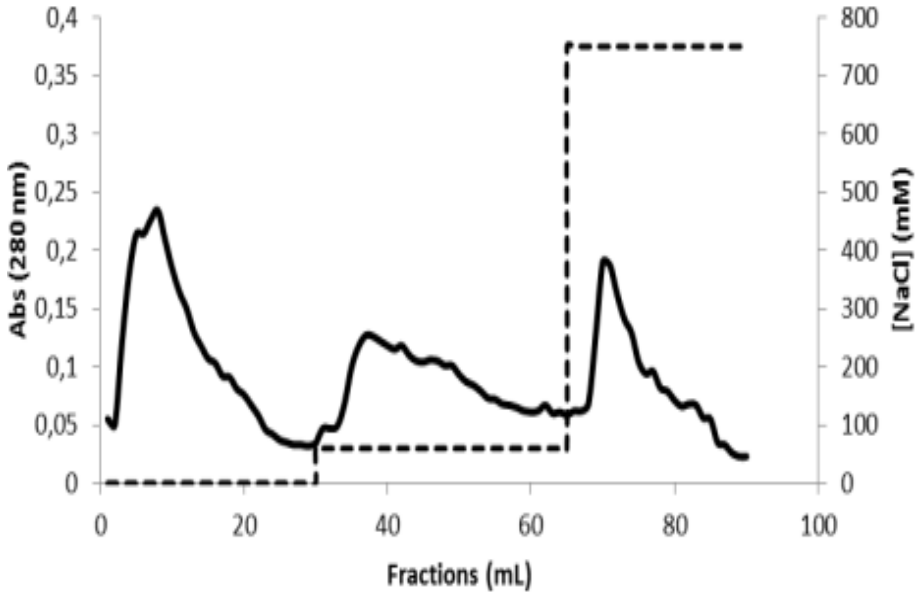

Figure 17 - Chromatografic profile obtained for BSA + α-chymotrypsin + lysozyme. The first step was performed in 25 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2, the second step was performed with 60 mM NaCl in 50 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2 and the third step was performed with 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2.

Figure 18 shows SDS-PAGE electrophoresis analysis of peak fractions collected in the chromatographic assay of combined model proteins BSA + α-chymotrypsin + lysozyme. Taking into account that lane V is accordingly to the BSA standard band (lane I) at 66 KDa, it is concluded that the protein eluted in first step was BSA. Lane VI is according to lane II, so it concluded that line VI corresponds to α-chymotrypsin. Finally, lane VII corresponds to lysozyme, because it was in accordance with line III, which correspond to its standard. Comparing with molecular weight standards are all in agreement.

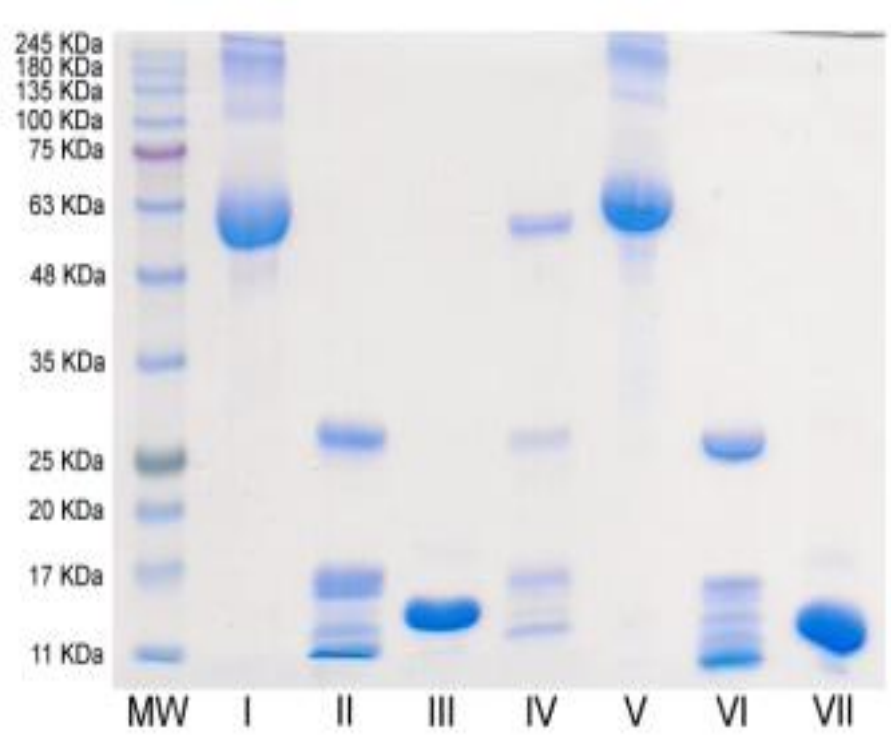

Figure 18 – SDS-PAGE electrophoresis analysis of the peak fractions collected in the chromatographic assays. MW – Molecular weight standards; Lane I – BSA standard; Lane II – α-chymotrypsin standard; Lane III – lysozyme standard; Lane IV – sample inject onto the column; Lane V – First peak from combined BSA + α-chymotrypsin + lysozyme assay; Lane VI – Second peak from combined BSA + α-chymotrypsin + lysozyme assay; Lane VII – Third peak from combined BSA + α-chymotrypsin + lysozyme assay.

The pH of buffers used in this assay was always the same, because under these conditions, proteins had different isoelectric points. Consequently, BSA had negative charge, whereas α-chymotrypsin and lysozyme had positive charge. Taking into account that the matrix had negative charge due to its anionic nature, it can differently interact with the proteins.

By agarose gel electrophoresis analysis (Figure 18) of chromatographic peaks represented in Figure 17, it was confirmed that BSA was eluted in the first step at 25 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2. Accordingly, BSA was the first protein to elute without NaCl in buffer, which means that this protein was not bound to the matrix. In the second step, NaCl and $ZnSO_4$ were increased in order to cause the elution of a protein and maintain the gel stability. Consequently, α-chymotrypsin was eluted, because the interaction of this protein with the matrix is weaker than interaction of the lysozyme with the matrix. In the third step, the NaCl concentration was again increased and the lysozyme, which promoted the stronger interaction to the gellan matrix, was eluted by salt competition.

4.5 Chromatographic assay with combined model proteins (BSA + α-chymotrypsin + lysozyme) with pH variation in the elution buffer

This chromatographic assay was made in order to study the possible protein elution by changes in pH of the buffer. Taking into account that the pH value affects protein isoelectric points, in this assay it was tested stepwise elution based on pH and NaCl concentration variations. Stepwise elution included three steps.

Figure 19 shows the resulting chromatogram for the separation of combined model proteins (BSA + α-chymotrypsin + lysozyme) with pH variation in the elution buffer. First, the column was equilibrated with 25 mM $ZnSO_4$ in 10 mM MES 6.2 and the sample was loaded onto the matrix under the same elution conditions, it being obtained a first peak in the flowthrough. In the second step, the pH was changed of 6.2 to 10 in 10 mM carbonate buffer (since a basic pH was intended) supplemented with50 mM $ZnSO_4$ only to maintain the gel stability, and a second peak was obtained. Finally, a last peak was obtained only by the change of NaCl concentration to 750 mM in 100 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10.

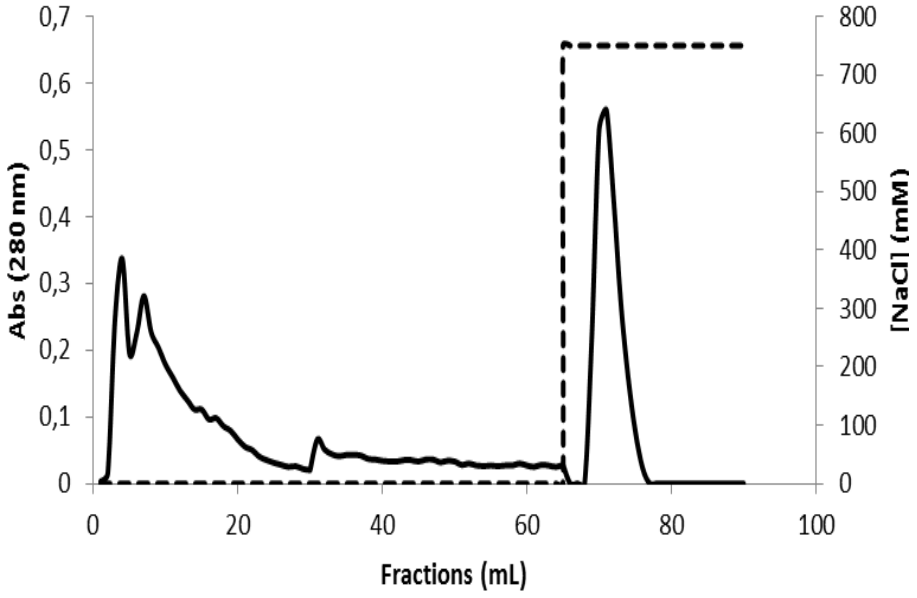

Figure 19 - Chromatografic profile obtained for BSA + α-chymotrypsin + lysozyme. The first step was performed in 25 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2, the second step was performed 50 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10 and the third step was performed with 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10.

Figure 20 shows SDS-PAGE electrophoresis of each peak obtained in the chromatographic assay with combined model proteins BSA + α-chymotrypsin + lysozyme with pH variation in the elution buffer.

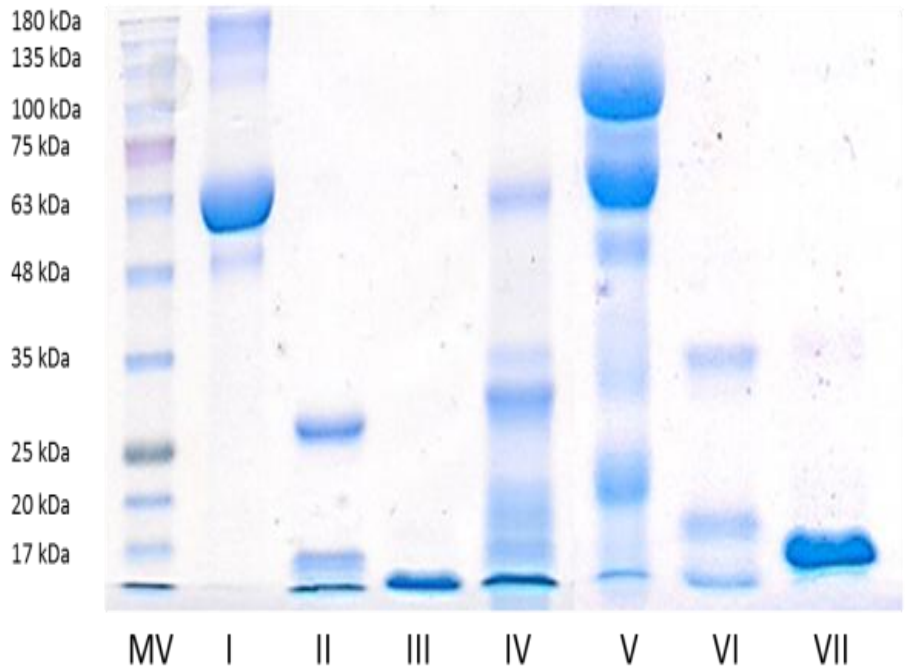

Figure 20 – SDS-PAGE electrophoresis analysis of the peak fractions collected in the chromatographic assays. MW – Molecular weight standards; Lane I – BSA standard; Lane II – α-chymotrypsin standard; Lane III – lysozyme standard; Lane IV – sample inject onto the column; Lane V – First peak from combined BSA + α-chymotrypsin + lysozyme with pH buffers variation assay; Lane VI – Second peak from combined BSA + α-chymotrypsin + lysozyme with pH buffers variation assay; Lane VII – Third peak from combined BSA + α-chymotrypsin + lysozyme with pH buffers variation assay.

Lane I, II and III correspond to BSA, α-chymotrypsin and lysozyme standard, respectively. Lane IV corresponds to the sample inject onto the column. Lane V corresponds to the collected fraction in the first peak when it was used 25 mM $ZnSO_4$ in 10 mM MES 6.2 and various bands were observed. Lane VI presents the collected fraction in the second peak when it was used 50 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10 and corresponds to α-chymotrypsin, because the band was according to the standard α-chymotrypsin in lane II. Lastly, lane VII corresponds to the collected fraction in the last chromatographic step when it was used 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10, corresponding to the lysozyme.

By agarose gel electrophoresis analysis (Figure 20) of both chromatographic peaks represented in Figure 19, it was confirmed that various bands were observed in lane V, which means that there is not a complete retention of both α-chymotrypsin and lysozyme proteins, which were partially eluted with the BSA. So, it was supposed that some interaction occurred between proteins, consequently forming aggregates that are not available to bind the matrix.

In the second step, the pH variation form 6.2 to 10 in the buffer induced the α-chymotrypsin elution, whereas the lysozyme remained bound. This behavior is justified by the isoelectric points of each protein. Given that α-chymotrypsin and lysozyme have the isoelectric points of 8.6 and 11 respectively, the elution buffer with pH 10 led to an change in the net charge of α-chymotrypsin from positive to negative that was eluted in the second peak, whereas the same did not happen to lysozyme. Under these elution conditions, lysozyme maintained a positive global charge. As it was aforementioned, gellan matrix is a negative polymer and proteins with the same charge are eluted. Finally, lysozyme was only eluted by the application of 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM carbonate buffer pH 10. In this step, only NaCl concentration was increased and it was not varied the buffer pH, because extreme conditions can lead to the loss of gel stability. However, with the increment in NaCl concentration, interactions between lysozyme and matrix were weakened by competition, resulting in protein elution.

After evaluating the last chromatograms and the respective SDS-PAGE electrophoresis, it was concluded that gellan matrix revealed a behavior of cation exchange support that allows the protein elution either by salt or pH manipulation.

5. Dynamic Binding Capacity

In order to characterize gellan gum as a chromatography matrix, it was studied the dynamic binding capacity parameter.

For the determination of dynamic binding capacity, gellan gel was prepared according to experimental design conditions and it was equilibrated with 25 mM $ZnSO_4$ in 10 mM MES buffer pH 6.2. Thereafter, the matrix was overloaded with 0.5 mg/mL of lysozyme in 25 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2, at a flow rate of 1 mL/min. Afterwards, the elution of the bound lysozyme was achieved by increasing the ionic strength to 750 mM NaCl in 100 mM $ZnSO_4$ and 10 mM MES buffer pH 6.2.

Dynamic binding capacity values were then obtained by subtracting the value obtained under non-binding conditions, according follow equation:

$$DBC = \frac{(VL - Vo) \times Cp}{Vc} \quad (1)$$

Where DBC is a dynamic binding capacity (mg/mL), VL corresponds to volume loaded up to the breakthrough point (mL), Vo is a void volume of the column (mL) and Cp corresponds to concentration of protein (mg).

Determination of dynamic binding capacity was carried out by recording breakthrough curves and calculating the amount of bound lysozyme per mL support at 10% and 50% of

breakthrough curve. Figure 20 represents the values of dynamic binding capacity of the gellan matrix. Thus, values of dynamic binding capacity obtained at 10% and 50% were 3.9 mg/mL and 17.4 mg/mL, respectively. The capacity values obtained for gellan matrix are satisfactory and strengthen the applicability of this matrix.

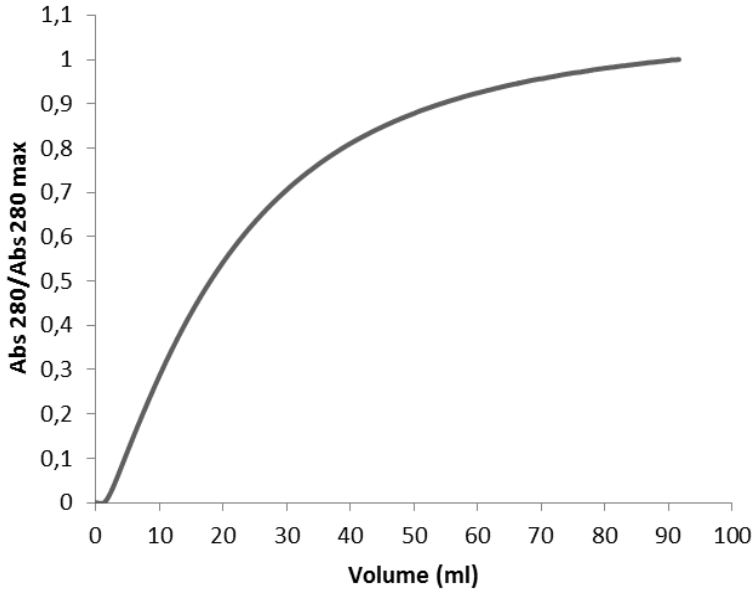

Figure 21 - Dynamic binding capacity of gellan gum for 0.5 mg/mL solution lysozyme at 1 mL/min flow rate.

Table 8 shows dynamic binding capacity values of several chromatographic matrices with different structural properties and different ligands, previously referred. Thus, for cation exchange chromatographic matrices, values varied from 18 to 37 mg/mL, at 10% and from 30 to 60 mg/mL at 50%. For matrices used in affinity chromatography range of values obtained at 10% and 50% were from 4 to 20 mg/mL and from 5 to 24 mg/mL, respectively. Comparing these values with the values of the gels used for cation exchange chromatography (Fractogel SE HICAP (M), MacroPrep CM and SP Toyopearl 650m), it was concluded that are lower. Moreover, comparing the values of the gels used in affinity chromatography (Heparin Sepharose FF, Heparin and Heparin Toyopearl 650m Ceramic HYPERD M), it was concluded that the values of dynamic binding capacity of the gellan gum to 10% and 50% are similar [115,116].

Table 8 – Dynamic binding capacity of cation-exchange resins and heparin for 0.5 mg/ml solution lysozyme.

Resin	Flow rate (ml/min)	Lysozyme concentration (mg/ml)	Dynamic Binding Capacity (mg/ml)	
			10 %	50%
Fractogel SE HICAP (M)	1.3	1.0	37.0	60.0
MacroPrep CM	2.5	1.0	18.0	30.0
SP Toyopearl 650m	1.3	1.0	30.0	42.0
Heparin Sepharose FF	0.6	1.0	4.0	5.0
Heparin Toyopearl 650m	1.3	1.0	17.0	21.0
Heparin Ceramic HyperD M	4.0	1.0	20.0	24.0
Gellan Gum	1.0	0.5	3.9	17.4

As in some cases, these values reflect the functionality of the immobilized ligands at each support, the modification of gellan matrix by specific ligand immobilization can be a good possibility to increase the support binding capacity in the future.

Chapter V - Conclusion

Over the years, the development of new techniques for the purification of biomolecules has been a topic of great importance and continuous progress. Chromatography represents part of these purification methods and has played a key role in the biotechnological industry.

Gellan gum is a natural polysaccharide that revealed great potential to become a chromatographic matrix, due to its gelling ability. After testing different gel formulations, ideal conditions (0.75% gellan, 48 mM $ZnSO_4$, 0 % DMF, 25ºC, and 0.5 hours) were found with experimental design application.

The applicability of gellan gun matrix was tested with three model proteins (BSA, α-chymotrypsin and lysozyme) and consequently their separation was accomplished, depending on their charge and taking into account the concept of cation exchange chromatography. The interaction of the matrix with the proteins was established in different levels. Therefore, BSA did not interact with the matrix, while the others two proteins (lysozyme and α-chymotrypsin) were retained to the column, it being eluted at different NaCl concentrations. In fact, this matrix revealed different interaction forces with proteins positively charged, which it is reflected in a characteristic chromatographic behavior.

In order to better characterize this new matrix, determination of the dynamic binding capacity was estimated. Dynamic binding capacity values were satisfactory for an innovative chromatographic matrix and are also according to the commercial resins values.
As this study represents only the beginning of the formulation, characterization and application of the gellan gum in chromatographic processes, learning and adapting the matrix to specific objectives must continue to subsequently get the best benefit from its use.

Chapter VI – Futures perspectives

Chromatography is one of the most studied methods, due to its simplicity, versatility and high reproducibility, to separate and purify biomolecules that can have therapeutic or industrial interest.

Gellan gum application as chromatographic matrix is a new approach in this area. Therefore, various chromatographic parameters have yet to be studied since the present study is an initial work. For the purpose of characterize this matrix, ionic and electrostatic interactions, binding strength, compressibility and pore size distribution should be made. The success of these concepts depends on the intrinsic characteristics of the gellan gum. Evaluation of o these basic concepts about the matrix properties must allow to increase the stability and optimization for a particular separation purpose.

In order to increase gel stability, the preparation of gel formulations with other divalent cations such as magnesium and strontium should be done. Nickel can be a good choice, to use in gel formulation, because these ions being chelated form reversible bonds with certain amino acid residues such as imidazole of histidine. In this way, would be interesting to test more complex samples, namely cellular extracts with recombinant proteins containing histidine tails (tag) incorporated. Consequently, depending on the results, gellan gum can be explored as chromatographic matrix in others chromatography types, such as IMAC. The gellan matrix modification with specific ligands should also be explored, since it would give a versatility of interactions to this innovative matrix with countless target molecules with different characteristics.

In the future, if studies indicate that matrix stability is high and efficient, it could lead to different applications and possibly to scale-up manufacturing.

References

[1] G. Healthcare, *Strategies for protein purification*. Uppsala, 2010.

[2] R. Hutti-Kaul and B. Mattiasson, *Isolation and Purification of proteins*. Marcel Dekker, Inc, New York, 2003.

[3] B. R. Markham and J. D. Smith, "Chromatographic Studies of Nucleic Acids," *Biochemical Journal*, vol. 49, pp. 294–298, 1951.

[4] A. Sousa, F. Sousa, and J. a Queiroz, "Differential interactions of plasmid DNA, RNA and genomic DNA with amino acid-based affinity matrices." *Journal of separation science*, vol. 33, pp. 2610–8, 2010.

[5] A. Biosciences, *Protein Purification*. Amersham Biosciences AB, Uppsala, 2001.

[6] A. Sousa, F. Sousa, and J. a Queiroz, "Advances in chromatographic supports for pharmaceutical-grade plasmid DNA purification." *Journal of separation science*, vol. 35, pp. 3046–58, 2012.

[7] J. a Queiroz, C. T. Tomaz, and J. M. Cabral, "Hydrophobic interaction chromatography of proteins." *Journal of biotechnology*, vol. 87, pp. 143–59, 2001.

[8] S. H. AE Karlström, *Chromatographic methods for protein purification*. Stockholm, 2006.

[9] S. R. Narayanan, "Review Preparative affinity chromatography of proteins." *Journal of chromatography. A*, vol. 658, pp. 237-258, 1994.

[10] R. Prasad, "Purification, reconstitution, and functional characterization of zinc transporter from rat renal brush border membranes." *Methods in molecular biology*, vol. 228, pp. 195–221, 2003.

[11] E. R. Francotte, "Enantioselective chromatography as a powerful alternative for the preparation of drug enantiomers." *Journal of chromatography. A*, vol. 906, pp. 379–97, 2001.

[12] A. Jungbauer, "Review Preparative chromatography of biomolecules." *Journal of chromatography. A*, vol. 639, pp. 3–16, 1993.

[13] E. Biotec, "Theoretical aspects of affinity chromatography." Journal of Biotechnology, vol. 11, pp. 143–160, 1989.

[14] L. Janson, J. C. e Rydén, *Protein Purification: Principles, High Resolution Methods, and Applications*. Uppsala, 1989.

[15] S. Harris, E L. V. Angal, *Protein Purificacion Methods: A Practical Appoach*. Oxford, 1989.

[16] S. M. Cramer and G. Jayaraman, "Preparative chromatography in biotechnology." *Current opinion in biotechnology*, vol. 4, pp. 217–25, 1993.

[17] G. Guiochon, "Preparative liquid chromatography." *Journal of chromatography. A*, vol. 965, pp. 129–61, 2002.

[18] J. F. K. H. A. Weltrli, V. Hermann, "Effect of phase system selectivity in preparative column liquid chromatography." *Journal of chromatography*, vol. 125, pp. 59–70, 1976.

[19] E. M. V. Garcia, F.A.P. Pires, *Recovery Processes for Biological Materials*. New York, 1993.

[20] S. Ghose and S. M. Cramer, "Characterization and modeling of monolithic stationary phases: application to preparative chromatography." *Journal of chromatography. A*, vol. 928, pp. 13–23, 2001.

[21] L. Miller, "Evaluation of non-traditional modifiers for analytical and preparative enantioseparations using supercritical fluid chromatography." *Journal of chromatography. A*, vol. 1256, pp. 261–6, 2012.

[22] G. Briefs and R. Kula, "Fast protein chromatography on analytical and preparative scale using modified." *Chemical Engineering Science*, vol. 47, pp. 141–149, 1992.

[23] S. A. Carr, M. E. Hemling, M. F. Bean, and G. D. Roberts, "Perspective: analytical biotechnology Integration of Mass Spectrometry in Analytical Biotechnology." *Analytical Chemistry*, vol. 63 pp. 2802–2824, 1991.

[24] S. Fekete, E. Oláh, and J. Fekete, "Fast liquid chromatography: the domination of core-shell and very fine particles." *Journal of chromatography. A*, vol. 1228, pp. 57–71, 2012.

[25] D. Hess, T. C. Covey, R. Winz, R. W. Brownsey, and R. Aebersold, "Analytical and micropreparative peptide mapping by high performance liquid chromatography/electrospray mass spectrometry of proteins purified by gel

electrophoresis." *Protein science : a publication of the Protein Society*, vol. 2, pp. 1342–51, 1993.

[26] F. Sousa, D. M. F. Prazeres, and J. a Queiroz, "Affinity chromatography approaches to overcome the challenges of purifying plasmid DNA." *Trends in biotechnology*, vol. 26, pp. 518–25, 2008.

[27] M. M. Diogo, J. a. Queiroz, and D. M. F. Prazeres, "Chromatography of plasmid DNA." *Journal of Chromatography A*, vol. 1069, pp. 3–22, 2005.

[28] X. Geng and L. Wang, "Liquid chromatography of recombinant proteins and protein drugs." *Journal of chromatography. B,* vol. 866, pp. 133–53, 2008.

[29] A. Biosciences, *Gel filtration*. Amersham Biosciences AB, Uppsala, 2002.

[30] M. Hutta, R. Góra, R. Halko, and M. Chalanyova, "Some theoretical and practical aspects in the separation of humic substances by combined liquid chromatography methods." *Journal of chromatography. A*, vol. 1218, pp. 8946–57, 2011.

[31] C. Laurent and J. K. Llander, "A theory of gel filtration and its experimental verefication." *Journal of chromatography. A*, vol. 14, pp. 317–330, 1964.

[32] D. Glick, *Methods of Biochemical Analysis*. John Wiley & Sons, Inc.vol. 18. USA, 1970.

[33] H. S. Carola Hunte, Gebhard von Jagow, *Membrane protein purification and crystallization*. Academic press, Frankfurt, 2003.

[34] G. Healthcare, *Hydrophobic Interaction and Reversed Phase Chromatography*. Uppsala, 2006.

[35] P. Jandera, "Stationary and mobile phases in hydrophilic interaction chromatography: a review." *Analytical chimical acta*, vol. 692, pp. 1–25, 2011.

[36] C. Horvath and W. Melander, "Liquid Chromatography with Hydrocarbonaceous Bonded Phases; Theory and Practice of Reversed Phase Chromatography" *Journal of Chromatographic Science*, vol. 15, pp. 393–404, 1977.

[37] D. S. Hage, "High-performance affinity chromatography: a powerful tool for studying serum protein binding." *Journal of chromatography. B,* vol. 768, pp. 3–30, 2002.

[38] D. S. Hage, "Affinity chromatography: a review of clinical applications." *Clinical chemistry*, vol. 45, pp. 593–615, 1999.

[39] I. Tadeu, L. Bresolin, E. Alves, S. Maria, and A. Bueno, "Immolilized metal-ion affinity chromatography (IMAC) of biomolecules: fundamental aspects and technological applications." vol. 32, pp. 1288–1296, 2009.

[40] H. Block, B. Maertens, A. Spriestersbach, N. Brinker, J. Kubicek, R. Fabis, J. Labahn, and F. Schäfer, "Immobilized-metal affinity chromatography (IMAC): a review." *Methods in enzymology*, vol. 463, pp. 439–73, 2009.

[41] A. Jungbauer and R. Hahn, "Ion-exchange chromatography." *Methods in enzymology*, vol. 463, pp. 349–71, 2009.

[42] A. Biosciences, *Ion Exchange Chromatography & Chromatofocusing*. Amersham Biosciences AB, Uppsala, 2004.

[43] T. Ishihara and S. Yamamoto, "Optimization of monoclonal antibody purification by ion-exchange chromatography." *Journal of Chromatography A*, vol. 1069, pp. 99–106, 2005.

[44] S. Yamamoto and T. Ishihara, "Resolution and Retention of Proteins near Isoelectric Points in Ion-Exchange Chromatography. Molecular Recognition in Electrostatic Interaction Chromatography." *Separation Science and Technology*, vol. 35, pp. 1707–1717, 2000.

[45] P. Gagnon, "Technology trends in antibody purification." *Journal of chromatography. A*, vol. 1221, pp. 57–70, 2012.

[46] S. Yamamoto, P. K. Watler, D. Feng, and O. Kaltenbrunner, "Characterization of unstable ion-exchange chromatographic separation of proteins." *Journal of chromatography. A*, vol. 852, pp. 37–41, 1999.

[47] L. a Passarinha, M. J. Bonifácio, P. Soares-da-Silva, and J. a Queiroz, "A new approach on the purification of recombinant human soluble catechol-O-methyltransferase from an Escherichia coli extract using hydrophobic interaction chromatography." *Journal of chromatography. A*, vol. 1177, pp. 287–96, 2008.

[48] B. V. Ayyar, S. Arora, C. Murphy, and R. O'Kennedy, "Affinity chromatography as a tool for antibody purification." *Methods*, vol. 56, pp. 116–29, 2012.

[49] J. Billen and G. Desmet, "Understanding and design of existing and future chromatographic support formats." *Journal of chromatography. A*, vol. 1168, pp. 73–99; discussion 71–2, 2007.

[50] A. Jungbauer, "Chromatographic media for bioseparation." *Journal of Chromatography A*, vol. 1065, pp. 3–12, 2005.

[51] J. J. Stickel and a Fotopoulos, "Pressure-flow relationships for packed beds of compressible chromatography media at laboratory and production scale." *Biotechnology progress*, vol. 17, pp. 744–51, 2001.

[52] A. Sousa, F. Sousa, and J. A. Queiroz, "Impact of lysine-affinity chromatography on supercoiled plasmid DNA purification." *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, vol. 879, pp. 3507–15, 2011.

[53] N. R. Deshmukh and A. M. Lali, "Adsorptive purification of pDNA on superporous rigid cross-linked cellulose matrix." *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, vol. 818, pp. 5–10, 2005.

[54] D. Kendall, G. J. Lye, and M. S. Levy, "Purification of plasmid DNA by an integrated operation comprising tangential flow filtration and nitrocellulose adsorption." *Biotechnology and bioengineering*, vol. 79, pp. 816–22, 2002.

[55] P. E. Gustavsson, R. Lemmens, T. Nyhammar, P. Busson, and P.-O. Larsson, "Purification of plasmid DNA with a new type of anion-exchange beads having a non-charged surface." *Journal of Chromatography A*, vol. 1038, pp. 131–140, 2004.

[56] R. Giovannini and R. Freitag, "Continuous Isolation of Plasmid DNA by Annular Chromatography." Biotechnology and Bioengineering, vol. 77, pp. 445–454, 2002.

[57] T. R. Strick, J. F. Allemand, D. Bensimon, and V. Croquette, "Behavior of supercoiled DNA." *Biophysical journal*, vol. 74, pp. 2016–28, 1998.

[58] A Kurganov, Y. U. Puchkova, V. Davankov, and F. Eisenbeiss, "Polyvinylpyrrolidone-coated silica packings for chromatography of proteins and peptides.," *Journal of chromatography. A*, vol. 663, pp. 163–74, Mar. 1994.

[59] I. Perçin, E. Sağlar, H. Yavuz, E. Aksöz, and A. Denizli, "Poly(hydroxyethyl methacrylate) based affinity cryogel for plasmid DNA purification." *International journal of biological macromolecules*, vol. 48, pp. 577–82, 2011.

[60] M. Wu, R. Kempaiah, P.-J. J. Huang, V. Maheshwari, and J. Liu, "Adsorption and Desorption of DNA on Graphene Oxide Studied by Fluorescently Labeled Oligonucleotides." J*ournal of surfaces and colloids*, pp. 2731–2738, 2011.

[61] T. Rodrigues, M. J. T. Carrondo, P. M. Alves, and P. E. Cruz, "Purification of retroviral vectors for clinical application: biological implications and technological challenges." *Journal of biotechnology*, vol. 127, pp. 520–41, 2007.

[62] T. B. Tennikova and F. Svec, "High-performance membrane chromatography: Highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes." *Journal of Chromatography A*, vol. 646, pp. 279–288, 1993.

[63] A. Jungbauer and R. Hahn, "Monoliths for fast bioseparation and bioconversion and their applications in biotechnology." *Journal of Separation Science*, vol. 27, pp. 767–778, 2004.

[64] E. Müller, "Properties and Characterization of High Capacity Resins for Biochromatography." *Chemical Engineering & Technology*, vol. 28, pp. 1295–1305, 2005.

[65] H. L. Knudsen, R. L. Fahrner, Y. Xu, L. a Norling, and G. S. Blank, "Membrane ion-exchange chromatography for process-scale antibody purification." *Journal of chromatography. A*, vol. 907, pp. 145–54, 2001.

[66] A. Sousa, D. Bicho, C. T. Tomaz, F. Sousa, and J. a Queiroz, "Performance of a non-grafted monolithic support for purification of supercoiled plasmid DNA." *Journal of chromatography. A*, vol. 1218, pp. 1701–6, 2011.

[67] R. E. F. Boto, U. Anyanwu, F. Sousa, P. Almeida, and J. a Queiroz, "Thiacarbocyanine as ligand in dye-affinity chromatography for protein purification. II. Dynamic binding capacity using lysozyme as a model." *Biomedical chromatography : BMC*, vol. 23, pp. 987–93, 2009.

[68] Y.-K. Chang and I. P. Chang, "Method development for direct recovery of lysozyme from highly crude chicken egg white by stirred fluidized bed technique." *Biochemical Engineering Journal*, vol. 30, pp. 63–75, 2006.

[69] I. Mihelic, A. Podgornik, and T. Koloini, "Temperature influence on the dynamic binding capacity of a monolithic ion-exchange column." *Journal of chromatography. A*, vol. 987, pp. 159–68, 2003.

[70] A. H. Krauland, V. M. Leitner, and A. Bernkop-Schnürch, "Improvement in the in situ gelling properties of deacetylated gellan gum by the immobilization of thiol groups." *Journal of pharmaceutical sciences*, vol. 92, pp. 1234–41, 2003.

[71] K. Urayama, Y. Taoka, K. Nakamura, and T. Takigawa, "Markedly compressible behaviors of gellan hydrogels in a constrained geometry at ultraslow strain rates." *Polymer*, vol. 49, pp. 3295–3300, 2008.

[72] R. E. González-Cuello, E. G. Ramos-Ramírez, A. Cruz-Orea, and J. A. Salazar-Montoya, "Rheological characterization and activation energy values of binary mixtures of gellan." *European Food Research and Technology*, vol. 234, pp. 305–313, 2011.

[73] T. P. West, "Improved Polysaccharide Production Using Strain Improvement." *Methods in Biotechnology*, vol. 18, 2005.

[74] H. Lee, S. Fisher, M. S. Kallos, and C. J. Hunter, "Optimizing gelling parameters of gellan gum for fibrocartilage tissue engineering." *Journal of biomedical materials research. Part B, Applied biomaterials*, vol. 98B, pp. 238–45, 2011.

[75] R. Mao, J. Tang, and B. G. Swanson, "Texture properties of high and low acyl mixed gellan gels." *Carbohydrate Polymers*, vol. 41, pp. 331–338, 2000.

[76] I. B. Bajaj, S. A. Survase, P. S. Saudagar, and R. S. Singhal, "Gellan Gum : Fermentative Production , Downstream Processing and Applications." vol. 45, pp. 341–354, 2007.

[77] H. N. Cheng and T. G. Neiss, "Solution NMR Spectroscopy of Food Polysaccharides." *Polymer Reviews*, vol. 52, pp. 81–114, 2012.

[78] Y. Huang, J. Tang, B. G. Swanson, and B. a. Rasco, "Effect of calcium concentration on textural properties of high and low acyl mixed gellan gels." *Carbohydrate Polymers*, vol. 54, pp. 517–522, 2003.

[79] E. R. Morris, K. Nishinari, and M. Rinaudo, "Gelation of gellan – A review." *Food Hydrocolloids*, vol. 28, pp. 373–411, 2012.

[80] A. M. Fialho, L. M. Moreira, A. T. Granja, A. O. Popescu, K. Hoffmann, and I. Sá-Correia, "Occurrence, production, and applications of gellan: current state and perspectives." *Applied microbiology and biotechnology*, vol. 79, pp. 889–900, 2008.

[81] E. Yabuuchi, I. Yano, H. Oyaizu, Y. Hashimoto, T. Ezaki, and H. Yamamoto, "Proposals of Sphingomonas paucimobilis gen. nov. and comb. nov., Sphingomonas parapaucimobilis sp. nov., Sphingomonas yanoikuyae sp. nov., Sphingomonas adhaesiva sp. nov., Sphingomonas capsulata comb. nov., and two genospecies of the genus Sphingomonas." *Microbiology and immunology*, vol. 34, pp. 99–119, 1990.

[82] A. M. Fialho, L. O. Martins, M. Donval, J. H. Leitão, M. J. Ridout, A. J. Jay, J. Victor, I. Sá-correia, N. I. O. M. Fialho, V. J. Morris, and I. Sa, "Structures and Properties of Gellan Polymers Produced bySphingomonas paucimobilis ATCC 31461 from Lactose Compared with Those Produced from Glucose and from Cheese Whey Structures and Properties of Gellan Polymers Produced by Sphingomonas paucimobilis ATC." *Applied and environmental microbiology*, vol. 65, pp. 2485–2491, 1999.

[83] I. B. Bajaj, P. S. Saudagar, R. S. Singhal, and A. Pandey, "Statistical approach to optimization of fermentative production of gellan gum from Sphingomonas paucimobilis ATCC 31461." *Journal of bioscience and bioengineering*, vol. 102, pp. 150–6, 2006.

[84] K. M. Nampoothiri, R. R. Singhania, C. Sabarinath, and A. Pandey, "Fermentative production of gellan using Sphingomonas paucimobilis." *Process Biochemistry*, vol. 38, pp. 1513–1519, 2003.

[85] N. Requirements, "Studies on the Exopolysaccharide from Sphingomonas paucimobilis-GSl: Nutritional Requirements and Precursor-Forming Enzymes." vol. 31, pp. 234–238, 1995.

[86] D. Lobas, S. Schumpe, and W. Deckwer, "The production of gellan exopolysaccharide with Sphingomonas paucimobilis E2 (DSM 6314)." *Applied Microbiology and Biotechnology*, vol. 31 pp. 411–415, 1992.

[87] H. Jin, N.-K. Lee, M.-K. Shin, S.-K. Kim, D. L. Kaplan, and J.-W. Lee, "Production of gellan gum by Sphingomonas paucimobilis NK2000 with soybean pomace." *Biochemical Engineering Journal*, vol. 16, pp. 357–360, 2003.

[88] B. Manna, a. Gambhir, and P. Ghosh, "Production and rheological characteristics of the microbial polysaccharide gellan." *Letters in Applied Microbiology*, vol. 23, pp. 141–145, 2008.

[89] I. R. I. C. Dreveton, D. I. Ric, D. Ballerini, J. Lecourtier, I. Francais, A. De Bois, R. Malmaison, and I. Cedex, "Effect of Mixing and Mass Transfer Conditions on Gellan Production by Auromonas elodea." vol. 77, pp. 642–649, 1994.

[90] I. Giavasis, L. M. Harvey, and B. McNeil, "The effect of agitation and aeration on the synthesis and molecular weight of gellan in batch cultures of Sphingomonas paucimobilis." *Enzyme and Microbial Technology*, vol. 38, pp. 101–108, 2006.

[91] D. Rho, A. Mulchandani, J. H. T. Luong, and A. Leduy, "Applied Microbiology Biotechnology Oxygen requirement in pullulan fermentation." *Applied Microbiology and Biotechnology*, vol. 28 pp. 361–366, 1988.

[92] U. Ran, E. Gura, E. Olszewski, and F. Wagner, "Enhanced glucan formation of filamentous fungi by effective mixing, oxygen limitation and fed-batch processing." vol. 9, pp. 19–25, 1992.

[93] R. M. Banik and a Santhiagu, "Improvement in production and quality of gellan gum by Sphingomonas paucimobilis under high dissolved oxygen tension levels." *Biotechnology letters*, vol. 28, pp. 1347–50, 2006.

[94] N. B. Vartak, C. Lin, J. M. Cleary, M. J. Fagan, and M. H. Saier, "Glucose metabolism in 'Sphingomonas elodea' : pathway engineering via construction of a glucose-6-phosphate dehydrogenase insertion mutant." *Microbiology*, pp. 2339–2350, 1995.

[95] I. Sá-Correia, a M. Fialho, P. Videira, L. M. Moreira, a R. Marques, and H. Albano, "Gellan gum biosynthesis in Sphingomonas paucimobilis ATCC 31461: genes, enzymes and exopolysaccharide production engineering." *Journal of industrial microbiology & biotechnology*, vol. 29, pp. 170–6, 2002.

[96] D. A. K. Wataru, M. Shozo, "Oral sustained delivery of paracetamol from in situ-gelling gellan and sodium alginate formulations." *International Journal of Pharmaceutics*, vol. 258, pp. 55–64, 2003.

[97] D. M. De Souza and C. H. Garcia-cruz, "Produção fermentativa de polissacarídeos extracelulares por bactérias." Ciências Agrárias, vol. 25, pp. 331–340, 2004.

[98] I. Camelin, C. Lacroix, C. Paquin, H. Prévost, R. Cachon, and C. Divies, "Effect of chelatants on gellan gel rheological properties and setting temperature for immobilization of living bifidobacteria." *Biotechnology progress*, vol. 9, pp. 291–7, 1993.

[99] I. Bajaj and R. Singhal, "Gellan gum for reducing oil uptake in sev, a legume based product during deep-fat frying." *Food Chemistry*, vol. 104, pp. 1472–1477, 2007.

[100] Y. D. Sanzgiri, S. Maschib, V. Crescenzib, E. M. Topp, and V. J. Stella, "Gellan-based systems for ophthalmic sustained delivery of methylprednisolone." vol. 26, pp. 195–201, 1993.

[101] S. Suri and R. Banerjee, "In vitro evaluation of in situ gels as short term vitreous substitutes." *Journal of Biomedical Materials Research Part,* vol. 79, pp. 650–664 2006.

[102] D. F. Coutinho, S. V Sant, H. Shin, J. T. Oliveira, M. E. Gomes, N. M. Neves, A. Khademhosseini, and R. L. Reis, "Modified Gellan Gum hydrogels with tunable physical and mechanical properties." *Biomaterials*, vol. 31, pp. 7494–502, 2010.

[103] G. P. Okiror and C. L. Jones, "Effect of temperature on the dielectric properties of low acyl gellan gel." *Journal of Food Engineering*, vol. 113, pp. 151–155, 2012.

[104] J. M. Oliveira, S. G. Caridade, J. T. Oliveira, R. A. Sousa, and J. F. Mano, "Gellan gum-based hydrogels for intervertebral disc tissue-engineering applications." *Journal of Tissue Engineering and Regenerative Medicine,* vol. 5, pp. 97–107, 2011.

[105] K. S. Kang, G. T. Veeder, P. J. Mirrasoul, T. Kaneko, and I. W. Cottrell, "Agar-like polysaccharide produced by a pseudomonas species: production and basic properties." *Applied and environmental microbiology*, vol. 43, pp. 1086–91, 1982.

.[106] E. Murano, "Use of natural polysaccharides in the microencapsulation techniques." *Journal of Applied Ichthyology*, vol. 14, pp. 245–249, 1998.

[107] P. Moslemy, S. R. Guiot, and R. J. Neufeld, "Activated sludge encapsulation in gellan gum microbeads for gasoline biodegradation." *Bioprocess and biosystems engineering*, vol. 26, pp. 197–204, 2004.

[108] H. Grasdalen and O. Smidsrod, "Gelation of Gellan Gum." *Carbohydrate Polymers,* vol. 7, pp. 371–393, 1987.

[109] M. H. Lau, J. Tang, and A. T. Paulson, "Texture profile and turbidity of gellan/gelatin mixed gels." *Food Research International,* vol. 33, pp. 665–671, 2000.

[110] T. Funami, S. Noda, M. Nakauma, S. Ishihara, R. Takahashi, S. Al-Assaf, S. Ikeda, K. Nishinari, and G. O. Phillips, "Molecular structures of gellan gum imaged with atomic force microscopy in relation to the rheological behavior in aqueous systems in the presence or absence of various cations." *Journal of agricultural and food chemistry*, vol. 56, pp. 8609–18, 2008.

[111] J. Tang, R. Mao, M. . Tung, and B. . Swanson, "Gelling temperature, gel clarity and texture of gellan gels containing fructose or sucrose." *Carbohydrate Polymers*, vol. 44, pp. 197–209, 2001.

[112] A. Tecante, "Dynamic viscoelastic behavior of gellan- -carrageenan and gellan-xanthan gels." *Food Hydrocolloids,* vol. 13, pp. 59–64, 1999.

[113] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature*, vol. 227, pp. 680–5, 1970.

[114] L. A. Passarinha, M. J. Bonifácio, and J. A. Queiroz, "Comparative study on the interaction of recombinant human soluble catechol-O-methyltransferase on some hydrophobic adsorbents." *Biomedical Chromatography,* vol. 438, pp. 430–438, 2007.

[115] A. Verma and J. K. Pandit, "Comparative evaluation of Ca^{++} and Zn^{++} cross-linked gellan gum based floating beads." *Archives Des Sciences,* vol. 65, pp. 75–84, 2012.

[116] F. Sousa, D. M. F. Prazeres, and J. A. Queiroz, "Dynamic binding capacity of plasmid DNA in histidine – agarose chromatography." *Biomedical Chromatography,* vol. 998, pp. 993–998, 2007.

[117] R. R. Rueckert, "Observations on Molecular on Polyacrylamide Gel Weight." Journal of Biological Chemistry, vol. 244, pp. 5074–5080, 1969.

[118] M. L. Bender, F. J. Kézdy, and F. C. Wedler, "Alpha-chymotrypsin: enzyme concentration and kinetics." *Journal of chemical education*, vol. 44, pp. 84–8, 1967.

[119] J. A. G. K. J. Palmer , M. Ballantyne, "The Molecular Weight of Lysozyme Determined by the X-Ray Diffraction Method." *Journal of the American Chemical Society*, vol. 70, pp. 906–908, 1948.

[120] A. Staby, M.-B. Sand, R. G. Hansen, J. H. Jacobsen, L. a. Andersen, M. Gerstenberg, U. K. Bruus, and I. H. Jensen, "Comparison of chromatographic ion-exchange resins." *Journal of Chromatography A*, vol. 1069, pp. 65–77, 2005.

[121] A. Staby, J. H. Jacobsen, R. G. Hansen, U. K. Bruus, and I. H. Jensen, "Comparison of chromatographic ion-exchange resins V. Strong and weak cation-exchange resins." *Journal of chromatography. A*, vol. 1118, pp. 168–79, 2006.