UNIVERSIDADE DA BEIRA INTERIOR
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Capture strategies of human catechol-*O*-methyltransferase from *Komagataella pastoris* lysates by gellan microspheres

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Aos meus pais, irmã e avós maternos:

as pessoas mais importantes da minha vida

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Resumo alargado

A catecol-*O*-metiltransferase (COMT) tem sido descrita como um importante alvo biofarmacêutico devido à sua possível associação a algumas doenças neurológicas, nomeadamente a doença de Parkinson (PD), onde o desenvolvimento de novos inibidores da COMT tem levado a melhorias no tratamento da doença. Para o desenvolvimento de inibidores com um melhor perfil terapêutico é fundamental ter disponibilidade de amostras de COMT recombinante com elevado grau de pureza. De modo a satisfazer os requerimentos da indústria farmacêutica, torna-se imperativo implementar novas estratégias de isolamento e purificação céleres e com reprodutibilidade. O método de *Batch* surge como uma alternativa a técnicas cromatográficas para o isolamento de proteínas, sendo um método simples, rápido e de baixo custo, que tem a peculiaridade de ser altamente flexível em relação à natureza do material que constitui as partículas de captura. A goma de gelana é um exopolissacarídeo aniónico que tem a capacidade de formar géis com diferentes conformações e estrutura em função da concentração de polímero, temperatura, ambiente aquoso e presença de catiões mono e divalentes. Apesar da goma de gelana possuir inúmeras aplicações na indústria alimentar e biofarmacêutica, entre outras, a sua aplicação como uma matriz cromatográfica só começou a ser investigada recentemente devido não só às propriedades já mencionadas, mas também à sua porosidade, hidrofilicidade, elevada capacidade de ligação e aptidão para formar géis resistentes à temperatura e a condições acídicas extremas. Assim, o principal objetivo deste trabalho foi desenvolver estratégias de captura da isoforma solúvel humana recombinante da COMT (hSCOMT) obtida de lisados de *Komagataella pastoris* (*K. pastoris*) através de um método de *Batch* com microesferas de gelana, reforçando a sua aplicabilidade no isolamento de biomoléculas e clarificação de amostras. Para atingir tal objetivo, os lisados de hSCOMT foram obtidos de culturas de *K. pastoris* induzidas por metanol, enquanto que as microesferas de gelana foram formuladas pela técnica de emulsão de água em óleo e reforçadas com uma solução de iões níquel ou magnésio. As microesferas de gelana com níquel e magnésio como *crosslinkers* foram caracterizadas quanto ao diâmetro médio, morfologia e presença dos iões usados no *crosslinking* através de microscopia semiótica, microscopia eletrónica de varrimento e espetroscopia de raio X de energia dispersiva, respetivamente. As duas formulações de microesferas apresentaram uma estrutura uniforme e consistente de forma esférica. No entanto, as microesferas de gelana com magnésio como *crosslinker* apresentaram um diâmetro médio menor que aquelas formuladas com o ião níquel, uma vez que o magnésio tem um raio atómico maior, ocupando mais espaço entre as moléculas de gelana. Pela mesma razão, o conteúdo de magnésio foi menor do que o de níquel na análise elementar. De seguida, as duas formulações de microesferas de gelana foram aplicadas num método *Batch* para capturar a proteína hSCOMT através de duas estratégias desenvolvidas à temperatura ambiente (RT) e a 4 ºC: a primeira estratégia foi baseada na interação entre as cargas negativas das microesferas

de gelana e os resíduos positivos da proteína hSCOMT (estratégia iónica) e a segunda na afinidade da hSCOMT para os iões divalentes das microesferas de gelana (estratégia de afinidade). Após otimização, as condições que deram origem aos melhores resultados de captura com ambas as formulações de microesferas de gelana na estratégia iónica foram a ligação da hSCOMT a pH 4.0 e eluição com 500 mM e 100 mM NaCl, pH 6.2, a RT e a 4 ºC, respetivamente. Na estratégia de afinidade ainda com as microesferas de gelana com níquel como *crosslinker* foram capturados elevados níveis de hSCOMT a pH 7.5 contendo 10 mM imidazole, e foi maioritariamente recuperada com 150 mM NaCl e 300 mM imidazole, pH 7.5, a 4 ºC. No caso das microesferas de gelana com magnésio como *crosslinker*, níveis elevados da proteína hSCOMT foram capturados a pH 5.2, e eluídos com 100 mM NaCl, pH 5.2, a RT, ou com 250 mM NaCl e 500 mM $MgCl_2$, pH 5.2, a 4 ºC. De forma geral, todas as estratégias demonstraram ótimos resultados tanto à temperatura ambiente como a 4 ºC, permitindo uma retenção quase completa da proteína hSCOMT e a sua recuperação praticamente num único passo, dando origem a amostras bastante clarificadas e enriquecidas em hSCOMT através da eliminação de contaminantes noutros passos. Extrapolando a aplicação destas microesferas na captura de outras proteínas recombinantes, a estratégia de afinidade com as microesferas de gelana com níquel como *crosslinker* é mais adequada para proteínas que sejam produzidas com cauda de histidinas. Por outro lado, proteínas que possuam afinidade natural para iões divalentes, estes podem ser incorporados nas esferas, desenvolvendo-se assim uma estratégia de bioafinidade como foi o caso das microesferas com magnésio no presente trabalho. Em conclusão, este trabalho demostrou a versatilidade da goma de gelana na formulação de microesferas para captura da hSCOMT,que permitiu não só extrapolar uma vertente de trocador catiónico devido às moléculas aniónicas de gelana, mas também extrapolar diferentes interações de afinidade com os iões divalentes usados no reforço das microesferas, permitindo assim o design de estratégias de captura mais específicas, eficientes e adequadas à molécula alvo.

Abstract

Catechol-*O*-methyltransferase (COMT) has become an interesting biopharmaceutical target due to its possible association to some disorders, particularly to Parkinson's disease (PD), where the development of new COMT inhibitors lead to an improvement in clinical treatments, which requires great quantities of this enzyme in a highly purified form, preserving its enzymatic activity. Since the 1960s, recombinant human soluble COMT (hSCOMT) has been extensively subjected to several purification strategies. However, some of them presented significant losses of hSCOMT activity and yield as the main drawbacks. Thus, the present work consists of developing retention and elution strategies to capture hSCOMT from complex *Komagataella pastoris* lysates through a batch method using gellan microspheres as the main capture method. Briefly, hSCOMT lysate was recovered from *K. pastoris* methanol-induced cultures and the gellan microspheres were formulated by water-in-oil emulsion technique and reinforced with a nickel or magnesium ion solution. The hSCOMT capture on gellan microspheres was performed through the batch method at room temperature (RT) and 4 ºC, using two strategies: the first one was based on the interaction between negative charges of gellan microspheres and hSCOMT positive residues (ionic strategy) and the second one was founded on the affinity of the hSCOMT to the divalent ions of gellan microspheres (affinity strategy). After optimization, the conditions that allowed the best outcomes with both microspheres formulations in the ionic strategy were the hSCOMT binding at pH 4.0 and elution with 500 mM and 100 mM NaCl, pH 6.2, at RT and 4 ºC, respectively. In the affinity strategy with nickel-crosslinker gellan microspheres, high levels of hSCOMT were captured at pH 7.5, containing 10 mM imidazole, which was mainly recovered with 150 mM NaCl and 300 mM imidazole, pH 7.5, at 4 ºC. In the affinity strategy with magnesium-crosslinked gellan microspheres, hSCOMT was practically all retained at pH 5.2, and eluted with 100 mM NaCl, pH 5.2, at RT, or with 250 mM NaCl and 500 mM $MgCl_2$, pH 5.2, at 4 ºC. In conclusion, the present work demonstrated the gellan gum versatility in the microspheres formulation for the capture of hSCOMT, not only extrapolating a cation exchanger strand due to the anionic gellan molecules, but also extrapolating different affinity strategies with the divalent ions used in the microspheres reinforcement, thereby allowing the design of capture strategies more specific, efficient and adequate to the target molecules.

Keywords

Catechol-*O*-methyltransferase, Gellan microspheres, Batch method, Affinity.

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List of acronyms

3,5-DNC	3,5-dinitrocatechol
AC	Affinity chromatography
AOX	Alcohol oxidase I gene
BSA	Bovine serum albumin
Co^{2+}	Cobalt ion
COMT	Catechol-*O*-methyltransferase
Cu^{2+}	Copper ion
D-Glc	ß-D-glucose
D-GlcA	ß-D-glucoronic acid
DNA	Deoxyribonuleic acid
DNase	Deoxyribonuclease I
DTT	Dithiotreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediamine tetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FDA	Food and Drug Administration
GF	Gel filtration
GFP	Green fluorescent protein
GRAS	Generally recognized as safe
HCl	Hydrochloric acid
HIC	Hydrophobic interaction chromatography
His-tagged proteins	Histidine-tagged proteins
hSCOMT	human soluble catechol-*O*-methyltransferase
IEC	Ion exchange chromatography
IMAC	Immobilized metal ion affinity chromatography
L-Rha	α-L-rhamnose
K_2HPO_4	Potassium phosphate dibasic
KH_2PO_4	Potassium phosphate monobasic
MBCOMT	Membrane-bound catechol-*O*-methyltransferase
MES	4-Morpholineethanesulfonic acid
Mg^{2+}	Magnesium ion
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
NaCl	Sodium chloride
Ni^{2+}	Nickel ion
$NiCl_2.6H_2O$	Nickel chloride hexahydrate

$OD_{560\ nm}$	Optic density at 560 nm
PD	Parkinson's disease
PFC	Prefrontal cortex
pI	Isoelectric point
PSA	Ammonium persulphate
RNA	Ribonucleic acid
RNase	Ribonuclease A
RPC	Reversed phase chromatography
RT	Room temperature
SAM	*S*-adenosyl-*l*-methionine
SAH	*S*-adenosyl-*l*-homocysteine
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SCOMT	Soluble catechol-*O*-methyltransferase
SEM	Scanning electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
UV	Ultraviolet-visible
WB	Western blot
W_R	Working reagent
YNB	Yeast nitrogen base
Zn^{2+}	Zinc ion

List of communications

Oral communication in the XIII Annual CICS-UBI Symposium, Covilhã, July 2018: Gonçalves C, Sousa Â, Passarinha LA, A new formulation of gellan microspheres to capture human soluble catechol-*O*-methyltransferase from *Komagataella pastoris* lysates.

Poster presentation and **short oral communication** in the 38th International Symposium on the Purification of Proteins, Peptides and Polynucleotides (ISPPP), Berlin, November 2018: Gonçalves C, Sousa Â, Passarinha LA, Soluble catechol-*O*-methyltransferase capture from a *Pichia pastoris* X33 lysate through a new batch method with gellan microspheres.

Chapter 1 – Introduction

1.1. Catechol-*O*-methyltransferase (COMT)

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6.), the enzyme responsible for the *O*-methylation of catecholamines and other catechols, was discovered by Axelrod and Tomchick in 1958 and partially purified and characterized by the same group [1].

The basic research of the enzyme lasted several years after the discovery and consisted of studies to understand the physiological role and pharmacological aspects of COMT. At this time, some COMT properties, such as molecular weight and different isoforms of the enzyme, its distribution at cellular and subcellular levels, its enzymatic role on the *O*-methylation mechanism of catecholamines, among other findings, became clear and known. Furthermore, some methods for evaluation of enzymatic activity of COMT were explored and the first inhibitors of COMT were introduced. In 1975, Guldberg and Marsden put together all the obtained data on an extensive review [2].

In the late 1980s, Männistö e Kaakkola developed new effective and selective COMT inhibitors, the second generation of COMT inhibitors, remarking the rebirth of interest in COMT [3]. Within two years, the COMT gene and the two protein isoforms were characterized, culminating in the polypeptide cDNAs cloning [4–6].

1.1.1. Isoforms of COMT and tissue distribution

COMT is an intracellular enzyme almost ubiquitously distributed throughout the different human tissues, meaning that it can be found in both peripheral tissues and the central nervous system [7]. COMT can be found, practically, in all the peripheral tissues (lung, stomach, intestines, heart, muscles, red blood cells, etc.), but it is abundantly expressed in the liver and kidney. Moreover, the highest enzymatic activity in peripheral tissues is found in the liver, which is significantly higher than in the brain. Here, COMT is similarly distributed in different brain areas [8].

The enzyme appears as two distinct molecular isoforms in human tissues, a soluble form (SCOMT) and a membrane-bound form (MBCOMT). SCOMT isoform is a nonglycosylated protein [9], mainly present in the cytoplasm, and contains 221 amino acid residues and a molecular weight of 24.7 kDa [9–11]. On the other hand, MBCOMT, as the name suggests, is an integral membrane protein and can be found mostly associated with rough endoplasmic reticulum membrane. Comparatively to SCOMT, the MBCOMT isoform has a molecular weight of 30 kDa, since this protein comprises more 50 amino acid residues in it's amino-terminal, which 21 are hydrophobic amino acid residues, constituting the membrane anchor region [9, 10]. In most

human tissues, the levels of SCOMT greatly exceed the levels of MBCOMT, except for the brain, where prevails the MBCOMT isoform [8].

1.1.2. Physiological functions of COMT

Catechol-*O*-methyltransferase is a monomeric magnesium-dependent enzyme that catalyzes the *O*-methylation of catechol substrates, represented in Figure 1. Here, the cofactor *S*-adenosyl-*l*-methionine (SAM) donates a methyl group to catechol substrate's hydroxyl group, yielding, as reaction products, the *O*-methylated catechol and the *S*-adenosyl-*l*-homocysteine (SAH) [9–11]. Typically, the physiological catechol substrates of COMT include catecholamines (dopamine, epinephrine and norepinephrine), their hydroxylated metabolites, catecholestrogens, ascorbic acid, and dietary and medicinal compounds, such as triphenols and substituted catechols [7, 12].

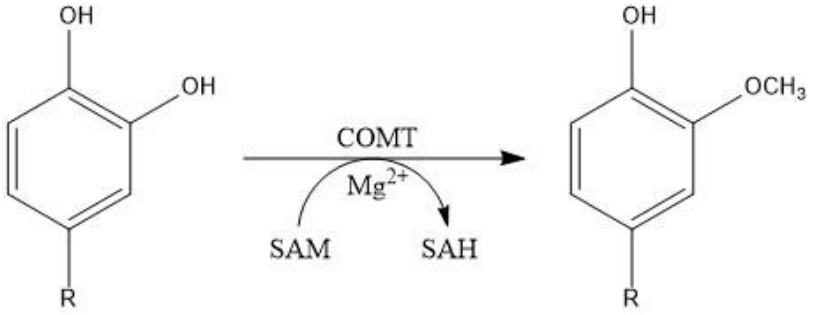

Figure 1. The enzymatic reaction catalysed by COMT. In the presence of magnesium (Mg^{2+}), COMT transfers a methyl group from the cofactor SAM to a hydroxyl group of the catechol substrate.

The main physiological function of COMT is the metabolic inactivation of endogenous catechol neurotransmitters and xenobiotic substance [8]. This role allows COMT to protect the placenta and the developing embryo during pregnancy and act as an enzymatic detoxicating barrier between blood and other tissues. COMT may also be involved in the regulation of active dopamine quantity in the kidney, intestinal tract and brain, which may be associated with other cognitive processes [7]. Of special importance is the collapse of dopamine levels in the prefrontal cortex (PFC), where COMT appears to have an important role in the regulation of dopamine (Figure 2) [13].

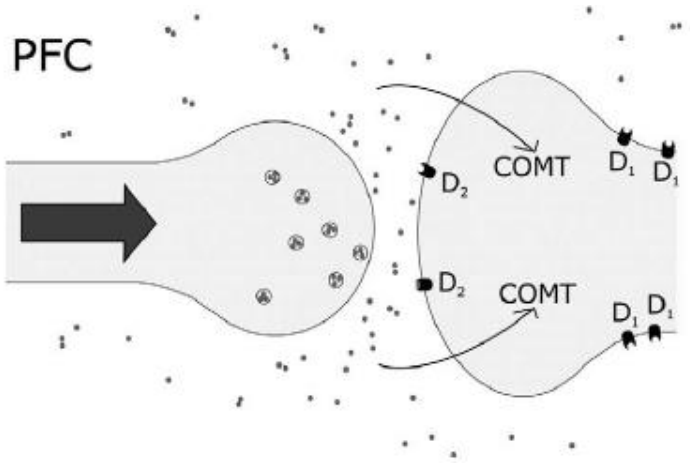

Figure 2. Dopaminergic transmission in the prefrontal cortex (PFC). The scarcity of the dopamine transporter in the synapse means that COMT plays a prominent role in inactivating dopamine (adapted from [13]).

1.1.3. Three-dimensional structure of COMT

In 1994, Vidgren and collaborators solved the three-dimensional structure of rat recombinant SCOMT in complex with Mg^{2+}, the co-substrate SAM and a competitive inhibitor 3,5-dinitrocatechol (3,5-DNC) by X-ray crystallography [14]. Rutherford and colleagues described the crystal structure of human SCOMT (hSCOMT) [15]. The analysis of both human and rat SCOMT crystal structures revealed a similar structural fold [8, 16]. Besides that, all the residues involving catechol binding and catalysis are conserved, which means that rat enzyme should be a good model for the structure prediction of the human enzyme [17].

The three-dimensional structure of COMT enzyme comprehend one single domain with a characteristic α/β-folded assembly, containing a seven-stranded central β-sheet core and eight α-helices arranged around that central nucleus, more precisely five α-helices on one side and three α-helices on the other side [8, 12, 16], as represented in Figure 3.

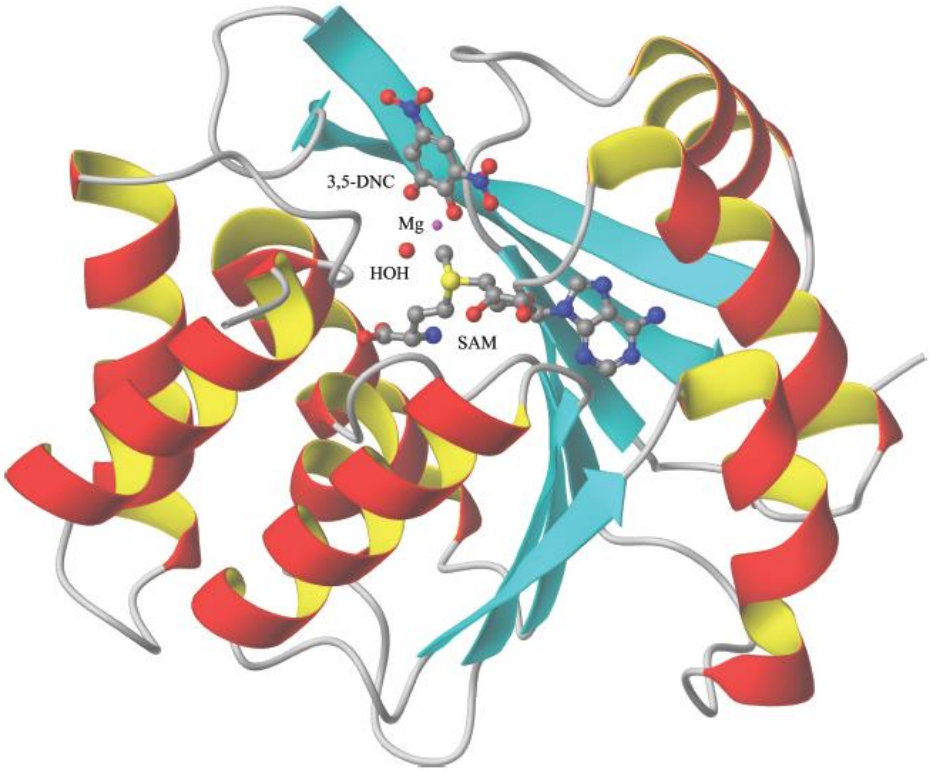

Figure 3. Schematic representation of the three-dimensional structure of COMT. The S-adenosyl-*l*-methionine (SAM), the inhibitor 3,5-dinitrocatechol (3,5-DNC), the magnesium ion, and coordinated water molecules are depicted [12].

The active site of COMT, shown in Figure 4, is compound by two regions: the SAM-binding domain and the catalytic region, which binds the magnesium ion and the catechol substrate. The methylation reaction follows a sequentially ordered mechanism, wherein SAM is the first ligand to bind the enzyme, followed by the magnesium ion and finally by the catechol substrate [8, 12]. Figure 4 also shows most of molecular interactions present in the protein. The adenine ring of SAM forms hydrogen bonds with serine and glutamine residues (Ser119 and Gln120) and Van der Waals interactions with isoleucine, alanine and tryptophan residues (Ile91, Ala118 and Trp143) [8]. In the centre of the catalytic site, the magnesium ion has no contact with SAM and is octahedrally coordinated to one asparagine residue (Asn170), two aspartic acid residues (Asp141 and Asp169), and one water molecule. The remaining coordination sites are destined to the hydroxyl groups belonging to the catechol substrate, meaning that Mg^{2+} secures the substrate or inhibitor molecules in a catalytic position. The ion has the ability to reduce substrate pK_a, facilitating the deprotonation of the hydroxyl groups by Lys144, yielding to a phenolate anion that may be rapidly methylated by SAM. The hydroxyl groups are also hydrogen bonded to residues Glu199 and Lys144. Last of all, the hydrophobic "gatekeeper" residues (Trp38, Trp143, Pro174, and Leu198) provide the additional interactions with the planar catechol ring, maintaining the substrate correctly oriented for methylation [8, 12].

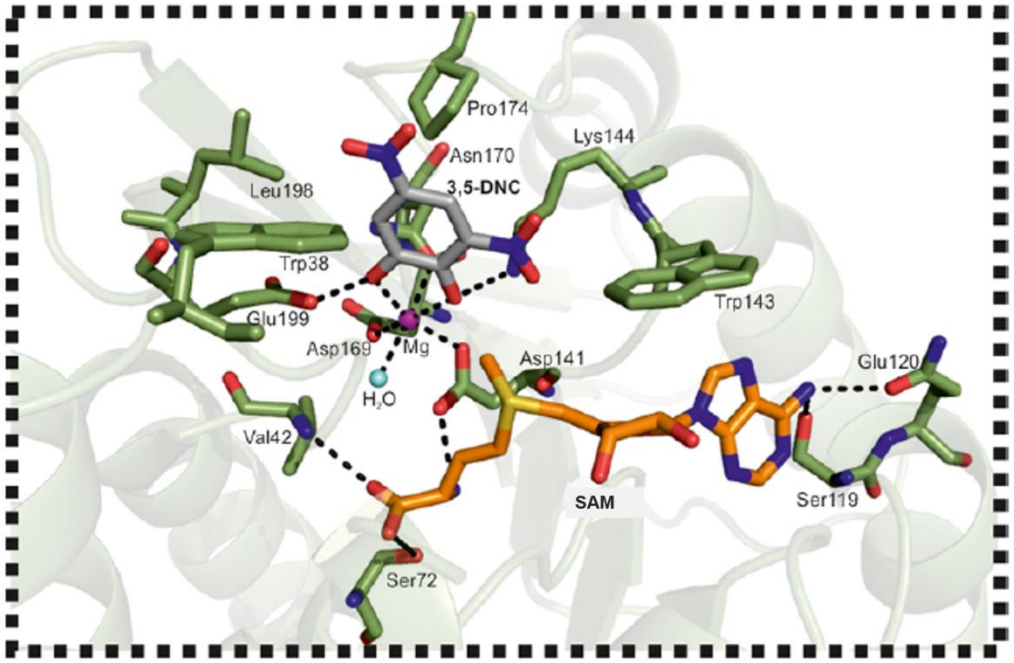

Figure 4. View of the catalytic site of SCOMT in complex with methyl donor SAM, the Mg^{2+} and the inhibitor 3,5-DNC. The figure was drawn using program PyMOL (adapted from [8]).

1.1.4. COMT inhibitors on Parkinson's disease

During the last decades, COMT has become an interesting biopharmaceutical target due to its involvement in some human disorders, for example, cardiovascular diseases [18], estrogen-induced cancers [19] and neurologic disorders [12]. Among these, the role that COMT plays in Parkinson's disease (PD) is the finest documented [11].

Parkinson's disease is the most prevalent chronic neurodegenerative disorder that affects motor coordination. The gradual loss of dopaminergic neurons, mainly in the *substantia nigra*, and the decrease of dopamine levels in the brain are the main pathological characteristics of PD [20]. Indeed, motor neurons control the muscles and are stimulated by the neurotransmitter dopamine. So, if dopamine production is depleted, the motor nerve cells become incapable to control movement and coordination [8]. Actually, the most common PD symptoms include tremor, rigidity, slowness, postural instability and serious difficulty or inability to coordinate physical movements [8, 12]. The clinical symptoms of this disease normally appear when around 60% of all dopaminergic neurons have already been lost. The primary symptoms arise at 60 years of age and the disease normally progresses slowly during the next 10 to 20 years [12]. So far, no cure has been found for PD, however, the symptoms can be controlled with drug administration [8]. In fact, dopamine replacement therapy with levodopa in combination with an aromatic amino acid decarboxylase inhibitor and a COMT inhibitor remains the most effective treatment of this neurological disorder [11].

In the late 1950s, several classes of COMT inhibitors were identified, becoming the first-generation of COMT inhibitors, which were reviewed by Guldberg and Marsden, in 1975 [2]. These compounds are classical competitive substrates of COMT since they contain a catechol structure or some related bioisosteric portion. Among these compounds, derivatives of pyrogallol and catechols, such as gallic acid, caffeic acid, U-0521, and flavonoids, were identified. Furthermore, noncatecholic compounds, like ascorbic acid, tropolones, and derivatives of 8-hydroxyquinolines, were also described as COMT inhibitors [12]. Despite their use as *in vitro* tools, some of the early COMT inhibitors showed low efficacy *in vivo*, poor selectivity, and toxicity [7].

Later, in the 1980s, a new generation of potent, selective [7, 12, 20], reversible [20], and orally active COMT inhibitors were investigated [7]. This second-generation of COMT inhibitors consists of a new class of di-substituted catechols, namely nitrocatechols such as tolcapone and entacapone [12, 20]. These last two COMT inhibitors were both introduced into clinical practice for treatment of PD in the late 1990s, after extensive clinical trials in patients with PD symptoms [20]. From the pharmacological point of view, tolcapone presents higher potency and high action time comparatively to entacapone, after oral administration to rats and humans [12, 20]. Unpleasantly, these available COMT inhibitors also show side effects, particularly discolouration of the urine, and sometimes diarrhoea occurring until 2-4 months following treatment initiation [20].

Thus, it is extremely necessary becomes clearly important the development of new and more effective drugs for COMT inhibition in order to improve the efficacy pf PD therapy and new systems capable of obtaining large quantities of the protein in a highly purified form [11]. A way for the study of new COMT inhibitors goes through the recombinant production of this protein in adequate host strains, such as *Komagataella pastoris*, and subsequently its recovery and purification, thereby allowing the required structural and inhibition studies to accomplish the main goal.

1.2. Recombinant protein biosynthesis

The main human proteins that have an important pharmaceutical value are difficult to obtain from their natural sources. The recombinant technology has been offering a very potent tool for production of proteins by relatively easy, rapid and inexpensive procedures [21]. Over the years, the recombinant hSCOMT has been produced through different expression systems, such as transfected mammalian cells [22], insect cells (via mammalian and baculovirus vectors) [23], plant cells (via a potyvirus) [24] and prokaryotic cells, such as *Escherichia coli* [25, 26]. More recently, yeasts have gained an industrial importance as hosts for biopharmaceutical production, extending their traditional applications in food and fermentation processes [27].

Yeasts provide advantages in biopharmaceutical recombinant production as unicellular eukaryotic microbial host cells, namely the combination of individual advantages of prokaryotic systems and mammalian host cells [28]. For instance, yeasts allow an ideal conjugation of robust growth on simple media in large-scale bioreactors (characteristic of prokaryotic cells) with the introduction of the desired post-translational modifications and genetic modifications (characteristics of the mammalian cells) [29]. In general, yeasts are an attractive eukaryotic host for the recombinant protein biosynthesis due to their fast growth rates coupled with high cell densities and the well-developed diversity of genetic tools [11, 30]. In addition, some yeast species present extra desirable physiological characteristics, such as faster growth on cheap carbon sources and higher secretion capacity [28].

Over the last two decades, one of the most frequently used expression systems for recombinant protein biosynthesis was the methylotrophic *Komagataella pastoris* (*K. pastoris*) [9] that was generally recognized as safe (GRAS) organism by Food and Drug Administration (FDA) in 2006 [29]. *K. pastoris* was initially described by Philips Petroleum Company for the production of single cell protein and nowadays is considered a nonconventional yeast successfully used in laboratory and large-scale fermentation procedures for recombinant production [31]. *K. pastoris*, as a yeast, is an unicellular microorganism that is easy to manipulate and cultivate and, as an eukaryotic cell, is capable of introducing post-translational modifications [11, 30]. Other benefits of this system include high expression level of heterologous proteins, efficient secretion of extracellular proteins and the presence of a promoter derived from the alcohol oxidase I gene (AOX) [9]. In addition, *K. pastoris* strongly prefers a respiratory metabolism, even at high cell density processes, avoiding the accumulation of secondary metabolites [32]. Our research group has already reported the biosynthesis of both SCOMT and MBCOMT isoforms through cultures of *K. pastoris* [11, 30]. After the recombinant production of the target protein, it is fundamental to proceed for its recovery and purification, thereby acquiring high quantities of COMT in a high purity form, which is required for further structural and inhibition studies.

1.3. Chromatography

Chromatography is the name commonly used to represent a family of analytical techniques that can be applied to a large diversity of mixtures of solids, liquids, and gases. These techniques are related to separating the substances of complex mixtures into their individual components, in order to be thoroughly analysed or used [33].

By other words, molecules present in a mixture are separated from each other between a mobile and a stationary phase. The stationary phase is composed by a solid material or a viscous liquid adsorbed on the surface of a solid support, whereas the mobile phase is a liquid solution or a gas, carrying the analyte mixture with it. While the mobile phase moves through the stationary phase, some components of the analyte stay longer in the stationary phase, while others flow quickly through it, leaving the chromatographic system faster. The elution order is

based on different characteristics of components in the analyte, such as affinity or molecular weights, allowing the separation of the different compounds along the way [33, 34].

Since proteins have different characteristics like size, shape, net charge, these properties combined with the binding capacity of the stationary phase, can be applied to promote the purification of these compounds by different chromatographic methods [34]. Among these methods, Ion Exchange Chromatography (IEC), Size Exclusion Chromatography (SEC), Hydrophobic Interaction Chromatography (HIC), Reversed-Phase Chromatography (RPC) and Affinity Chromatography (AC), such as Immobilized Metal Ion Affinity Chromatography (IMAC) are frequently applied for the purification of biomolecules, namely proteins [34–36]. The action principles and protein properties explored in these chromatographic methods are briefly described in Table 1.

Table 1. Action principles and protein properties used in different types of protein chromatography [35, 36].

Method	**Action principle**	**Protein property**
IEC	Ionic binding	Charge
SEC	Size exclusion	Size
HIC	Hydrophobic complex formation	Hydrophobicity
RPC	Hydrophobic complex formation	Hydrophobicity
AC	Specific adsorption/desorption	Specific ligand recognition
IMAC	Complex formation with transition metals	Metal ion binding

1.3.1. Ion Exchange Chromatography (IEC)

Ion exchange chromatography has been applied on biomolecules purification since the 1960s and nowadays is one of the most popular techniques for separation and purification of proteins, peptides, nucleic acids, among other charged biomolecules. In addition, IEC separates molecules with different net surface charge, offering high-resolution separation with high sample loading capacity [37].

IEC is a technique based on the reversible electrostatic interactions established between charged groups on the protein surface, and oppositely charged groups on the solid support (matrix) [34]. This principle of action is shown in Figure 5. Considering the isoelectric point (pI) of protein, the surface charge varies according to the solution pH. Typically, pH above its pI, the protein becomes negatively charged and interacts with a positively charged matrix (anion exchange matrix). In the opposite case, pH below its pI, the protein acquires a positive charge and binds to a negatively charged matrix (cation exchange matrix) [34, 36–38].

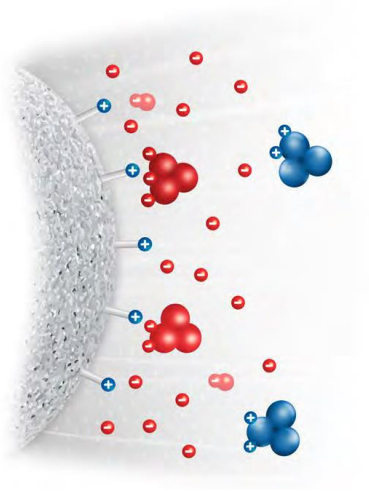

Figure 5. Representation of the ion exchange chromatography [37].

In the same way, after the proteins bind to the matrix, they need to be eluted by increasing of its net charge in order to be collected in a purified fraction. This separation can be achieved by changing the pH or the ionic strength in the elution buffer. When the ionic strength increases, the salt ions compete with the bounded proteins by the charges on the matrix surface and, eventually, elute. Thus, at a specific pH, the proteins with the lowest net charge are the first ones to elute, and the proteins with the higher net charge are the last ones. Summing up, the higher the protein net charge, the higher the ionic strength required for elution [37, 38]. For the separation by pH changing, the best way to elute the protein is by using a condition that disfavour the electrostatic interaction previously established, according to the isoelectric point (pI) of the protein, by decreasing or raising the pH of the elution buffer. So, in the case of an anion exchanger, it should be used a pH below the pI of the protein, while for a cation exchanger, it must be applied a pH above the pI of the protein [39].

1.3.2. Size Exclusion Chromatography (SEC)

Since the late 1960s, size exclusion chromatography, also called Gel Filtration (GF), has been playing a crucial role in the separation and purification of proteins and enzymes, polysaccharides, nucleic acids, and other biological macromolecules [40]. Mostly, this method is used to determine the molecular weights of proteins, and also to sample desalinization [34]. In the last years, SEC proved to be the most favourable technique applied to monitor the protein aggregation, due to its speed and reproducibility, since the aggregates can have a potential effect on efficacy and immunogenicity of protein-based therapies [41].

SEC consists of a simple, highly versatile and non-binding separation technique based on the different sizes of analytes in solution [36, 40], which action principle is shown in Figure 6. The stationary phase consists of chemically and physically stable molecules with small pores and properties that minimize adsorption of biomolecules [40]. The mobile phase carries the analyte solution with a constant flow rate through the matrix. Molecules that are larger than the pore

size cannot enter to the matrix. These molecules are eluted together as they move rapidly through spaces between the porous particles. Molecules that are smaller than the pore size are diffused into pores, and, subsequently, are separated and elute in order of decreasing size [34].

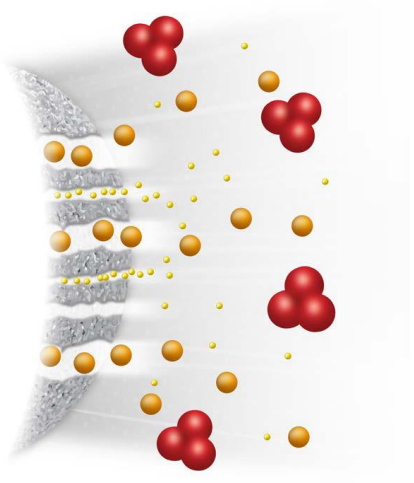

Figure 6. Representation of the size exclusion chromatography [40].

Unlike other techniques such as IEC or AC, molecules do not bind to the matrix, which means that the concentration of the sample components does not directly affect the separation resolution. In addition, separations in the SEC can be performed within a broad pH, ionic strength, and temperature range, and in the presence of essential ions, cofactors, detergents, urea, or guanidine hydrochloride [36, 40].

1.3.3. Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography is often used as a complementary step to other separation techniques, becoming a powerful technique in modern biotechnology for the downstream processing of several biomolecules. Among these, HIC can be applied to all proteins, adapting to the specific hydrophobic requirements of each protein [42]. Moreover, this procedure can be used as a first, an intermediate, or the final purification step.

HIC separates proteins with differences in hydrophobicity. Specifically, the separation principle is based on the interaction established between hydrophobic residues on the protein surface and the hydrophobic ligands immobilized on the stationary phase, as shown in Figure 7. The presence of high salt concentrations removes the hydration water and consequently improves the attraction of protein non-polar residues to the hydrophobic ligands on the matrix, thereby increasing the hydrophobic interactions between them [43]. For these reasons, HIC is well suited when samples have been previously subjected to precipitation with ammonium sulfate or elution with high ionic strength during IEC. In these cases, the sample already contains high salt concentrations and, consequently, can be directly applied to the HIC [44].

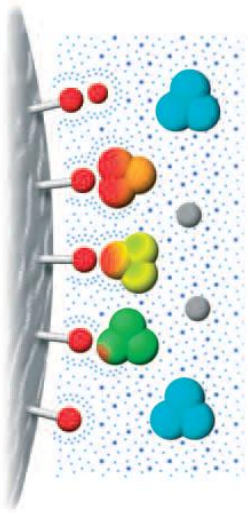

Figure 7. Representation of the hydrophobic interaction chromatography [44].

In general, most of sample components bind to a HIC matrix in the presence of high salt concentrations, usually ammonium sulfate or sodium chloride [36, 44]. Then, the elution is normally performed by decreasing the salt concentration, which allows the regression of the interaction, and the proteins elute by increasing order of hydrophobicity. Proteins with lower hydrophobicity are eluted in first place, being followed by proteins with the higher hydrophobicity, which require a more abrupt decrease of salt concentration to reverse the interaction [44]. Furthermore, the elution can also be performed by reducing eluent polarity, adding chaotropes or detergents, or changing pH or temperature [36].

1.3.4. Reversed-Phase Chromatography (RPC)

Reversed-phase chromatography has gained a crescent importance for high-resolution separation and purity checking analysis of proteins, peptides and nucleic acids, and is also widely used for final polishing of oligonucleotides and peptides when the protein impurities were mostly removed [44]. However, this technique requires the use of organic solvents, which promote the denaturation of several proteins, compromising the recovery of activity and native tertiary structure. Thus, RPC is particularly recommended for small target proteins that are denatured on a smaller scale by organic solvents [36].

RPC, such as HIC, separates proteins and peptides according to their hydrophobicity degrees, and explore the interactions between the hydrophobic groups on the surface of proteins and the hydrophobic ligands of the chromatographic matrix [44]. Nevertheless, both techniques are significantly different, essentially because RPC uses a hydrophobic or non-polar stationary phase and a hydrophilic or polar mobile phase [45]. The action principle of the RPC is shown in Figure 8.

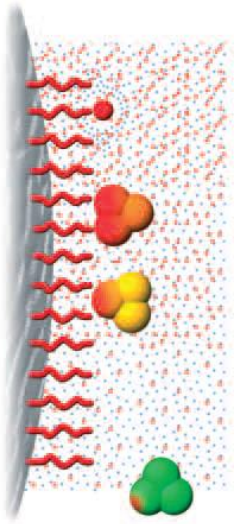

Figure 8. Representation of the reversed-phase chromatography [44].

Unlike HIC, RPC matrices have more and smaller hydrophobic groups attached, making the matrix more hydrophobic. So, the interactions are stronger and, consequently, for elution, the application of non-polar organic solvents such as acetonitrile and methanol are needed to decrease the water concentration in the mobile phase [46].

In general, when the sample is introduced into the chromatographic system, the hydrophobic molecules in the polar mobile phase bound to the hydrophobic stationary phase, allowing, initially the elution of the hydrophilic molecules. Then, increasing the concentration of organic solvents, the polarity of the mobile phase decreases, thereby reducing hydrophobic interactions between the proteins and the stationary phase [45]. At last, molecules are eluted according to the increasing hydrophobicity [46]. For instance, larger proteins are usually much more hydrophobic and therefore establish stronger hydrophobic interaction with the stationary phase. Thus, to elute them, a higher concentration of organic solvent in the mobile phase is required [45].

1.3.5. Affinity Chromatography (AC)

Affinity chromatography (AC), schematically represented in Figure 9, is the most versatile and selective biochemical separation technique, that relies on the reversible and specific interaction between a protein and its equivalent ligand, for example, binding of a hormone with its receptor, interaction of an enzyme with its substrate, or even binding of an antibody with its target antigen [47]. Indeed, AC is rather unique, since it is the only purification technique that works based on the biological function or the chemical structure of the biomolecule of interest. This procedure can be used to separate active biomolecules from denatured or functionally different forms, to isolate low concentrated substances in a complex sample, and to eliminate specific contaminants [48].

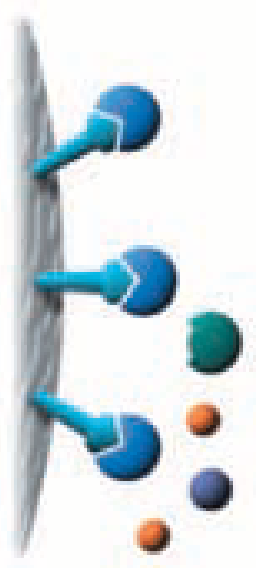

Figure 9. Representation of the affinity chromatography [48].

The interaction is specific since one of the interacting agents (ligand) is immobilized in the stationary phase [47], but it also must be reversible to allow the elution of the target biomolecule in the active form [48]. An AC matrix should be uniform, microporous, hydrophilic, chemically and mechanically stable, selective, with a basal non-specific adsorption, be insoluble in the purification solvent used, have optimal flow characteristics and exhibit a large surface area for ligand coupling [49].

First of all, the sample solution containing the target protein is applied under conditions that favour the specific binding of the target protein to the ligand attached to a chromatographic matrix, while other components move through [47]. These biological interactions can be an outcome of the electrostatic or hydrophobic interactions, Van der Waals forces and/or hydrogen bonding. The target is then eluted specifically by using a competitive ligand, or non-specifically by changing the pH, ionic strength or polarity [48].

1.3.5.1. Immobilized Metal Ion Affinity Chromatography (IMAC)

In AC, the interaction can also be biospecific, for example, a hormone binding its receptor, or non-biospecific, like histidine-containing proteins binding metal ion, as it happens in immobilized metal ion affinity chromatography [36].

Since 1975, the concept of IMAC is based on the affinity of transition metal ions like Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} to histidine, cysteine and tryptophan residues in aqueous solutions. Then, the idea was extended to the use of metal ions immobilized on a support to fractionate and purify proteins [50, 51]. In this way, the already mentioned amino acid residues present in the target protein bind specifically to the metal ions coordination sites [51], as shown in Figure 10.

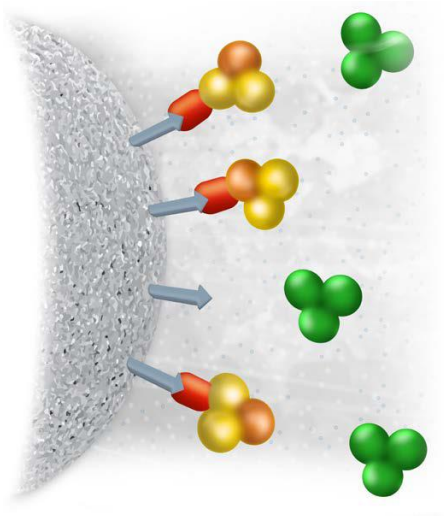

Figure 10. Representation of the immobilized metal ion affinity chromatography [52].

The best-known application of IMAC is the purification of histidine-tagged proteins (His-tagged proteins) [53, 54]. These recombinant proteins are expressed in fusion with an epitope containing at least six histidine residues [50]. Because of their extra histidine residues that have a high selective affinity for Ni^{2+} and several others metal ions, these proteins hold a higher affinity and are usually the strongest binders in a complex sample, whereas other proteins do not bind or bind weakly [36]. Therefore, in a single IMAC purification step, it can be obtained enough purity degree of the target protein for a lot of applications [50].

Normally, the sample involving the His-tagged protein of interest is applied with a binding buffer containing a low concentration of imidazole to reduce nonspecific binding of host cell proteins. Next, the target His-tagged protein binds to the metal ions in the chromatographic matrix and the unbound components of the sample are washed away. At last, the elution of the bound His-tagged protein is carried out by increasing the concentration of imidazole, which competes with proteins for binding to the metal ion in the chromatographic matrix, decreasing the binding of His-tagged proteins [52]. Besides the use of imidazole as a competitive displacement agent, the bound His-tagged proteins can also be eluted by lowering the buffer pH, or by using strong chelating compounds, such as EGTA and EDTA. At low pH, the imidazole ring of the histidine residues present in target proteins becomes protonated, and consequently, loses the bind to the metal ions and is eluted. In the case of chelating agents, they extract the metal ions from the chromatographic matrix, causing a protein/ion elution [51, 52].

The most common tag contains six histidine residues. However, a longer histidine-tag, for example, one with 10 histidine residues, will bind more strongly and, equivalently, a shorter histidine-tag promotes a weak interaction. This difference in binding strength allows the manipulation of imidazole concentrations in the binding buffer to prevent unwanted proteins

from binding and to facilitate the elimination of contaminants that otherwise could be copurified with a shorter tagged protein [52].

Although the best-known application of IMAC is the purification of His-tagged proteins, this technique is now also applied in the purification of nucleic acids, antibodies, natural metal-binding proteins and phosphorylated proteins, and it can be conjugated with other chromatographic techniques [50, 51]. Another application can be the immobilization of such metal ions in spheres, forming metallic spheres to be applied in capture procedures, similar to what happens in a batch method.

1.3.6. Batch Method

The Batch method is a simplest alternative chromatographic technique and is well suited for the capture of biomolecules, such as proteins [55].

In practical terms, the material of the chromatographic matrix, normally with a particulate or beaded form, is put in a vessel. The smaller the particles, the larger is the contact surface area, and, consequently, higher amounts of protein can binding to them [56].

This method is characterized by three essential steps, as shown in Figure 11: the binding step, the washing step and the elution step. In the first one, the sample containing the target biomolecule is mixed with the chromatographic particles and these biomolecules bind to the ligands present in the particles. The washing step is characterized by the washout of the contaminants that did not interact with the particles, by using the equilibrium buffer. Finally, the adsorbed biomolecules in the particles are dissociated in the elution step by applying a buffer that disfavour the interaction [55].

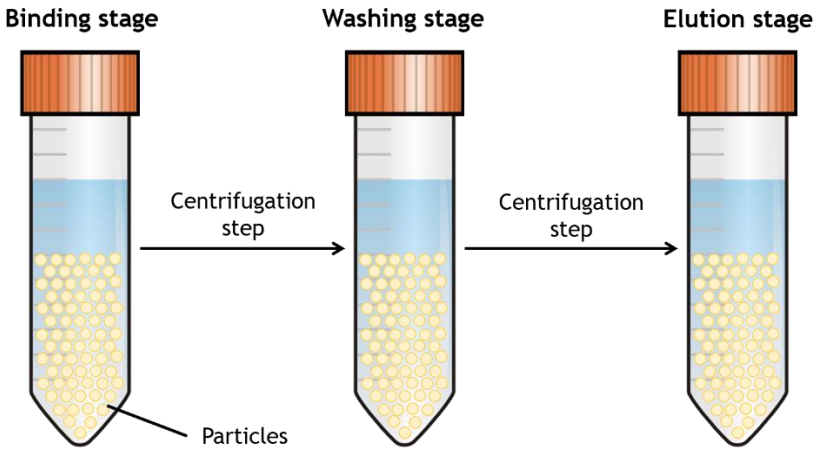

Figure 11. Schematic representation of the Batch Method.

These steps are carried out under constant stirring during a certain period of time in order to suspend the particles in the stirred sample solution [55, 56]. If the particle is too large or too

dense, higher stirring rates could be required, and thereby the protein denaturation becomes a potential danger. The risk of protein denaturation is also increased in the particles that are too irregular and containing sharp edges [55].

The three steps are isolated from each other by a centrifugation step or a magnetic field to separate and remove the supernatant from the particles. The protein concentration in the supernatants can be assessed by several methods, including ultraviolet-visible (UV) and fluorescence spectroscopies, colourimetric methods and many others, which constitutes an advantage of the batch method [55]. Besides being simple, the batch method promotes a low-cost and rapid isolation of proteins [57]. In addition, this procedure presents a huge flexibility regarding the nature of the material of the solid particles [56].

Generally, polymeric matrices present considerable advantages like their high adsorption capacity and selectivity. Recently, several materials have gained interest by the scientific community, namely biopolymers that can be obtained in large quantities and which are inoffensive to nature [58]. For instance, alginate is an anionic polysaccharide extracted from the cell walls of brown seaweed, that is widely used due to its abundant availability, biocompatibility and non-toxic properties [59]. Furthermore, the alginate molecules can be crosslinked with multivalent cations to form spherical gel beads [60] with different characteristics, depending on some variables such as alginate and cation concentrations, temperature and pH [61]. An example of application was the use of alginate beads crosslinked with zinc ions on an IMAC strategy to purify soybean trypsin inhibitor from a crude aqueous extract of soybean flour [62].

1.4. Gellan gum

A polymer with properties and characteristics similar to alginate is the gellan gum, discovered in 1978 in the laboratory of the Kelco Division of Merck and Co. California, USA. Gellan gum is a microbial exopolysaccharide with high molecular weight produced by the bacterium *Sphingomonas paucimobilis*, formerly referred to as *Pseudomonas elodea*. Although it was discovered in 1978, the gellan gum was only approved by the FDA for use as a food additive in 1992 [63, 64].

Microbial exopolysaccharides are widely applied in the food, pharmaceutical and other industries as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents, due to their unique structure and physical properties [64]. Thereby, these biopolymers have gained an industrial and economic importance, competing with natural gums produced from marine algae and other plants. Usually, microbial polysaccharides are ionic or non-ionic water-soluble polymers, whose repeating units are regular, branched or unbranched, and are connected by glycosidic linkages [63].

1.4.1. Structure of Gellan gum

Gellan gum is an anionic linear exopolysaccharide [65], which chemical structure consists of a tetrasaccharide repeating unit, shown in Figure 12. This sequence contains two residues of ß-D-glucose (D-Glc), one of ß-D-glucuronic acid (D-GlcA) and one of α-L-rhamnose (L-Rha). The percentage of these three constituents is approximately: glucose 60%, rhamnose 20% and glucuronic acid 20% [64].

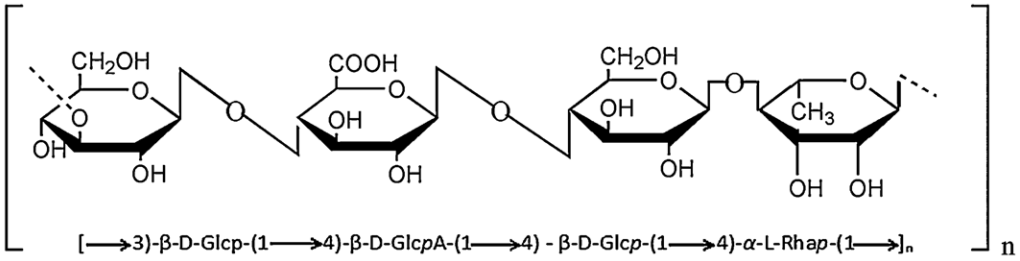

Figure 12. Tetrasaccharide repeating unit of the chemical structure of gellan gum [64].

After its biosynthesis, the native gellan structure has additionally two acyl substituents, an acetyl group and a glyceryl group on the C6 and C2, respectively, of the glucose residue, and can also be referred to as high-acyl gellan [66]. Moreover, a considerable amount of non-polysaccharide material, such as cell protein and ash, is found in native gellan gum. [66]. In commercial gellan, alkaline hydrolysis is used to remove the acyl residues, resulting in deacetylated gellan, also called low-acyl gellan [65]. The chemical structure of both high-acyl and low-acyl gellan forms is shown in Figure 13.

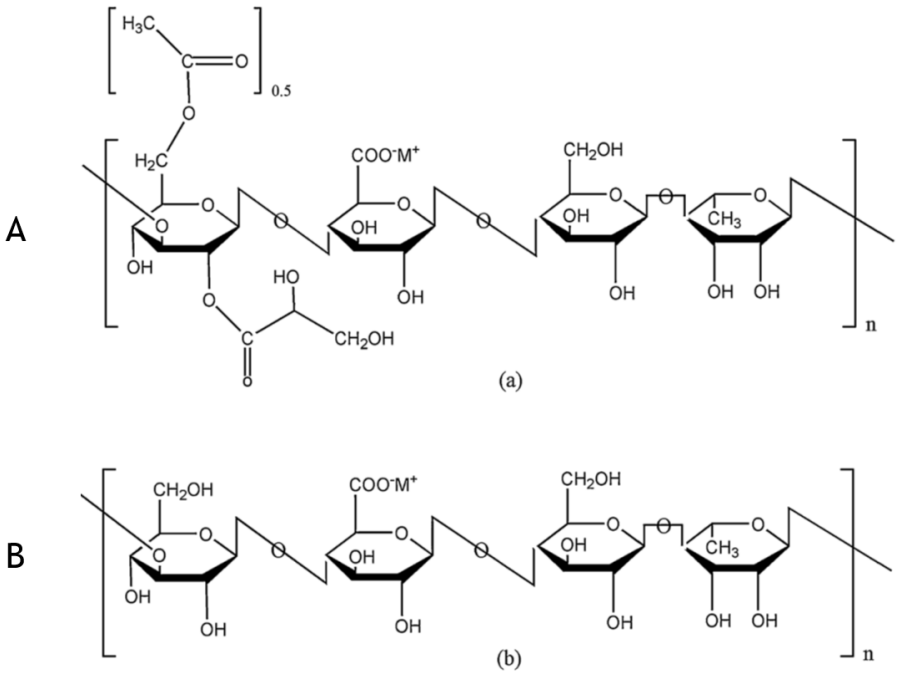

Figure 13. Tetrasaccharide repeating unit of the chemical structure of native (A) and deacetylated (B) gellan gum (adapted from [64]).

1.4.2. Physicochemical and gelation properties

Gellan gum can form hydrogels in the presence of cations [65]. The gellan conformation and structure obtained are functions of polymer concentration, temperature, aqueous environment, and presence of monovalent or divalent cations in the solution [66]. The gel formation consists of heating and cooling gellan solutions, which leads to conformation changes of the polymer chains, inducing coil-to-helix transition [65]. This thermally reversible transition, shown in Figure 14, is performed through helix-formation, followed by association of helices and culminating with the formation of junction zones [67]. Briefly, at high temperatures, gellan presents a random coil conformation [67] with a structure of single-stranded polysaccharide, which notably reduces the viscosity of the solution. Then, upon cooling, gellan forms ordered helices of double strands that aggregate and bind to one another at the junction zones [66], involving weak interactions such as Van der Waals forces and hydrogen bonds. Gel-promoting ions protect the electrostatic repulsions between the helices, promoting further aggregation of the helices, which results in a large three-dimensional network [68].

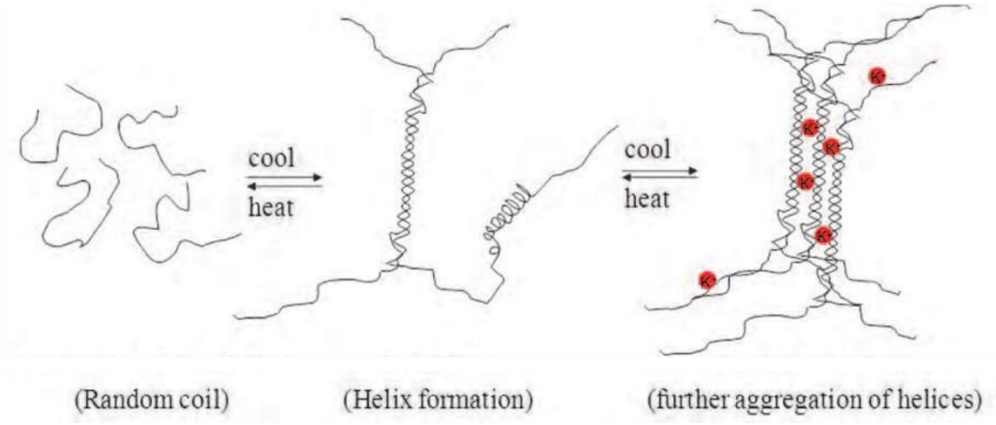

Figure 14. Representation of the coil-to-helix transition in the gelation process (adapted from [67]).

In the presence of monovalent or divalent cations added during cooling, the amount of salt bridges at the junction zones is bigger, making them more resistant to heating and therefore the gelling potential of gellan gum is enhanced [69]. Though the presence of cations is indispensable for the gelation process of gellan gum, the gellan gum reactivity with divalent cations is generously higher than with monovalent cations [70].

There are several factors that affect the gel strength and functionality [63, 66]. The most relevant is the acetyl content since gels present different properties if the acetyl content of the gellan gum solutions is also different. Generally, gellan gum with high acetyl content, like native gellan gum, forms soft, elastic and nonbrittle thermoreversible gels, while low acetyl gellan, such as deacetylated gellan gum, provides firm and brittle thermoreversible gels [68].

The type and concentration of ions, as previously mentioned, have an impact on gel strength and brittleness, namely divalent cations that even in gellan gels of very low concentration can achieve a high gel strength. In contrast, to produce a similar gel strength with monovalent cations, higher concentrations of gellan are required. In fact, monovalent cations had lower gel strength even with high salt concentration [63, 66].

The gel strength can also be influenced by the gel pH. Typically, the gel strength is enhanced within a pH range of 3.5 to 8, which is in accordance with the pH range in which the gellan is stable, 2 to 10 [71].

Another factor that affects the gel strength is the presence of hydrophilic ingredients that apparently are dependent on cation concentration [63]. Normally, the addition of hydrophilic ingredients, like sucrose, has the capacity of reducing the ion concentration required for great gellan gel strength [63, 66].

The temperature stability and the flexibility of the melting point are the final factors discussed as gel strength influencers [63, 66]. Gellan gum is stable at high temperatures, preserving its strength at 90ºC [71]. The melting point is highly flexible and depends on the conditions of gel formation, mainly on the concentration of cations present in the gels, such as mentioned earlier, increase the number of junction zones, making them more heat resistant [63].

In addition to all these functional properties, gellan gum is also relatively nontoxic, presents dispersibility (because it is easily and completely dissolved in water), compatibility (since it can be combined with other polymers), providing a wider spectrum of functional properties and applications, and flavour release (which is possibly due to the water binding properties of gellan gum) [66].

1.4.3. Applications

As previously mentioned, gellan gum is widely applied in the food, pharmaceutical and other industries as emulsifiers, stabilizers, binders, gelling agents, coagulants, thickening and suspending agents, due to its unique structure and numerous properties [64].

Gellan gum is been applied in the food industry since 1992 and thereby is the most well-known application of gellan [63]. This polymer is used as a highly versatile additive in different types of food. Gellan gum can be used in confectionery and bakery products to provide structure and texture for the reduction of the setting time. Additionally, gellan gum can prevent moisture fluctuations in sugary foods, icings and toppings [63, 66]. Gellan gum is also used in the preparations of water-based desserts and aspics, such as meat and vegetable aspic, mostly because gellan addition can increase the melting point of the products, helping them to remain soft and juicy with their visual appearance intact [63]. In the case of jams and jellies, gellan gum can be used to replace pectins as texturizer agents, being effective at lower

concentrations. Pie fillings and puddings have a firm consistency conventionally acquired from starches [66], which can be replaced by gellan gum, a structuring agent. Gellan gum combined with modified starches can also be used as a stabilizer and water-binding agent, averting starches from having the "blunting effect" on food flavour. Since the gellan gum offers a high thermal resistance during the pasteurization process, preserving shape under the processing conditions, this polymer can be applied in the category of fabricated foods, like fruit pieces or meat chunks. Dairy products are another type of foods in which gellan gum can be applied. For instance, in cheese making process, the added gellan interacts with milk proteins, increasing the water retention and the total yield of cheese, and reducing the solid losses, especially whey proteins [63, 66].

In the pharmaceutical industry, gellan gum is applied in various areas, namely oral drug delivery, ophthalmic and nasal formulations. Some of these applications are summarized in Table 2. Briefly, in oral drug delivery, gellan is mainly used as a disintegrating agent or as a matrix-forming vehicle for sustained release. In ophthalmic formulations, gellan can be applied as a thickening and gelling agent to extend the drug release, and in nasal formulations, gellan improves epithelial transport and shows higher bioavailability [65].

Table 2. Some examples of oral, ophthalmic and nasal formulations based on gellan gum [65].

Formulation type	**Drug used**	**Action**
Oral formulations		
Gellan capsules and beads; gellan beads; *in situ* gelling system	Theophylline	Phosphodiesterase inhibitor
Gellan beads	Propranolol hydrochloride	Beta-blocker
***In situ* gelling system**	Paracetamol	Analgesic, antipyretic
Gellan beads	Cephalexin	Antibiotic
Gellan granules	Ephedrine hydrochloride	Sympathomimetic
***In situ* gelling floating gellan system; gellan macrobeads; gellan beads coated with chitosan**	Amoxicillin	Antibiotic
Carboxymethyl gellan beads	Metformin	Antidiabetic
Gellan beads gelated by Al^{3+} and crosslinked by glutaraldehyde	Glipizide	Type 2 diabetes mellitus
Microcapsules of gellan gum and egg albumin	Diltiazem-resin complex	Antihypertensive
Ophthalmic formulations		
***In situ* gelling ophthalmic solution**	Sezolamide, dorzolamide	Anti-glaucoma
Soluble bioadhesive ocular insert	gentamicin	Antibiotic for veterinary use

Table 3 (cont.). Some examples of oral, ophthalmic and nasal formulations based on gellan gum [65].

Formulation type	**Drug used**	**Action**
***In situ* gelling ophthalmic solution; albumin nanoparticles with gellan for ophthalmic use**	Pilocarpine	Anti-glaucoma
***In situ* gelling ophthalmic solution**	Indomethacin	Anti-inflammatory
	Timolol maleate	Anti-glaucoma
	Piroxicam	Anti-inflammatory
	Gatifloxacin	Bacterial conjunctivitis
***In situ* gelling ophthalmic nanoemulsion**	Terbinafine hydrochloride	Fungal keratitis
Nasal formulations		
***In situ* nasal gel**	Fluorescein dextran	Epithelial uptake testing
	Momethasone furoate	Anti-inflammatory
Intranasal microparticles	Metoclopramide hydrochloride	Antiemetic
***In situ* nasal gel based on thiolated gellan**	Dimenhydrinate	Motion sickness prevention

Gellan gum can be used for personal care in cosmetics. Lotions and creams, make-up, face masks, hair care products, toothpaste, and air freshener gels, are the type of products included in this category, where gellan gum can be used as stabilizer and suspending agent (for example in shampoo and conditioner products), and as stabilizer and emulsifying agent (for instance in creams and lotions, especially in suntans and sunscreens). Also, due to its binding properties, reversible structure and flavour release, gellan gum is useful in tooth-paste formulations [64].

More recently, gellan gum has also been investigated in the biomedical field, mostly in tissue engineering, surgery and wound healing [65]. In tissue engineering, gellan is used mainly as a material for cartilage reconstruction, through the incorporation/encapsulation of autologous cells, for example, human nasal chondrocytes or bone marrow cells, in a gellan matrix. The results are promising since most cases showed a good cell viability during packing in the polymer matrix and preliminary cell proliferation [72, 73]. Moreover, as a biocompatible and non-toxic polymer, gellan can be used in novel wound dressings in order to prevent postsurgical adhesion and scar formation [65]. An example is a series of thick, water-insoluble films of low-acyl gellan crosslinked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), that showed to be non-toxic and has antiadhesive properties, with satisfactory effects [74].

In the biotechnology industry, gellan gum can be used as an alternative to agar in culture media for growth of microorganisms and plants. Due to its thermostable property, gellan is particularly useful for the culture of thermophilic microorganisms. In addition, properties like water clarity

and high purity are advantages in both microbiological media and plant tissue culture applications. In the last one, gellan gum also presents the benefits of being resistant to contamination by moulds and allowing the clear observation of the tissue development [64]. In a secondary field, like molecular biology, gellan gum can be used in gel electrophoresis, for example, as an agarose replacement. In this application, gellan gels combined with a second polymer work as a matrix to separate DNA fragments based on size [63].

Finally, our research group has been exploring the application of gellan gum in the purification of biomolecules, and why not? Gellan gum is negatively charged, which is perfect to establish ionic interactions with positive groups of biomolecules. Besides, as previously said, in the presence of cations forms thermo-resistant gels, which are stable within a vast pH range. In addition, gellan gum has properties like porosity, hydrophilicity and high binding capacity that are considered extremely important to improve the performance of a chromatographic matrix [75].

Chapter 2 – Objectives

The major aim of this project consists of reinforcing the recent applicability of gellan gum in the purification of biomolecules, thereby using a new formulation of gellan microspheres to capture the hSCOMT protein from *Komagataella pastoris* complex lysates. So, in order to achieve this goal, some assignments need to be performed.

The first assignment comprehends the gellan microspheres production by a water-in-oil emulsion technique, which is an easy and low-cost method, followed by a reinforcement of the target microspheres through the addition of a divalent cation solution, namely Ni^{2+} or Mg^{2+} solutions. The nickel ion was chosen based on the best results obtained in previous works of our research group, and magnesium ion because it is a hSCOMT cofactor, having a natural affinity for the enzyme.

The second assignment consists of confirming the microspheres formation and the presence of the divalent cations used as crosslinkers, by semi-optical microscopy, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) analysis.

Finally, the last assignment is based on the application of these microspheres in a batch method to design and optimize capture strategies of hSCOMT from *Komagataella pastoris* lysates.

Chapter 3 – Materials and Methods

3.1. Materials

Ultrapure reagent-grade water used for the solutions was obtained from a Milli-Q system (Merck Millipore, Germany). Gellan gum (Gelzan ®), glass beads, deoxyribonuclease I (DNase), protease inhibitor cocktail and bromophenol blue were purchased from Sigma-Aldrich Co. (St Louis, USA). Nickel chloride hexahydrate ($NiCl_2.6H_2O$), glucose, agar, methanol, sodium chloride (NaCl), Tris-Base, hydrochloric acid (HCl) for glass beads wash, imidazole, citric acid, bovine serum albumin (BSA), glycine and Tween-20 were purchased from Thermo Fisher Scientific (Loughborough, UK). Zeocin was obtained from InvivoGen (Toulouse, France) and yeast extract from Biokar Diagnostics (France). Peptone was purchased from Becton, Dickinson Company (Sparks, MD), yeast nitrogen base (YNB) from Pronadisa (Malaysia), biotin from Roche (Basileia, Swiss) and glycerol was obtained from HIMedia Laboratories (Mumbai, India). Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) was obtained from labkem (Barcelona, Spain). 4-Morpholineethanesulfonic acid (MES), L-cysteine and trehalose were purchased from Acros Organics (Geel, Belgium). Potassium phosphate dibasic (K_2HPO_4), dithiothreitol (DTT) and sodium dodecyl sulphate (SDS) were obtained from PanReac AppliChem (Darmstadt, Germany). Potassium phosphate monobasic (KH_2PO_4) and acetic acid glacial were purchased from Chem-labs (Nairobi, Kenya). Bis-Acrylamide 30% was obtained from Grisp Research Solutions (Porto, Portugal). Sodium citrate, ß-mercaptoethanol and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Merck (Germany). Ammonium persulphate (PSA) was obtained from Eurobio (Courtaboeuf, France). Monoclonal rabbit anti-COMT antibody was purchased from Abcam (Cambridge, UK) and anti-rabbit IgG alkaline phosphatase secondary antibody were obtained from GE Healthcare Biosciences (Brondby, Denmark). Agarose was acquired from Hoefer (San Francisco, USA). Ribonuclease I (RNase) and Greensafe were purchased from NZYTech (Lisbon, Portugal).

3.2. Gellan microspheres production by water-in-oil emulsion

The gellan microspheres production by the water-in-oil emulsion technique was described by Narkar and co-workers [76] and optimized by our research group through an experimental design tool [77]. Firstly, a 1.41 % (w/v) gellan solution was prepared in water and, with a constant stirring of 300 rpm, was heated at 100ºC for 15 minutes. After gellan dissolution in water, the solution was transferred to a syringe attached to a laboratory support with a 21G needle (Medoject, UK). This syringe will leak dropwise of a 20 cm height at a flow rate of 75 µL/min programmed in a Harvard apparatus 11 plus syringe pump (Harvard Apparatus, UK) into a 100% vegetable cooking oil solution, under a constant sitting of 750 rpm and a temperature set point near 100 ºC.

Then, a 200 mM $NiCl_2$ or $MgCl_2$ (crosslinker) solution was added to the mixture at the same constant stirring of 750 rpm during 30 minutes at room temperature. Finally, the microspheres were washed with ethanol 70% and dried with water in a vacuum filtration with a filter paper (VWR, USA), and then recovered and stored in a 10 mM MES buffer solution, pH 6.2, at 4 ºC until further application.

3.2.1. Semi-optical microscopy analysis of gellan microspheres

The semi-optical microscopy was used to determine the average diameter of the gellan microspheres. Both nickel-crosslinked and magnesium-crosslinked formulations of the gellan microspheres, stored in the 10 mM MES buffer, pH 6.2, were put in microscope slides. The samples were visualized in a hydrated state at 10x magnification lens, and five different images (n=5) of each formulation were taken in order to determine the mean diameter of the gellan microspheres.

3.2.2. SEM analysis of gellan microspheres

The scanning electron microscopy (SEM) was applied to visualize the morphology of the gellan microspheres, through a Hitachi S-3400 N (Tokyo, Japan). The gellan microspheres of each formulation were removed from the storage MES buffer and transferred to an aluminium support with a carbon base, and then frozen at -20 ºC. The images were acquired at different magnifications, through a Bruker 129 eV (Bruker, USA).

3.2.3. EDX analysis of gellan microspheres

After the SEM analysis, the gellan microspheres were subjected to an energy-dispersive X-ray spectroscopy (EDX) analysis to identify the major chemical elements present in nickel-crosslinked and magnesium-crosslinked formulations and confirm the presence of the divalent ions. For this goal, the gellan microspheres still in a frozen state, after the SEM analysis, were analysed at 15 kV through a QUANTAX 400 (Bruker, USA).

3.3. Recombinant hSCOMT Biosynthesis

The recombinant production of human soluble catechol-*O*-methyltransferase isoform was performed as described by Pedro and contributors [30]. The *Komagataella pastoris* X33 cells were grown at 30 ºC in YPD plates (1% yeast extract, 2% peptone, and 2% glucose). Then, a single colony was inoculated in 50 mL of BMGH medium (100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base, 0.4 mg/L biotin, and 1% glycerol) in 250 mL shake flasks, where cells were grown at 250 rpm and 30 ºC overnight. Normally during this time, the cell density at 600 nm (OD_{600}) reaches 6.0. Since the initial OD_{600} of the main fermentation is fixed at 1.0, an aliquot of the inoculum was collected according to the following equation:

$$OD_{pre-inoculum} \times V_{pre-inoculum} = \left(V_{pre-inoculum} + V_{fermentation}\right) \times OD_{fermentation} \quad (1)$$

The aliquot was centrifuged at room temperature for 5 minutes and, ensuring that all glycerol was removed, the resulting pellet of cells was resuspended in the BMMH medium (100 mM potassium phosphate buffer, pH 6.0, 1.34% yeast nitrogen base, 0.4 mg/L biotin, and 0.5% methanol), which was added to a total volume of 100 mL within 500 mL shake-flasks. The fermentations were performed at 30 ºC and 250 rpm for 120 hours. Every 24 h, 1% (v/v) methanol was added to the cultures in order to induce the promotor AOX, which stimulates the expression of the recombinant hSCOMT. At last, the cells were centrifuged at 1500 g and 4 ºC for 10 minutes, and then stored at -20 ºC until use.

3.3.1. Recombinant hSCOMT Recovery

The recovery of the recombinant human soluble catechol-*O*-methyltransferase was performed partially as described previously [30]. Briefly, cells were mechanically lysed in a proportion of 1:2:2, corresponding to 1 g of cells, 2 g of glass spheres and 2 mL of lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM DTT, 1 mM $MgCl_2$, pH 8.0), respectively. It was also added a cocktail of protease inhibitors (1 mM PMSF, 5.0 µg/mL leupeptin, and 0.7 µg/mL pepstatin A). The mechanical lysis of this mixture was made through 7 vortex cycles of 1 minute, which were intercalated by incubations of 1 minute on ice. Afterwards, the mixture was centrifuged at 500 *g* and 4 ºC for 5 minutes, and the supernatant was discarded. The resultant pellet was resuspended in the lysis buffer along with DNase (1 mg/mL) and RNase (1 mg/mL) solutions.

3.4. Batch method for the capture of hSCOMT

The batch method was applied with different capture strategies in order to isolate the hSCOMT from complex *K. pastoris* lysates by using gellan microspheres with nickel or magnesium as a crosslinker. Briefly, the batch method consisted of an initial equilibration of gellan microspheres, followed by three characteristic steps of the batch method interleaved by a centrifugation step: the binding step to promote the interaction of hSCOMT with the gellan microspheres, the washing step to washout the contaminants that did not bind to the microspheres, and finally the elution step where the dissociation of the hSCOMT adsorbed in the gellan microspheres take place. All the steps were performed under constant stirring during a certain period.

Several assays were performed in order to optimize this method to achieve the best performance for hSCOMT adsorption. This optimization included the manipulation of various parameters such as the volume of gellan microspheres and the hSCOMT lysate concentration, the duration of binding and elution steps, the temperature set point, the ionic strength and pH of the binding and/or elution buffers and the application of hSCOMT stabilizers.

In general, two strategies were optimized with each microspheres formulation. Starting with gellan microspheres containing nickel as crosslinker, where the major optimization of the batch method was made, the first strategy consisted in the binding of the hSCOMT to the gellan

microspheres with 10 mM imidazole in 10 mM Tris buffer, pH 7.5. The elution was done with increasing concentrations of NaCl (150 mM and 250 mM) and imidazole (300 mM and 500 mM) in 10 mM Tris buffer, pH 7.5. In the second strategy, the hSCOMT bound to gellan microspheres with 10 mM citrate buffer, pH 4.0, and was eluted with increasing NaCl concentrations (100 mM, 200 mM and 500 mM) in 10 mM MES buffer, pH 6.2.

Regarding the gellan microspheres with magnesium as a crosslinker, the first strategy was similar to the second strategy by using gellan microspheres with nickel as a crosslinker. Briefly, the hSCOMT was bound to the gellan microsphere with 10 mM citrate buffer, pH 4.0, and eluted with increasing NaCl concentrations (100 mM, 200 mM and 500 mM) within 10 mM MES buffer, pH 6.2. In the second strategy, the binding step was performed with 10 mM MES buffer, pH 5.2 and the elution with increasing NaCl concentrations (100 mM and 500 mM) and 500 mM $MgCl_2$ in 10 mM MES buffer, pH 5.2.

3.4.1. Total Protein Quantification

The protein content in the samples, both hSCOMT lysate and recovered supernatants, was measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, USA), according to manufacturer's instructions. Briefly, 10 µL of each sample in triplicates were prepared and transferred to the wells of a 96 well microplate. Depending on sample concentration, a sample dilution between 1:10 and 1:30 could be required. Then, 200 µL of working reagent (W_R, provided by the manufacturer) were added to each well and homogenized. The microplate was incubated in the dark at 37ºC for 30 minutes and, after, the absorbance was measured at 562 nm in an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, USA).

Calibration curves were made for each buffer presented in the samples, by using bovine serum albumin (BSA) as standard. Solutions of different concentrations within a range of 0.025 to 2.0 mg/mL were prepared in triplicates applying the corresponding buffer solution. Therefore, four standard curves were constructed: the calibration curve of lysis buffer (Figure 15), the calibration curve of citrate buffer (Figure 16), the calibration curve of MES buffer (Figure 17) and the calibration curve of Tris buffer (Figure 18).

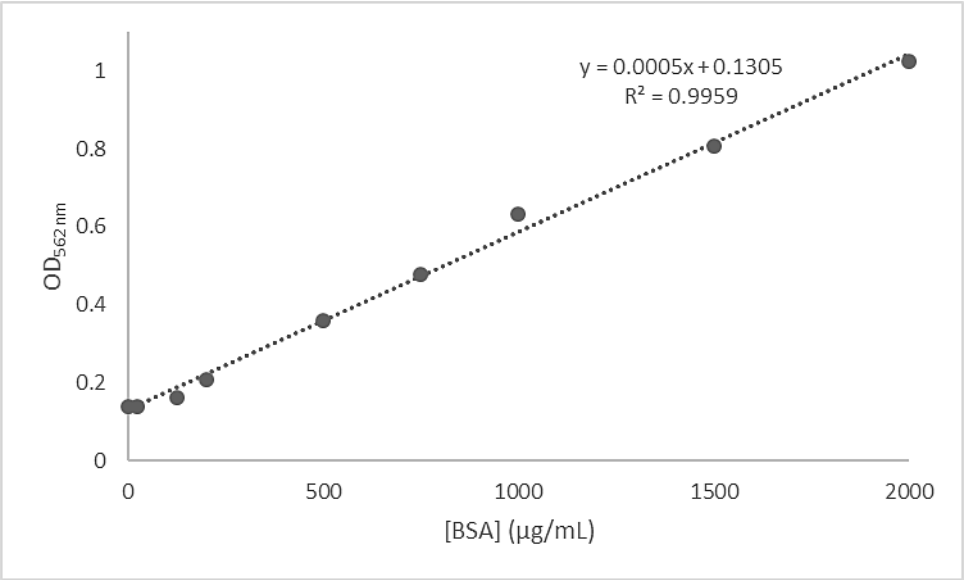

Figure 15. Standard curve of the relationship between BSA concentration and optic density at 562 nm, for the lysis buffer.

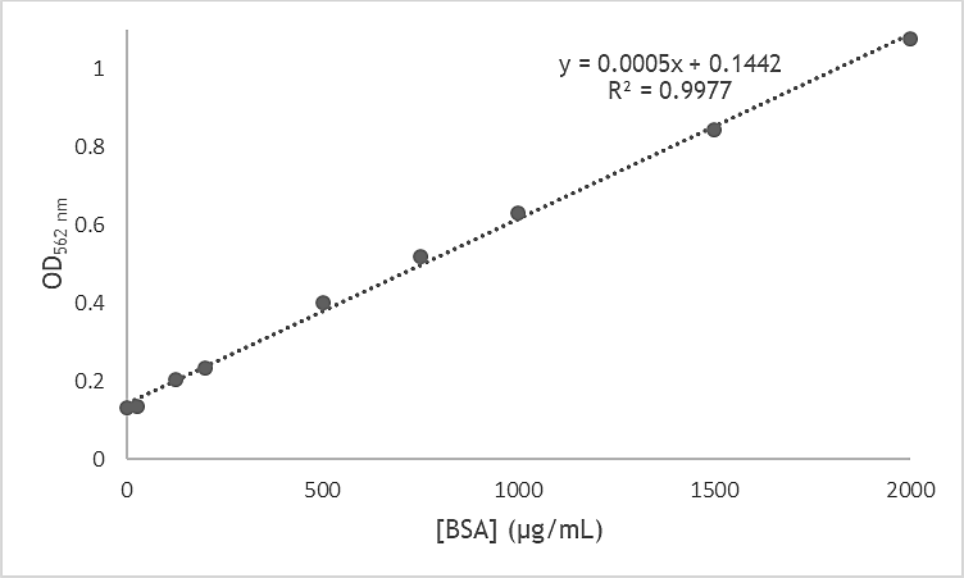

Figure 16. Standard curve of the relationship between BSA concentration and optic density at 562 nm, for the Tris buffer.

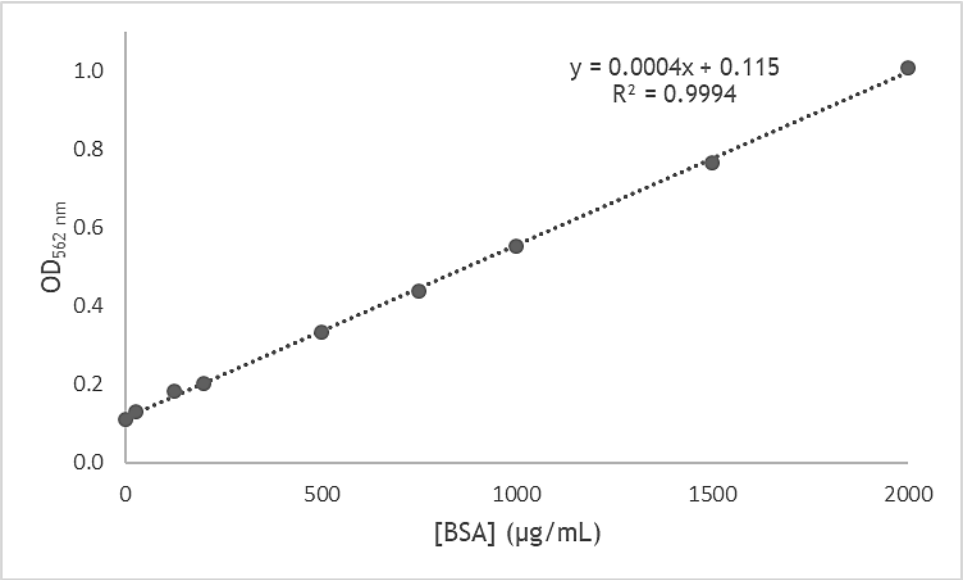

Figure 17. Standard curve of the relationship between BSA concentration and optic density at 562 nm, for the MES buffer.

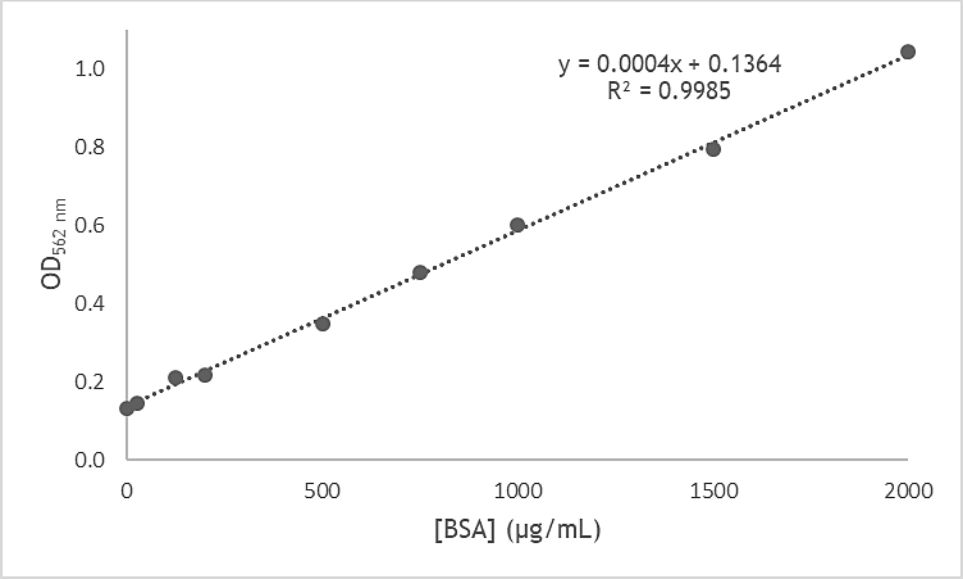

Figure 18. Standard curve of the relationship between BSA concentration and optic density at 562 nm, for the Citrate buffer.

When the stabilizers solution was used, the samples were precipitated by using the Compat-Able Protein Assay kit (Thermo Fisher, USA) before the quantification by the Pierce BCA Protein Assay Kit. The precipitation kit removes salts, detergents, reducing agents and other substances from protein samples, thereby eliminating interference with protein assays. Specifically, this extra precipitation in the quantification assay had the intuit to eliminate the interference of the cysteine, a reducing agent used as a stabilizer of the hSCOMT. Briefly, at 15 µL of each sample were added 150 µL of a precipitant reagent, followed by 150 µL of a co-precipitant.

Then, the mixtures were centrifuged at 14000 rpm for 10 minutes, and the supernatants discarded. The pellets were resuspended in 10 mM Tris buffer, pH 7.5 and quantified by the Pierce BCA Protein Assay Kit, according to the manufacturer's instructions. Since protein was precipitated, the use of protein mass is more reliable than the concentration. So, for this quantification, it was also made a calibration curve (Figure 19).

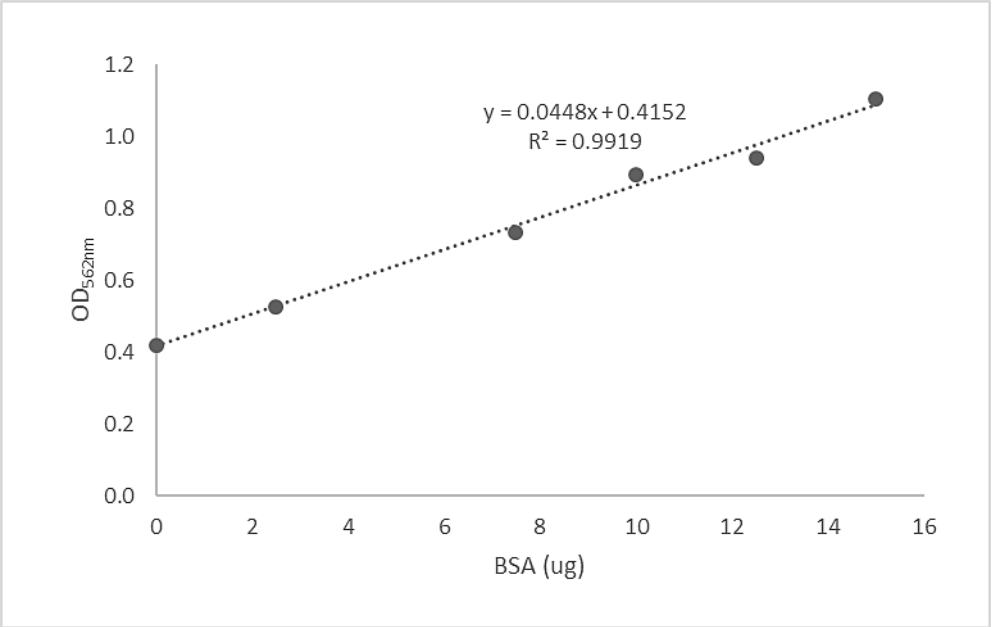

Figure 19. Standard curve of the relationship between BSA content and optic density at 562 nm, for the Tris buffer.

3.4.2. SDS-PAGE and Western blot analysis

The Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed according to the Laemmli method [78]. The samples normalized with 30 ug of total protein were prepared in a loading buffer (500 mM Tris-HCl, pH 6.8, 10 %(w/v) SDS, 0.02 %(w/v) bromophenol blue, 0.2 %(v/v) glycerol, 0.02 %(v/v) ß-mercaptoethanol) with a proportion of 3:1 (30 µL of normalized sample to 10 µL of loading buffer) and then denatured at 100ºC for 5 minutes. The electrophoretic assay was made on 4.5 % stacking and 12.5 % resolving gels at 120 V during approximately 1 hour and 40 minutes with a running buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS) at room temperature. After the running of two equal gels, one of them was used in the western blot assay, while the other one was stained with the colloidal blue staining.

In the western blot assay, the protein extracts in the gel were electrotransferred to a 6x9 cm PVDF membrane (GE Healthcare, UK) in an electrotransfer buffer (25 mM Tris, 192 mM glycine, 10 %(v/v) methanol, 10 %(v/v) SDS) at 750 mA during approximately 1 hour. Then, the membrane was immersed in a 5 % (w/v) non-fat milk in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20) for 1 hour with a constant stirring, to block non-specific sites. Next, the

membrane was washed in TBS-T and incubated overnight with a monoclonal rabbit anti-COMT antibody, diluted at 1:1000 in TBS-T, at 4 ºC and a constant stirring. Afterwards, the membrane was washed again in TBS-T and incubated with an anti-rabbit IgG alkaline phosphatase secondary antibody, diluted at 1:40 000, for 1 hour at room temperature under a constant stirring. Finally, the membrane was washed in TBS-T, subsequently exposed to ECL substrate for 5 minutes, and visualized on the Molecular Imager FX (BioRad, Hercules, USA).

The other gel obtained from the electrophoretic assay was initially fixed in a fixation solution (40 %(v/v) ethanol pure, 10 %(v/v) acetic acid glacial 99 %) for 1 hour at room temperature and constant stirring. Then, it was stained by Coomassie brilliant blue overnight also at room temperature and constant stirring. At last, the gel was transferred to a discolouration solution (1 %(v/v) acetic acid glacial 99%) for 1 hour at room temperature under a constant stirring.

3.4.3. Agarose gel electrophoresis

The nucleic acids content of the samples was analysed by an agarose gel electrophoresis as described in Nunes *et al*. [79]. Briefly, the agarose electrophoresis was run by an electric field in a horizontal gel electrophoresis using a 15 cm long gel of 1 %(w/v) agarose in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8.0), stained with Greensafe (1 µL/mL). The electrophoresis was carried out at 110 V for 30 minutes and then visualized in a UVITEC system (Cambridge, UK) using Firereader 1D Gel analysis software version 15.15 of UVITEC.

Chapter 4 – Results and Discussion

The development of new and different COMT inhibitors with a good and safe therapeutic profile relies on the availability of samples with high purity. To satisfy the requirements of the pharmaceutical industry, the implementation of new faster and reproducible isolation and purification strategies is imperative [80]. The batch method is a simple, rapid and low-cost alternative to sample precipitation and clarification strategies or even to chromatographic techniques for the isolation of proteins [57]. In addition, the batch method presents the peculiar advantage of huge flexibility regarding the nature of the material of the particles [56], which in this case is gellan gum. Gellan gum is an anionic microbial exopolysaccharide that can form gels with different conformations and structure depending on polymer concentration, temperature, aqueous environment, and presence of monovalent or divalent cations [81]. Despite of numerous applications of gellan gum in the food, pharmaceutical and other industries as emulsifiers, stabilizers, binders, thickening agents, etc [64], this polymer has been recently investigated as a chromatographic matrix not only due to the properties already mentioned but also to its porosity, hydrophilicity, high binding capacity, and capacity of forming gels, resistant to temperature and extreme acidic conditions [75]. Considering all these facts, the main goal of this research work consists of developing capture strategies to isolate hSCOMT from a complex *Komagataella pastoris* lysate through a batch method using gellan microspheres.

4.1. Gellan microspheres production by water-in-oil emulsion

Emulsions are techniques commonly used to produce polymeric microparticles and can be easily scaled-up, constituting an advantage for industrial applications [82]. By definition, the emulsion technique consists of two immiscible liquids in which small spherical droplets of one liquid, usually referred to as the “dispersed phase”, are dispersed in the other one, also called “dispersing phase”. In the case of a water-in-oil emulsion, the two immiscible liquids are water and oil, and consists of water droplets dispersed in an oil phase. The reduction of the droplets size in the emulsions is normally achieved by applying mechanical agitation to the liquid mixture [83]. Typically, the production of microparticles has a gelation step following the emulsion procedure. After the dispersion of the polymer solution in the organic phase, like the oil, the gelation step starts by cooling or with the addition of a gelling agent into the emulsion [82].

Gellan gum, as previously mentioned, has the ability to form hydrogels in the presence of divalent cations. The gelation of gellan is characterized by an initial formation of double helices from random coil chains and, subsequently, an aggregation of pairs of double helices, forming a three-dimensional network [84]. However, at low ionic strength, the negatively charged carboxylic groups on the gellan promote an electrostatic repulsion, impeding the aggregation

of double helices. In the presence of divalent ions, electrostatic interactions are established between them and the carboxylic groups of double helices, increasing the junction zones formation and, consequently forming a more stable and strength three-dimensional network [69].

The lab process of production and recovery of gellan microspheres is represented in Figure 20. Firstly, the gellan microspheres were produced by water-in-oil emulsion by using commercial gellan gum and conditions optimized through an experimental design tool by our research group [77]. An aqueous solution of gellan gum with a concentration of 1.41 %(w/v) was heated at 100ºC under a constant stirring of 300 rpm. The polymeric solution was dripped, through a 21G needle, into the oil solution previously heated with a constant stirring of 750 rpm, at a flow rate of 75 µL/min. Then, the emulsion mixture was transferred to a divalent ion solution at room temperature in order to reinforce and stabilize the gellan microspheres. At last, gellan microspheres were recovered by vacuum filtration of the mixture, washing with ethanol 70% and drying with water.

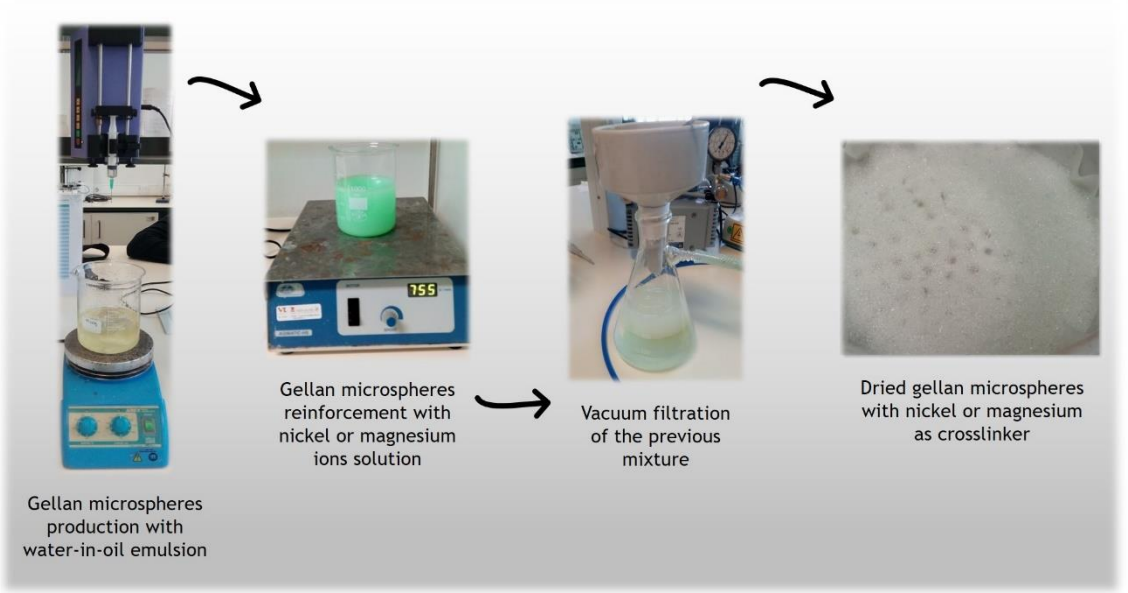

Figure 20. Schematic representation of the process for production and recovery of gellan microspheres.

The reinforcement solutions contained nickel chloride or magnesium chloride. The nickel ion was chosen according to a previous analysis performed by our research group. In this study, the gellan microspheres were produced using different divalent cations as crosslinkers and, then, analysed and compared on the basis of their mean diameter and morphology. The gellan microspheres with nickel as a crosslinker were selected due to their minimum mean diameter and their uniform spherical structure, in comparison with other microspheres [85].

The magnesium ion was additionally used as crosslinker, since beyond to be a divalent ion, it is also a biological cofactor of the COMT, and can establish specific interactions with the target

molecule. The catechols *O*-methylation catalysed by COMT is magnesium-dependent since this cation plays the important structural role of orienting the hydroxyl groups of catechol substrate and the methyl group of SAM into a reactive conformation, on the active site [86]. This fact suggests that COMT has a natural affinity for the magnesium ions.

After the production and recovery of gellan microspheres, both nickel-crosslinked and magnesium-crosslinked formulations were analysed by semi-optical microscopy, SEM and EDX to evaluate the mean diameter, the morphology and the chemical composition of the gellan microspheres, respectively.

4.1.1. Semi-optical microscopy analysis of gellan microspheres

The gellan microspheres after production ad recovery were analysed on a semi-optical microscope to assess the respective mean diameter. From the samples visualized on the semi-optical microscope, five images (n=5) were captured for each one of the crosslinker formulations used. These images were then treated and the average diameter of both formulations of gellan microspheres was calculated. Figure 21 shows an example of each formulation of gellan microspheres with the diameter measurements for the mean diameter determination.

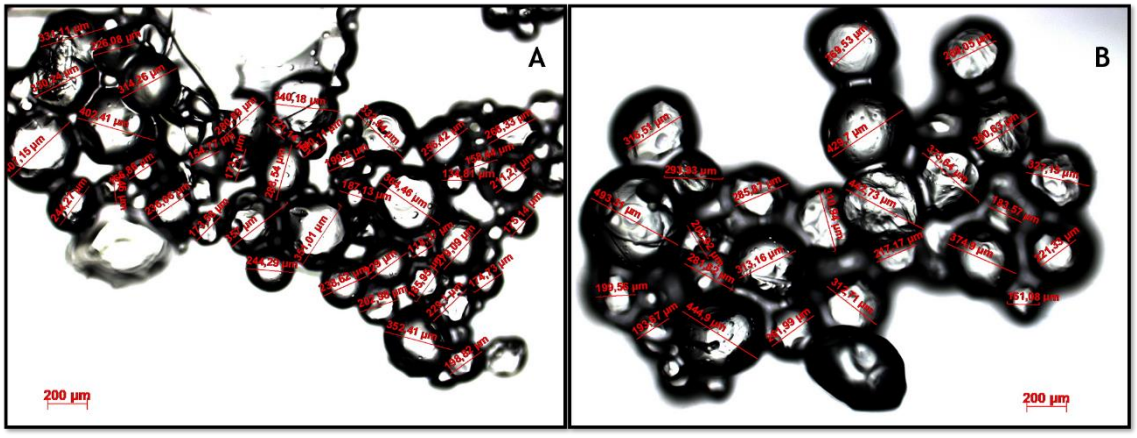

Figure 21. Images of gellan microspheres obtained from semi-optical microscopy. A – Gellan microspheres with nickel as crosslinker; B – Gellan microspheres with magnesium as crosslinker.

The mean diameter values of five different microscopic fields (n=5) of nickel-crosslinked and magnesium-crosslinked gellan microspheres are represented in Table 4. According to the values presented, the gellan microspheres with nickel as crosslinker showed a mean diameter of approximately 239 µm, which is a little bit smaller than the one of 300 µm calculated for the magnesium-crosslinked gellan microspheres. This result is in agreement with Figure 21, where this difference between the diameters of the two gellan microspheres formulation can be observed.

Table 4. Mean diameter of nickel-crosslinked and magnesium-crosslinked gellan microspheres.

Crosslinker ion	Nickel	Magnesium
Mean diameter of each replica (µm)	236.19	280.40
	235.80	307.61
	239.26	309.11
	249.47	306.13
	234.56	298.09
Average diameter of the replicas (µm)	239.06	300.27

For instance, alginate microspheres are typically prepared with calcium as crosslinker and have a diameter of 50-1000 µm [87, 88]. Being a polymer with similar properties to the alginate, the gellan microspheres are within the diameter range. The major advantage of producing microspheres with small dimensions for capture processes relies on the contact area increment. This is, as smaller the microspheres then the empty space between them is also smaller and, consequently, higher the contact surface area to promote the target molecule binding [89].

Regarding the difference on the mean diameter between the gellan microspheres produced, it can be due to the divalent ion used as crosslinker, namely its atomic radius. Knowing that the atomic radius of the magnesium (1.60 Å) is larger than nickel (1.28 Å) [90], it is supposed to fill a larger space between the gellan molecules, thereby resulting in microspheres with larger diameters [91].

4.1.2. SEM analysis of gellan microspheres

The gellan microspheres produced with different crosslinkers were frozen at -20 ºC for the SEM analysis. This technique was used to collect more information about the structure of the gellan microspheres, namely their morphology. The SEM images of gellan microspheres with nickel or magnesium as crosslinkers are illustrated in Figure 22 and Figure 23, respectively. These SEM images were captured obtained with different degrees of magnification.

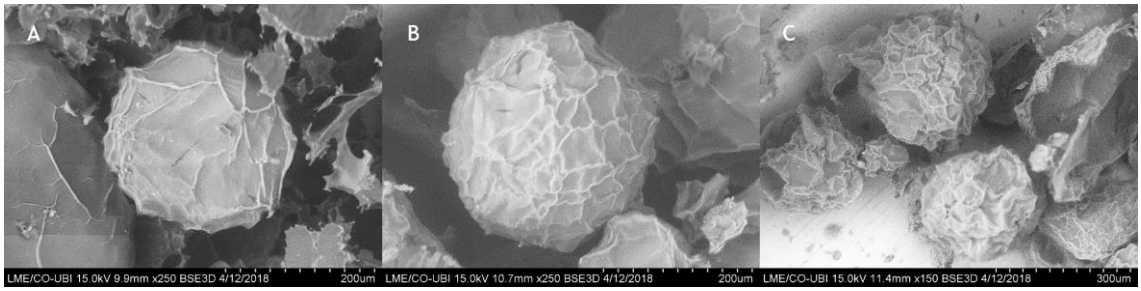

Figure 22. Representation of the gellan microspheres with nickel as crosslinker, obtained from SEM, with magnifications of x250 (A and B) and x150 (C).

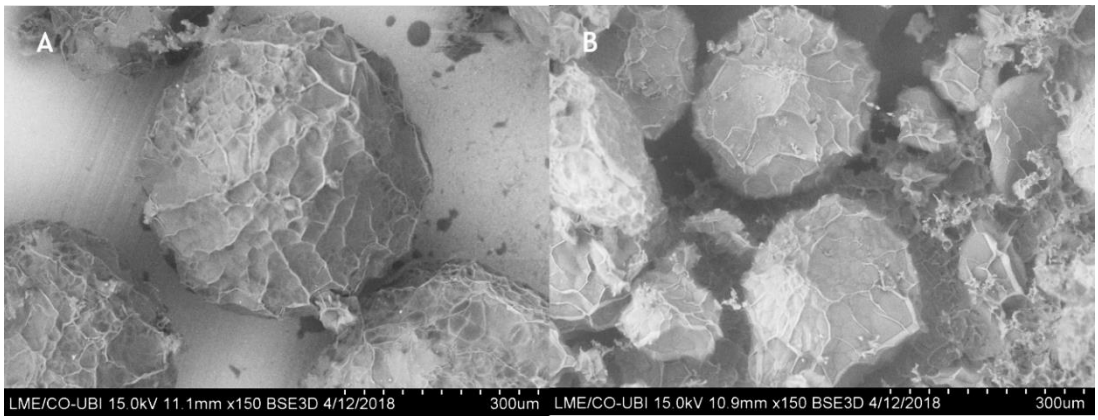

Figure 23. Representation of the gellan microspheres with magnesium as crosslinker, obtained from SEM, with a magnification of x150 (A and B).

The SEM images of both nickel-crosslinked (Figure 22) and magnesium-crosslinked (Figure 23) gellan microspheres show a consistent and uniform structure with a spherical shape. This morphology is in accordance with the results obtained in a previous work with other crosslinkers in which whatever the crosslinker, the gellan microspheres presented a similar uniform spherical structure [85]. In addition, gellan microspheres with calcium or magnesium as crosslinkers were also described to have a similar morphology to the ones obtained in this work [92].

4.1.3. EDX analysis of gellan microspheres

The same samples used on the SEM analysis were subjected to an elementary analysis by EDX in order to determine the major constituent of the gellan microspheres and, more importantly, confirm the presence of the crosslinker used to reinforce and stabilize those gellan microspheres. The outputs of this elementary analysis for the gellan microspheres with nickel and magnesium as crosslinkers are represented in Table 5 and Table 6, respectively. Those results are expressed in normalised concentration by weight percentage of each element (C norm. [wt. %]) and in atomic concentration by atomic percentage of each element (C Atom. [at. %]) and have also the error associated to the weight percentage at the 2 Sigma level (C Error (2 sigma) [wt. %]).

Table 5. Elementary analysis of gellan microspheres with nickel as crosslinker, through EDX.

Element	C norm. [wt. %]	C Atom. [wt. %]	C error (2 Sigma) [wt. %]
Carbon	39.19	47.13	8.91
Oxygen	57.73	52.12	12.84
Nickel	3.08	0.76	0.27
Total	100.0	100.0	

Table 6. Elementary analysis of gellan microspheres with magnesium as crosslinker, through EDX.

Element	C norm. [wt. %]	C Atom. [wt. %]	C error (2 Sigma) [wt. %]
Carbon	31.70	38.33	7.22
Oxygen	67.25	61.04	14.61
Magnesium	1.04	0.62	0.16
Total	100.0	100.0	

Both elementary analyses showed that the major components of the gellan microspheres are carbon and oxygen. This result was expected since the gellan gum is a polysaccharide composed by a tetrasaccharide repeat unit of two ß-D-glucose residues, one ß-D-glucuronic acid, and one α-L-rhamnose [93]. In addition, the crosslinker ions content proves that the reinforcement process was successful, and these ions are indeed present in the microspheres. However, the percentages differ in function of the crosslinker, suggesting that the binding capacity of different crosslinkers to the gellan microspheres is variable, which was also mentioned by our research group in previous work [85].

Like in the mean diameter determination of both gellan microspheres formulations, the difference between the magnesium and nickel contents can also be related to the atomic radius of magnesium and nickel. If nickel occupies a smaller space between the gellan molecules comparatively to magnesium, in a certain microsphere area, it is supposed to be found a higher amount of nickel than magnesium [91].

4.2. Capture strategies of hSCOMT through the batch method

The COMT protein is an important biopharmaceutical target that has been associated to some human disorders, in which new therapies for its treatment rely on structural and inhibition studies with COMT [2]. To accomplish this, high quantities of the enzyme in a high purity state, structural estability and preserved activity are constantly required. Several purification strategies have been applied for COMT isolation [10, 11, 26, 42] but some of them presented significant losses of COMT activity and yield [80, 94, 95]. Thus, it is of high interest the implementation of new faster capture strategies without aggressive conditions that can compromise the bioactivity of this enzyme.

The batch method is a simple, rapid and low-cost capture technique, which can also be used as an alternative chromatographic technique, with high potential for protein isolation. In this work, nickel-crosslinked and magnesium-crosslinked gellan microspheres were applied in a batch method to explore capture strategies of the hSCOMT isoform from a complex *Komagataella pastoris lysate*. Several parameters were tested and optimized for the capture of this enzyme, such as incubation times, amount of gellan microspheres, temperature, ionic

strength and pH of binding and elution buffers. To evaluate the performance of each strategy, the quantification of total protein in the supernatant of each binding and elution step was performed along with a SDS-PAGE and a Western Blot (WB) analysis.

4.2.1. Capture strategies of hSCOMT by using gellan microspheres with nickel as crosslinker

The gellan microspheres with nickel as a crosslinker were used in two major capture strategies. In the first strategy, it was taken advantage of the nickel (the positive divalent ion) present in gellan microspheres, that are known to have an affinity for histidine residues. Since the hSCOMT was recombinantly produced with a His-tag, a capture strategy similar to an IMAC purification was explored, which was named affinity strategy. The other one, which was called ionic strategy, was based on pH and ionic strength manipulation in order to explore ionic interactions between the negatively charged groups of the gellan polysaccharide and the positive groups on the protein. The optimization of both strategies are described in the next two subchapters.

4.2.1.1. Affinity strategy

The first assay was performed to study the capacity of hSCOMT protein bind to gellan microspheres when both are negatively charged. Knowing that the isoelectric point (pI) of hSCOMT is 5.2, the binding solution had a pH of 7.5 to create an effective negative charge on this protein.

Therefore, the assay 1 of the batch method consisted in 10 mL of gellan microspheres equilibrated in 10 mM Tris buffer, pH 7.5, and after the centrifugation of 3 minutes at 100 *g*, the supernatant was discarded and 3 mL of binding solution (750 µL of hSCOMT lysate with 2250 µL of 10 mM Tris buffer, pH 7.5) was added and incubated 1 hour with constant homogenization. After the incubation, the gellan microspheres were again centrifugated for 3 minutes at 100 *g* and the supernatant containing the molecules that did not bind to the gellan microspheres was collected. Then, the gellan microspheres were washed with binding buffer (10 mM Tris buffer, pH 7.5), centrifugated for 3 minutes at 100 *g*, and the supernatant was recovered. Finally, a single elution step was performed with 150 mM NaCl in 10 mM MES buffer, pH 5.2, with an incubation of 15 minutes, followed by another washing step with the elution buffer (150 mM NaCl in 10 mM MES, pH 5.2). The supernatant of these two steps was recovered after a centrifugation of 3 minutes at 100 *g*. The batch method assay was performed at room temperature (RT).

In order to evaluate the influence of the lysis buffer constituents on the binding step, the recovery of the recombinant hSCOMT was carried out separately with the binding buffer and with the lysis buffer, and thereby the assay 1 was performed twice, each one with a different buffer. The mechanical lysis was performed with an appropriate lysis buffer in order to increase the protein stability, thereby making the protein to expose its residues for interaction with the

gellan microspheres. In addition, the washing steps, that are normally discarded, were analysed as independent samples in order to evaluate the content of protein that could be lost on this step. In Table 7 are represented the total protein amount, expressed in µg, of the recovered supernatants from the binding, washing and elution steps. The WB and the SDS-PAGE of the same samples are illustrated in Figure 24 and Figure 25, respectively.

Table 7. Total protein amount of the recovered supernatants from assay 1 (10 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	**Total protein amount (µg)**	
	Lysis carried out with lysis buffer	Lysis carried out with binding buffer
Binding solution	15315	10004
hSCOMT lysate that did not bind to the microspheres in 10 mM Tris buffer, pH 7.5	2905	4221
Washing with 10 mM Tris buffer, pH 7.5	2885	2057
Elution of hSCOMT lysate with 150 mM NaCl in 10 mM MES buffer, pH 5.2	1954	1558
Washing with 150 mM NaCl in 10 mM MES buffer, pH 5.2	1125	510

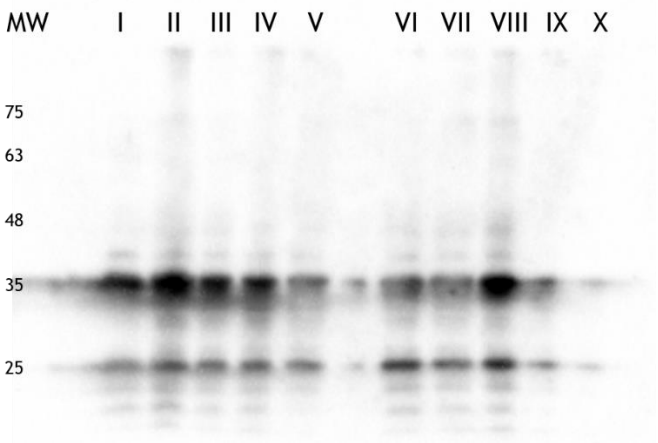

Figure 24. Western blot analysis of the recovered supernatants from assay 1 (10 mL of gellan microspheres with nickel as a crosslinker, through the batch method at RT). The I to V lines represent the assay with lysis carried out with lysis buffer and the VI to X lines illustrate the assay with lysis performed with the binding buffer. MW – Molecular weight; I and VI – hSCOMT lysate; II and VII – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III and VIII – Washing with 10 mM Tris buffer, pH 7.5; IV and IX – Elution of hSCOMT lysate with 150 mM NaCl in 10 mM MES buffer, pH 5.2; V and X – Washing with 150 mM NaCl in 10 mM MES buffer, pH 5.2.

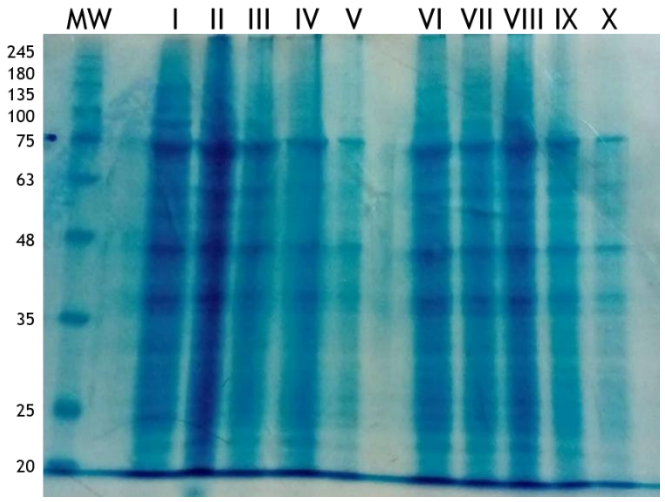

Figure 25. SDS-PAGE analysis of the recovered supernatants from assay 1 (10 mL of gellan microspheres with nickel as a crosslinker, through the batch method at RT). The I to V lines represent the assay with lysis carried out with lysis buffer and the VI to X lines illustrate the assay with lysis performed with the binding buffer. MW – Molecular weight; I and VI – hSCOMT lysate; II and VII – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III and VIII – Washing with 10 mM Tris buffer, pH 7.5; IV and IX – Elution of hSCOMT lysate with 150 mM NaCl in 10 mM MES buffer, pH 5.2; V and X – Washing with 150 mM NaCl in 10 mM MES buffer, pH 5.2.

The WB and SDS-PAGE methods were performed with samples containing the same quantity of total protein, 30 µg, in order to obtain bands that could be compared with each other. Using a specific antibody against COMT, two bands of 25 kDa and 35 kDa were detected in the western blot membrane. Typically, hSCOMT has a molecular weight of around 25 kDa [9, 10], so the band at approximately 35 kDa cannot be a result of protein aggregation. Our research group showed that the extra molecular weight on the 35 kDa band is due to an N-terminal fragment on the hSCOMT protein with approximately 8 kDa. In that study, it was proved that this fragment is not a peptide and, thereby, the hSCOMT protein has been correctly processed [11]. Thus, both 25 kDa and 35 kDa bands correspond to the hSCOMT protein.

By analysing the total protein concentration of samples in Table 7, it can be observed a higher concentration of proteins, when the lysis was performed with the lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM DTT, 1 mM $MgCl_2$, pH 8.0). Also, the amount of total protein that did not bind to the gellan microspheres is significantly lower in the same context. Moreover, the WB and SDS-PAGE analyses (Figure 24 and Figure 25, respectively) show that hSCOMT protein was poorly retained by the gellan microspheres (lines VII and VIII) in the assay with lysis carried out with binding buffer. These results suggested that the constituents of the lysis buffer, that are not present in the binding buffer, may help in the mechanical lysis thereby yielding in higher total protein concentrations and more stable hSCOMT protein. Thus, it was decided to continue the work recovering the recombinant hSCOMT protein with the lysis buffer.

Relatively to the washing samples, the total protein quantification results (Table 7) showed the presence of significant amounts of homologous host proteins with the presence of considerable

amounts of hSCOMT as showed in the WB (lines III, V, VIII and X of Figure 24). Thus, in the next assays, the supernatants recovered from the washing steps were analysed together with the ones recovered from the binding or elution steps.

Despite the significant losses of hSCOMT protein shown in lines II and III of Figure 24, some of this negatively charged protein was retained at pH 7.5 and then eluted by increasing the ionic strength at pH 5.2. This result suggests the involvement of ionic interactions between the negatively charged protein and the divalent cations (nickel) preset as crosslinkers in the gellan microspheres. Supporting this result, it was also obtained a similar result by our research group in a study for the hSCOMT recovery from Q-sepharose by an anion exchange chromatographic strategy. In this study, the hSCOMT was retained with 10 mM Tris, pH 7.8, and eluted with increasing NaCl concentrations, especially with 350 mM NaCl in 10 mM Tris buffer [10].

In addition, the total protein quantification (Table 7) showed that the initial sample had 15315 µg of total protein, that 5790 µg was not retained by gellan microspheres and that only 3079 µg was eluted. This means that the initial sample was not completely recovered, suggesting that some proteins stayed bound to the gellan microspheres. Thereby, the concentration of 150 mM NaCl does not seem to be enough for the elution of all sample bound to the gellan microspheres in a single elution.

The assay 2 consisted in the same conditions previously described but the elution step was conducted with 200 mM NaCl in 10 mM MES buffer, pH 5.2. Table 8 presents the total protein quantification of the recovered supernatants from the gellan microspheres, and the WB and the SDS-PAGE analyses of this assay are illustrated in Figure 26 (A) and (B), respectively.

Table 8. Total protein amount of the recovered supernatants from assay 2 (10 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	8082
hSCOMT lysate that did not bind to the microspheres in 10 mM Tris, pH 7.5	3561
Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2	1988

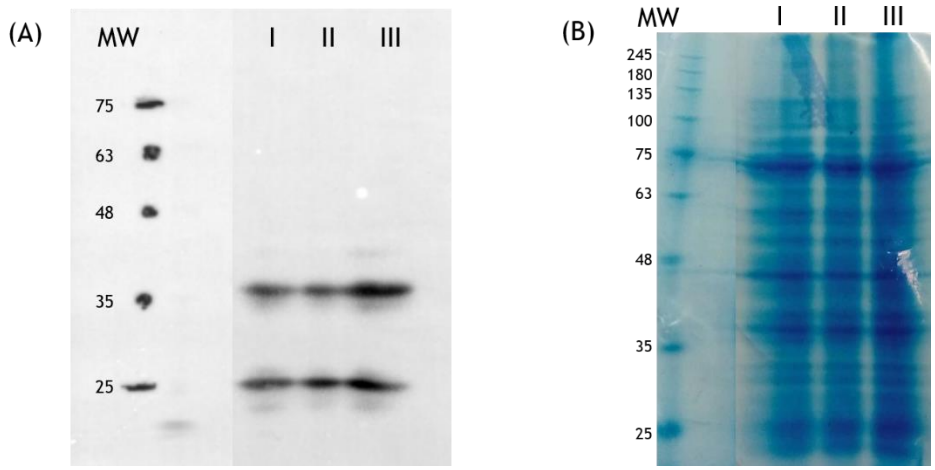

Figure 26. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 2 (10 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2.

The results of total protein quantification showed that in assay 2 (Table 8), the amount of protein eluted with 200 mM was slightly higher than the amount obtained in the assay 1 (Table 7)with 150 mM NaCl. Moreover, by analysing the WB of this assay (Figure 26 (A)) and comparing with the WB of the previous one (Figure 24), it seems that a salt concentration of 200 mM promotes an elution of higher amount of hSCOMT protein (line III of Figure 26 (A) and line IV of Figure 24). However, a considerable proportion of hSCOMT protein was not retained by gellan microspheres (line II of Figure 26 (A)).

So, in order to improve the binding step, the assay 3 consisted in the same conditions of ionic strength, pH and temperature of binding and elution steps of the previous batch method, but it was decided to duplicate the volume of gellan microspheres (20 mL) and the volume of binding solution (6 mL) but maintaining the volume of hSCOMT lysate (750 µL). It was also increased the duration of the binding step (2 hours), providing more time to promote protein binding to the gellan microspheres. In addition, to evaluate the content of nucleic acids in the samples, the next assay also included an agarose gel electrophoresis of samples. The total protein quantification of the recovered supernatants from the gellan microspheres is posted in Table 9, and the WB, SDS-PAGE and agarose gel electrophoresis are represented in Figure 27 (A), (B), and (C), respectively.

Table 9. Total protein amount of the recovered supernatants from assay 3 (20 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	3559
hSCOMT lysate that did not bind to the microspheres in 10 mM Tris, pH 7.5	1745
Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2	692

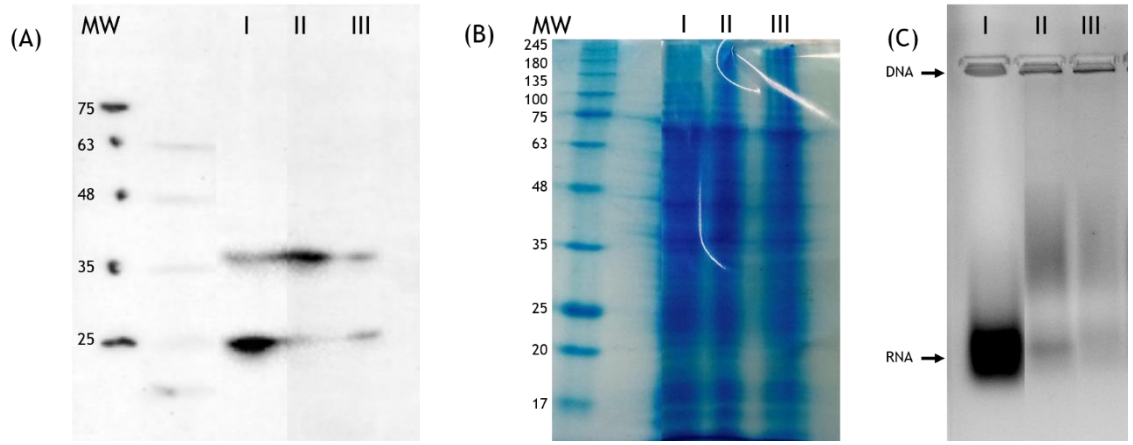

Figure 27. Western blot (A), SDS-PAGE (B), and agarose gel electrophoresis (C) of the recovered supernatants from assay 3 (20 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2.

By analysing the results of total protein quantification (Table 9) and comparing with the last assay (Table 8), it seems that a small increase was obtained in the protein retention. Also, the amount of total protein in the binding solution seems lower than the previous used in assay 2, which could be a result of the dilution of the hSCOMT lysate. This is, in the assay 2, hSCOMT lysate was diluted in a 1:4 proportion (750 µL of hSCOMT lysate with 2250 µL of 10 mM Tris buffer, pH 7.5), while in the assay 3, hSCOMT lysate was subjected to a higher dilution of 1:8 (750 µL of hSCOMT lysate with 5250 µL of 10 mM Tris buffer, pH 7.5). The WB (Figure 27 (A)) also seems to have been affected by this higher dilution, since the bands are more faded and difficult to compare than the previous ones. Therefore, the following assays were performed with 6 mL of binding solution, maintaining the dilution of hSCOMT lysate 1:4 (1500 µL of hSCOMT lysate with 4500 µL of 10 mM Tris buffer, pH 7.5).

Despite this, the line III of the WB (Figure 27 (A)) showed practically none elution of hSCOMT protein, which could happen due to the low duration of the elution step. Since the amount of microspheres and the duration of the binding step were doubled, it is likely that more time will

also be needed to elute all the hSCOMT protein that bound to the gellan microspheres. So, the elution step was performed during 1 hour in the assay 4.

Initially, at the end of the mechanical lysis, to recover the recombinantly produced hSCOMT protein, it was only added 25 µL of DNase (1 mg/mL) for each mL of lysis buffer. Thereby, the agarose gel electrophoresis was performed in order to evaluate the presence of nucleic acids in the samples, particularly the content of RNA. The result (Figure 27 (C)) showed some amount of DNA and a strong presence of RNA in samples, mostly in hSCOMT lysate (line I). As this strategy is based on the interactions between the negatively charged hSCOMT protein and the positive groups on the gellan microspheres, and knowing that the nucleic acids are also negatively charged [96], it was suggested that these biomolecules, essentially RNA, could be competing with the hSCOMT protein to bind to gellan microspheres. To overcome this hypothesis, 25 µL of RNase (1 mg/mL) for each mL of lysis buffer was added, along with the DNase, during the recovery of the recombinantly produced hSCOMT protein.

Thus, the conditions of temperature, ionic strength and pH of binding and elution steps of assay 3 and 4 were the same, but applying 6 mL of binding solution (1500 µL of hSCOMT lysate, previously treated with DNase and RNase, with 4500 µL of 10 mM Tris buffer, pH 7.5) and 1 hour of incubation in the elution step. Table 10 presents the total protein quantification of the recovered supernatants from the gellan microspheres. Also, the WB, SDS-PAGE and agarose gel electrophoresis are illustrated in Figure 28 (A), (B) and (C), respectively.

Table 10. Total protein amount of the recovered supernatants from assay 4 (20 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	12463
hSCOMT lysate that did not bind to the microspheres in 10 mM Tris, pH 7.5	6036
Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2	2868

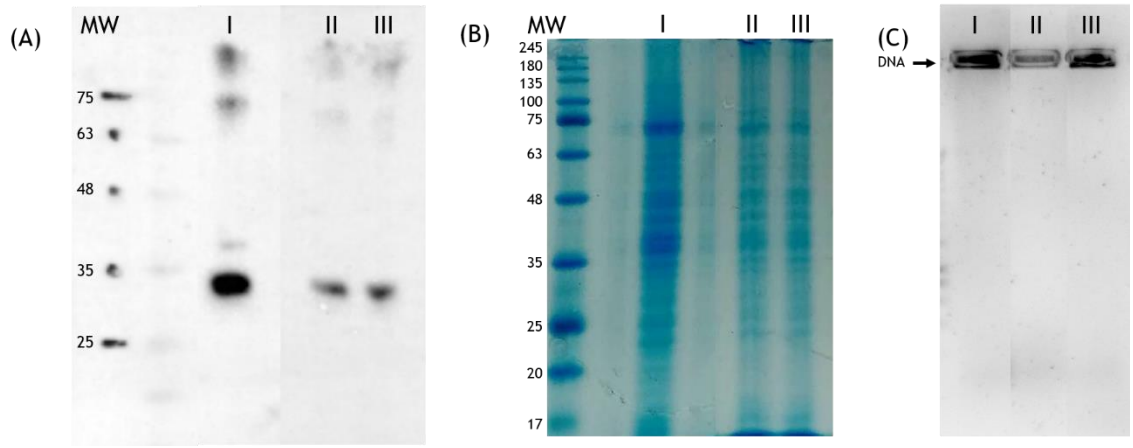

Figure 28. Western blot (A), SDS-PAGE (B), and agarose gel electrophoresis (C) of the recovered supernatants from assay 4 (20 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2.

By analysing the WB (line III of Figure 28 (A)), it suggested that a higher amount of hSCOMT protein was retained by the gellan microspheres, comparatively to the last assay (Figure 27 (A)). In addition, by comparing the agarose gel electrophoresis of this assay (Figure 28 (C)) and the last one (Figure 27 (C)), it seems that RNA was practically removed from samples. So, the RNA could actually be competing with the hSCOMT protein to bind to the gellan microspheres.

On the other hand, the total protein quantification results (Table 10) showed that a large concentration of proteins did not bind to the gellan microspheres, what was unexpected since the RNA was completely removed. However, the agarose gel electrophoresis (Figure 28 (C)) showed genomic DNA in samples, that could be interfering with the protein binding to the gellan microspheres similarly to what was suggested for the RNA. So, the initial volume of DNase added during the recovery of the recombinantly produced hSCOMT protein was duplicated in the assay 5. The RNase volume was also increased by 1.5 times in order to remove the residual RNA that remained in lysate samples.

In spite of this, hSCOMT protein was not completely retained. So, in view of achieving the complete bind of the hSCOMT protein to the gellan microspheres in assay 5, it was again increased the volume of gellan microspheres to 35 mL.

The SDS-PAGE analyses until now showed other proteins of different molecular weights mixed with the hSCOMT protein. Therefore, three elution steps with different salt concentrations (100 mM, 200 mM and 500 mM NaCl in 10 mM MES buffer, pH 5.2) were introduced in the batch method, in order to explore some selectivity by the hSCOMT protein and the contaminants separately. Thus, the assay 5 was carried out at RT and, after equilibrating the gellan microspheres with 10 mM Tris buffer, pH 7.5, 6 mL of a binding solution (1500 µL of hSCOMT lysate with 4500 µL of 10 mM Tris buffer, pH 7.5) was added to 35 mL of gellan microspheres

during 2 hours at constant agitation. Then, three elution steps with crescent NaCl concentrations were performed during 1 hour each one. Table 11 represents the total protein quantification of the recovered supernatants from the gellan microspheres. The WB and SDS-PAGE are shown in Figure 29 (A) and (B), respectively, and the agarose gel electrophoresis in Figure 30.

Table 11. Total protein amount of the recovered supernatants from assay 5 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	14239
hSCOMT lysate that did not bind to the microspheres in 10 mM Tris, pH 7.5	3376
Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 5.2	3470
Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2	3220
Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 5.2	3852

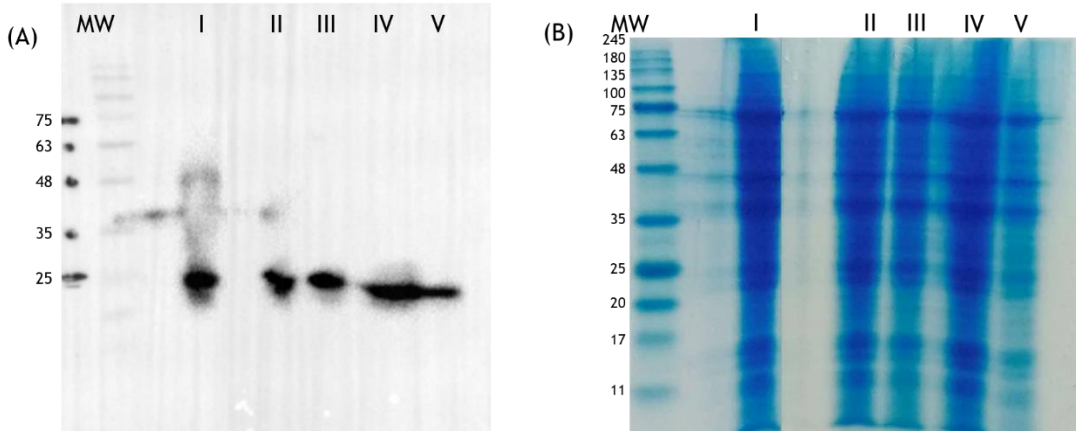

Figure 29. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 5 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 5.2; IV - Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 5.2.

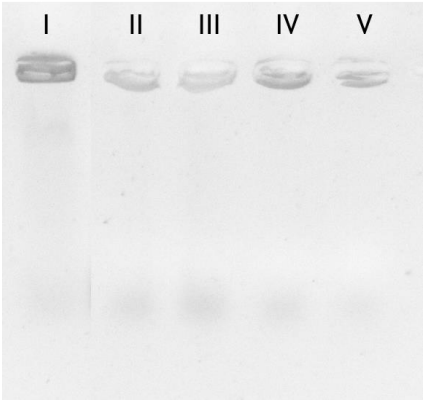

Figure 30. Agarose gel electrophoresis of the recovered supernatants from assay 5 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 5.2; IV - Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 5.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 5.2.

Starting with the analysis of the agarose gel electrophoresis (Figure 30), it seems that the DNase and RNase volumes added during the recovery of the recombinantly produced hSCOMT protein are sufficient for the removal of nucleic acids presented in samples. Comparing the total protein quantification of this assay (Table 11) with the last one (Table 10), it showed that more proteins bound to the gellan microspheres. These results suggest that a parallel increase of the gellan microspheres volume and/or removal of nucleic acids from samples promoted a higher retention of proteins.

On the other hand, by examining the WB (Figure 29 (A)), high amounts of hSCOMT protein were captured in the three elution steps (lines III to V), but the SDS-PAGE (Figure 29 (B)) showed low selectivity on the same lines. Thus, a capture strategy of hSCOMT protein based on the ionic interactions between the negatively charged protein and the positive groups on the gellan microspheres, namely nickel ions, does not seem to be the most appropriate one.

IMAC is a separation technique in which protein adsorption is based on the interaction between the metal ions immobilized on solid chromatographic supports and electron donor groups on the protein, mainly histidine residues. The transition metal ions, like Cu(II), Ni(II), Zn(II), Co(II) and Fe(III), are most frequently used [51]. Thus, another strategy was applied by taking advantage of nickel positive ions present in gellan microspheres, which are known to have an affinity for histidine residues. Since the hSCOMT was recombinantly produced with the His-tag, a capture strategy similar to an IMAC purification, which was called affinity strategy, could be a good alternative to improve the last assay achievement.

As the pK_a of the imidazole ring of histidine residues is about 6.0, the imidazole is unprotonated when pH is higher than this value and, thereby, able to bind to metal ions [51]. So, the binding buffer with pH 7.5 is favourable for the interaction between the histidine residues on the

surface of the hSCOMT protein and the metal ions in the gellan microspheres. Subsequently, for the elution of its proteins, it can be used a competitive displacement agent, like imidazole, that competes with the histidine residues for the binding to the immobilized metal ions [97].

Therefore, the assay 6 was conducted under previously optimized conditions, but the first elution step was made only by increase of NaCl concentration at the same pH (250 mM NaCl in 10 mM Tris, pH 7.5), to elute proteins that bound to the gellan microspheres by ionic interactions avoiding the destabilization of hSCOMT His-tag interactions, and two elution steps maintaining the first elution condition but increasing imidazole concentrations to 100 and 500 mM. The total protein quantification of the recovered supernatants from the gellan microspheres is presented in Table 12, and the WB and SDS-PAGE are illustrated in Figure 31 (A) and (B), respectively.

Table 12. Total protein amount of the recovered supernatants from assay 6 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	8208
hSCOMT lysate that did not bind to the microspheres in 10 mM Tris, pH 7.5	2651
Elution of hSCOMT lysate with 250 mM NaCl in 10 mM Tris buffer, pH 7.5	2609
Elution of hSCOMT lysate with 250 mM NaCl and 100 mM imidazole in 10 mM Tris buffer, pH 7.5	1793
Elution of hSCOMT lysate with 250 mM NaCl and 500 mM imidazole in 10 mM Tris buffer, pH 7.5	1446

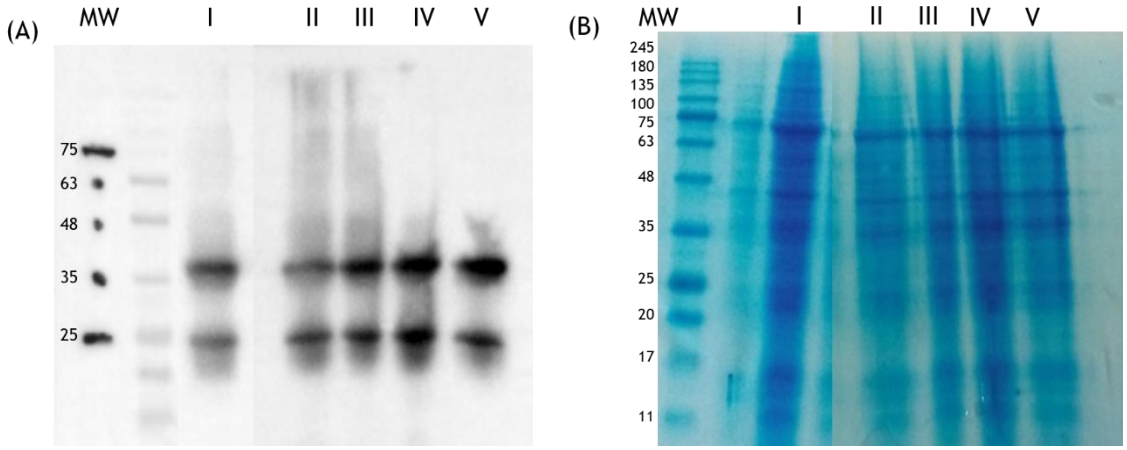

Figure 31. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 6 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 250 mM NaCl in 10 mM Tris buffer, pH 7.5; IV - Elution of hSCOMT lysate with 250 mM NaCl and 100 mM imidazole in 10 mM Tris buffer, pH 7.5; V - Elution of hSCOMT lysate with 250 mM NaCl and 500 mM imidazole in 10 mM Tris, pH 7.5.

By WB analysis (Figure 31 (A)), it was verified that the majority of the hSCOMT protein bound to the gellan microspheres (line II) and high amounts of contaminants were eliminated in the binding step and in the elution with 250 mM NaCl (Table 12). As expected, some proteins interacted electrostatically with the gellan microspheres, as denoted in total protein quantification (Table 12) and line III of the SDS-PAGE (Figure 31 (B)). These results also showed that a small amount of hSCOMT protein also bound by ionic interactions being eluted together with contaminants (line III of Figure 31 (A)). On the other hand, a higher amount of hSCOMT seems to interact with nickel ions on the surface of gellan microspheres by the His-tags, because it was eluted in the second and third elution steps (lines IV and V, respectively, of Figure 31 (A)), when the competitor agent imidazole was used. Similarly, Dieryck and co-workers purified His-tagged enhanced GFP (green fluorescent protein) by expanded-bed adsorption mode of IMAC, where 100 and 500 mM imidazole were used in the two elution steps. Target protein was mainly eluted with 100 mM imidazole, but some His-tagged EGFP was also eluted with 500 mM imidazole [98]. Moreover, Rashid and co-workers purified His-tagged protein A by $MnFe_2O_4$@SiO_2@Ni-Salen complex magnetic nanoparticles, where high amounts of the target protein were eluted in two elution steps with 100 and 250 mM imidazole, respectively, especially in the second one [99]. In addition, our research group also reported the purification of His-tagged hSCOMT by a prepacked IMAC stationary phase with nickel ions, and the protein elution was made in four steps with crescent imidazole concentrations (50, 70, 300 and 500 mM). In this study, the two first steps were used to eliminate host protein contaminants, the target protein was practically eluted with 300 mM imidazole and the last elution fraction had a little amount of proteins [11].

As the first elution step with imidazole shown higher content of the target protein, the assay 7 was adjusted in order to increase the recovery of the target protein in this step. Thus, the NaCl concentration of the first elution step was decreased to 150 mM to avoid the loss of the enzyme together with contaminants and the imidazole concentration of the second elution step was increased to 300 mM (in addition to the 150 mM of NaCl) to improve the hSCOMT recovery in this step. The third elution step was executed with 250 mM NaCl and 500 mM imidazole in 10 mM Tris buffer, pH 7.5. The idea was to elute as much as possible the hSCOMT protein in the second elution step and, thereby, eliminate contaminants in other two elution steps.

After this assay, during the concentration of the samples, a step that precedes the total protein quantification, it was added a solution of hSCOMT stabilizers (150 mM cysteine, 100 mM trehalose, 10 %(v/v) glycerol) with the intention of helping the preservation of the hSCOMT activity. According to the BCA kit manufacturer, cysteine highly interferes with the BCA kit used in total protein quantification. Therefore, the proteins were precipitated by using a precipitation kit before the quantification with the BCA kit. Figure 32 (A) and (B) illustrate the WB and SDS-PAGE, respectively, of the recovered supernatants from the gellan microspheres in this assay.

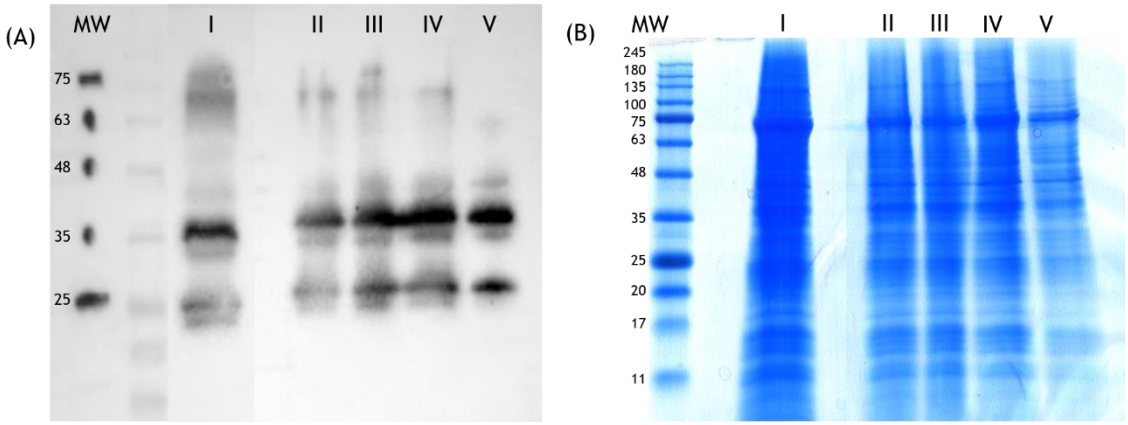

Figure 32. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 7 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 150 mM NaCl in 10 mM Tris buffer, pH 7.5; IV - Elution of hSCOMT lysate with 150 mM NaCl and 300 mM imidazole in 10 mM Tris buffer, pH 7.5; V - Elution of hSCOMT lysate with 250 mM NaCl and 500 mM imidazole in 10 mM Tris, pH 7.5.

The WB result (Figure 32 (A)) does not exactly show the expected. In fact, the results suggest that the hSCOMT promoted stronger affinity interactions, being necessary more than 150 mM NaCl combined with 300 mM imidazole to elute the protein. However, the line V of the SDS-PAGE (Figure 32 (B)) is more clarified and evidence fewer contaminants than other lines. The same line on the WB (Figure 32 (A)) shows a high amount of eluted hSCOMT protein, despite the content lost in previous elution steps (lines III and IV).

The WB (Figure 32 (A)) also showed in line II a small amount of hSCOMT protein that did not bind to the gellan microspheres. This could be due to host cell proteins with histidine residues on the surface, which are common in eukaryotic cells [100]. They could be competing with the protein of interest for the metal ions in the gellan microspheres, thereby impeding the complete retention of the hSCOMT protein. To overcome this interference, a small concentration of imidazole could be added to the binding buffer in order to remove those nonspecific interactions [52].

It is known that the hSCOMT is a sensitive and thermolabile protein and the temperature is a sensitive factor for the correct conformation of the hSCOMT and, consequently, for its stability [80, 101]. Previous works showed that low temperatures on the chromatographic system promote a correct fold of the protein and less aggregation that led subsequently to a significant upgrading in COMT activity results [80]. Thus, in order to optimize the recovery of the hSCOMT protein, maintaining its bioactivity, the assay 8 of the batch method was performed under the same conditions previously defined but at 4 ºC and introducing 10 mM of imidazole in the equilibrium and binding steps. The WB and SDS-PAGE of the recovered supernatants from the gellan microspheres are illustrated in Figure 33 (A) and (B), respectively.

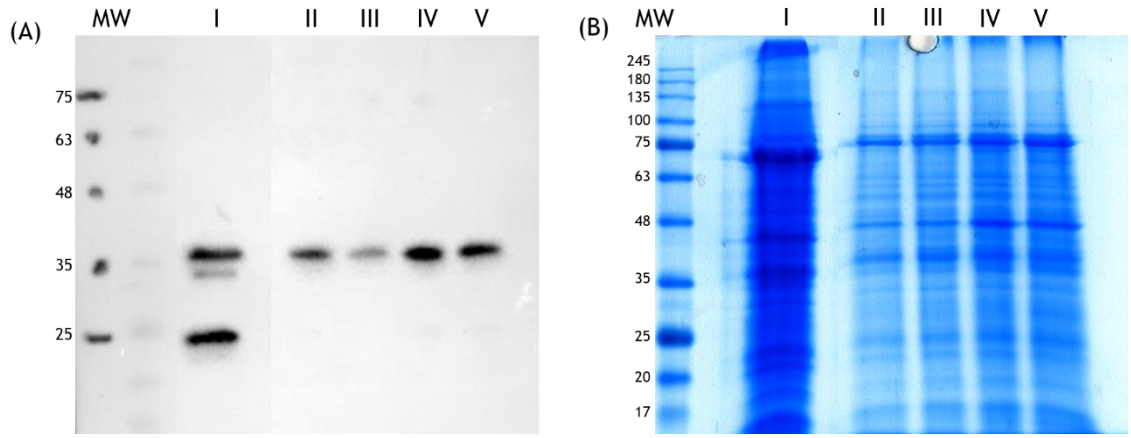

Figure 33. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 8 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at 4 ºC). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM Tris buffer, pH 7.5, containing 10 mM imidazole, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 150 mM NaCl in 10 mM Tris buffer, pH 7.5; IV - Elution of hSCOMT lysate with 150 mM NaCl and 300 mM imidazole in 10 mM Tris buffer, pH 7.5; V - Elution of hSCOMT lysate with 250 mM NaCl and 500 mM imidazole in 10 mM Tris, pH 7.5.

The removal of the nonspecific interactions by addition of 10 mM imidazole to the equilibrium and binding buffers, promoted an increased retention of hSCOMT protein because of a smaller immunoreactivity of the band in line II of Figure 33 was obtained compared to the Figure 31 (A) and Figure 32 (A). This means that this addition of imidazole decreased the binding of specific host cell proteins containing adjacent histidine residues, releasing binding sites on the gellan microspheres for the hSCOMT attachment [52, 100]. On the other hand, the results seem to suggest that temperature also has a significant role in the hSCOMT recovery. Comparing the elution step with 150 mM NaCl (line III) on the WB of this assay (Figure 33 (A)) and the previous one (Figure 32 (A)), it was observed a smaller loss of hSCOMT at 4 ºC, thereby resulting in the pleasant recovery of the hSCOMT on line IV. This result suggests that low temperatures improved the hSCOMT stability and subsequently stronger elution conditions were needed to disfavour the established interactions between hSCOMT protein and the gellan microspheres.

In general, this strategy not only promotes the almost complete capture of hSCOMT to nickel-crosslinked gellan microspheres, but also the recovery of high hSCOMT content in a clarified sample. In addition, it was achieved a considerable degree of selectivity by removal of contaminants with minimum hSCOMT loss before the recovery of the target protein. Therefore, this strategy seems to be a great affinity capture approach for the isolation of hSCOMT before a final purification step.

4.2.1.2. Ionic strategy

Another strategy, that was suggested for the capture of hSCOMT by gellan microspheres with nickel as crosslinker, was based on pH and ionic strength manipulation in order to explore ionic interactions between the negatively charged groups of gellan microspheres and the positive groups on the protein.

Knowing that the isoelectric point (pI) of hSCOMT is 5.2, the application of pH 4.0 in the binding solution can create a global positive charge on our target. Therefore, the assay 9 was performed at RT and started with 10 mL of gellan microspheres equilibrated in 10 mM citrate buffer, pH 4.0, followed by addition of 3 mL of binding solution (750 µL of hSCOMT lysate with 2250 µL of 10 mM citrate buffer, pH 4.0), that was incubated 1 hour with constant homogenization. Then, a single elution step was performed with 200 mM NaCl in 10 mM MES buffer, pH 6.2, during 15 minutes. Figure 34 (A) and (B) illustrate the WB and SDS-PAGE of the recovered supernatants from the gellan microspheres, respectively.

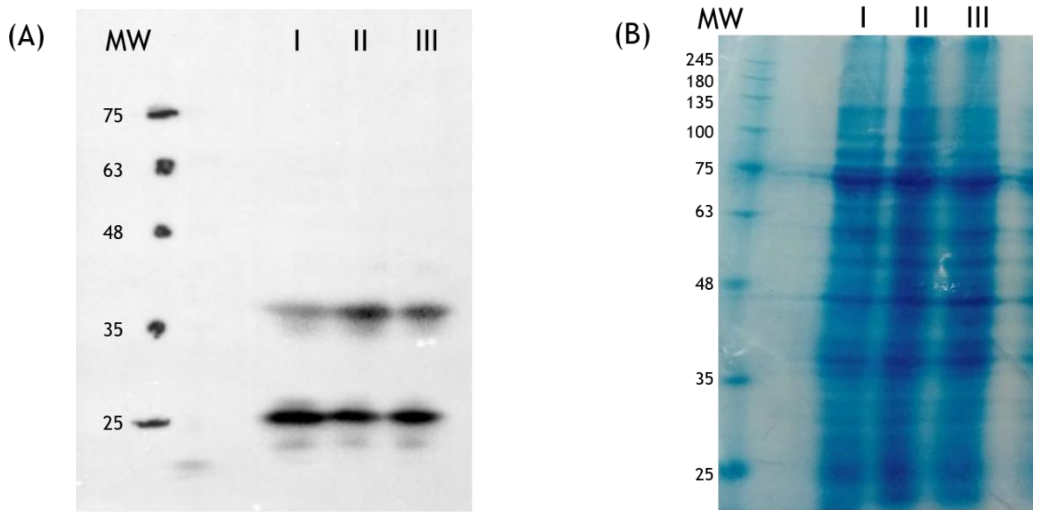

Figure 34. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 9 (10 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM citrate buffer, pH 4.0, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2.

By analysing the WB (Figure 34 (A)), it could be observed that some hSCOMT bound to the gellan microspheres and was eluted by changing the pH to 6.2 and increasing the ionic strength, where the protein acquires negative charge (line III). Therefore, it can be confirmed that indeed ionic interactions were established between positive groups on the protein and negative groups of gellan microspheres.

The WB (Figure 34 (A)) also showed a lower retention degree of hSCOMT protein (line II) similar to the last strategy. So, the batch method was optimized in a way quite similar to the affinity strategy. Briefly, in the assay 10, the volume of gellan microspheres was increased to 35 mL along with the binding solution volume (1500 µL of hSCOMT lysate, previously treated with DNase and RNase, with 4500 µL citrate buffer, pH 4.0). Increasing the available contact points (contact surface area), the duration of the binding step was also augmented to 2 hours, and each elution step to 1 hour. Trying to obtain some selectivity, it was performed an elution with three steps of increasing salt concentration (100 mM, 200 mM and 500 mM NaCl). The total protein quantification of the recovered supernatants from the gellan microspheres is

represented in Table 12, and the WB and SDS-PAGE are illustrated in Figure 35 (A) and (B), respectively.

Table 12. Total protein amount of the recovered supernatants from assay 10 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	14239
hSCOMT lysate that did not bind to the microspheres in 10 mM citrate buffer, pH 4.0	4393
Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2	2497
Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2	2327
Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2	2585

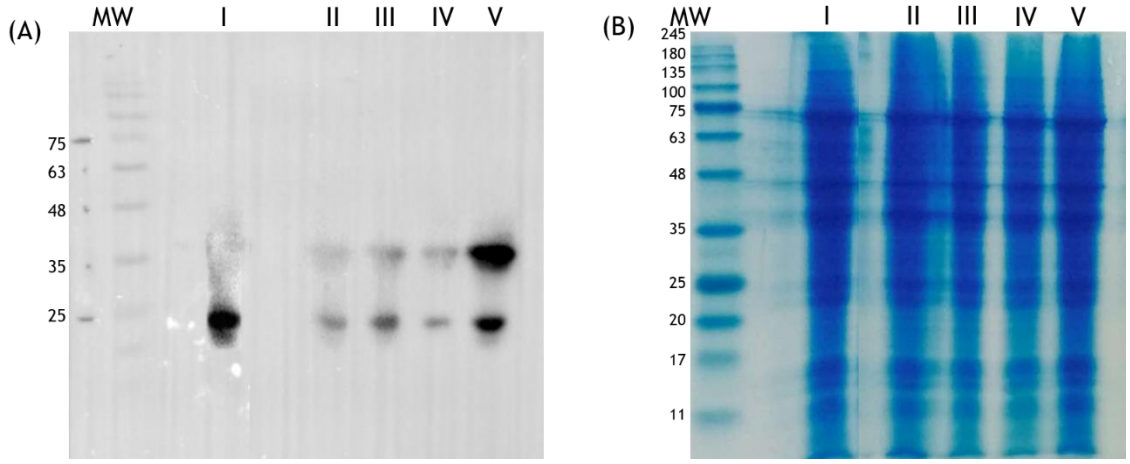

Figure 35. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 10 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM citrate buffer, pH 4.0, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2; IV - Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2.

By WB analysis (Figure 35 (A)), it could be observed that hSCOMT protein was practically all retained by the gellan microspheres (line II). In addition, practically no hSCOMT was lost in the two first elution steps with 100 and 200 mM NaCl (lines III and IV), being thereby recovered in the last elution step at 500 mM NaCl (line V). Although line V of SDS-PAGE (Figure 35 (B)) showed many contaminants, most of them were eliminated in the two previous elution steps (lines III and IV), thereby reaching some degree of selectivity.

On the other hand, in the last assay, the WB (Figure 34 (A)) showed that the first elution step with 200 mM NaCl (line III), directly after the binding step, could somehow destabilize the interaction of the hSCOMT protein with the gellan microspheres, being eluted earlier. Thus, the assay 10 showed the importance of intermediate elution steps with low salt concentrations, which allow the elimination of impurities and at the same time establishment of stronger interactions with specific proteins that are eluted only with higher ionic strength.

As this assay presented interesting results, through the batch method at room temperature, it was then transposed to 4 ºC with the stabilizers solution in order to optimize the recovery of the hSCOMT, preserving its bioactivity, like in the affinity strategy. So, the assay 11 was repeated under the same conditions but at 4 ºC and with stabilizers solution. In Figure 36 is represented the WB of the recovered supernatants from the gellan microspheres.

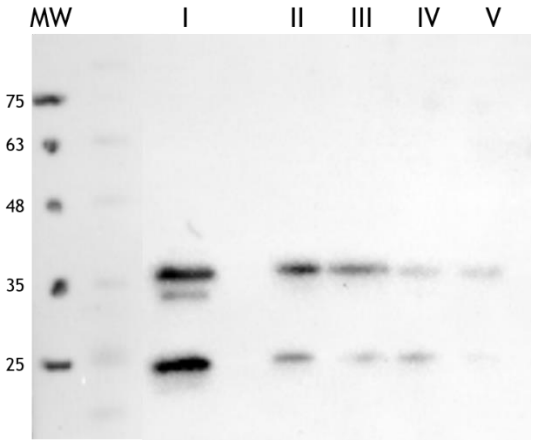

Figure 36. Western blot of the recovered supernatants from assay 11 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at 4 ºC). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM citrate buffer, pH 4.0, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2; IV - Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2.

By WB analysis (Figure 36), it showed that at 4 ºC a significant part of hSCOMT protein did not bind to the gellan microspheres (line II), indicating that ionic interactions are more favourable at room temperature than at low temperatures. Despite the absence of a theoretical global rule for the influence of temperature on the ionic interactions, it was found a study where the adsorption capacity was increased with higher temperatures [102]. Thus, in the assay 12, the contact time of hSCOMT protein and the gellan microspheres was increased for 4 hours. However, it was also showed that the hSCOMT elution occurred with less concentration of salt. Therefore, in the assay 12, the salt concentration of the second elution step was increased to 300 mM NaCl in order to elute more quantity of hSCOMT in this step. The WB and SDS-PAGE of the recovered supernatants from the gellan microspheres are illustrated in Figure 37 (A) and (B), respectively.

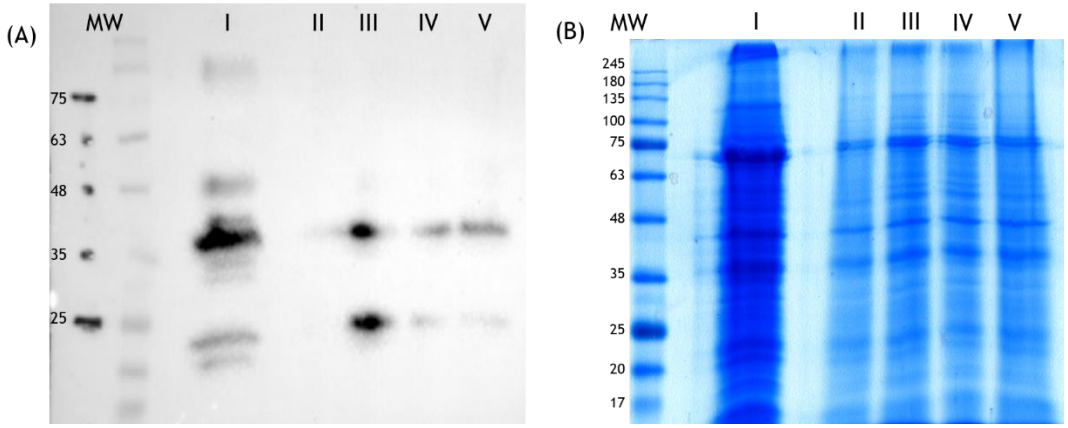

Figure 37. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 12 (35 mL of gellan microspheres with nickel as crosslinker, through the batch method at 4 ºC). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM citrate buffer, pH 4.0, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2; IV - Elution of hSCOMT lysate with 300 mM NaCl in 10 mM MES buffer, pH 6.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2.

By WB analysis (Figure 37 (A)), it can be observed that hSCOMT protein was completely retained by the gellan microspheres (line II), and most part eluted in a single elution step (line III). In addition, the SDS-PAGE ((Figure 37 (B)) showed that a lot of contaminants could subsequently be removed in other two elution steps (lines IV and V). So, this assay showed that a strategy where the ionic interactions between the positively charged protein and the anionic polymer on the gellan microspheres are explored can be interesting for the isolation of the hSCOMT protein with good expectations of yield sample clarification.

4.2.2. Capture strategies of hSCOMT by using gellan microspheres with magnesium as crosslinker

The gellan microspheres with magnesium as crosslinker were also applied in the capture of hSCOMT through two strategies. The first strategy was an ionic strategy similar to the one made with the gellan microspheres with nickel as crosslinker. In this capture strategy, the idea is to evaluate and confirm if the negatively charged groups of gellan microspheres maintain the ability to establish ionic interactions with positive groups on the protein by manipulating the pH and ionic strength. In the second strategy, it was taken advantage of magnesium ions present in gellan microspheres, that are known to be a cofactor of hSCOMT, thereby suggesting a natural affinity for the protein. So, in this capture strategy, the affinity between the magnesium ions and the active site of the hSCOMT protein was explored by manipulation of the ionic strength and magnesium concentration.

4.2.2.1. Ionic strategy

The assay 13 was performed by using the same conditions described in the ionic strategy with the gellan microspheres with nickel as crosslinker at RT (assay 10). The total protein

quantification of the recovered supernatants from the gellan microspheres is represented in Table 13. The WB and SDS-PAGE are illustrated in Figure 38 (A) and (B), respectively.

Table 13. Total protein amount of the recovered supernatants from assay 13 (35 mL of gellan microspheres with magnesium as crosslinker, through the batch method at RT).

Sample	Total protein amount (µg)
Binding solution	12258
hSCOMT lysate that did not bind to the microspheres in 10 mM citrate buffer, pH 4.0	3178
Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2	4050
Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2	2229
Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2	1167

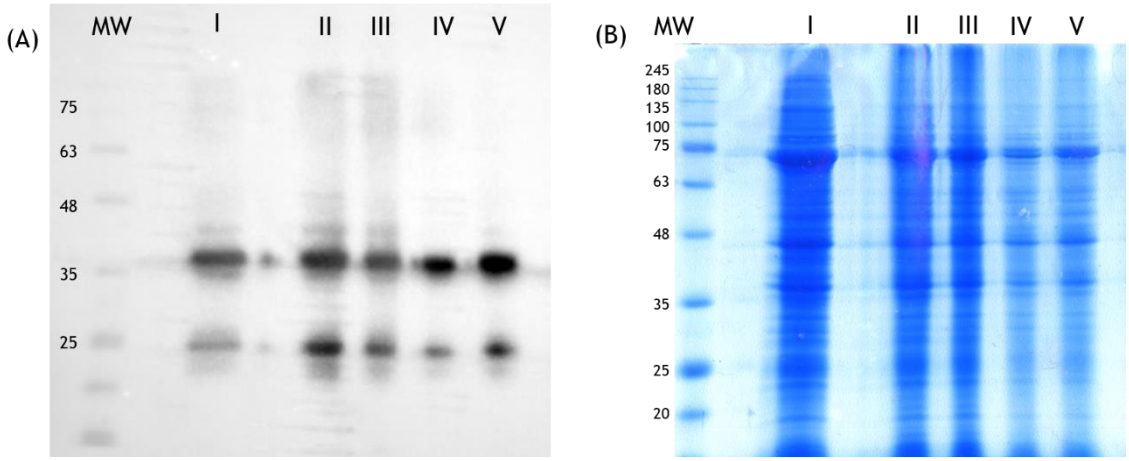

Figure 38. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 13 (35 mL of gellan microspheres with magnesium as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM citrate buffer, pH 4.0, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2; IV - Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2.

By WB analysis (Figure 38 (A)), the results obtained are similar to the gellan microspheres with nickel as crosslinker (assay 10), since the majority of hSCOMT protein was eluted in the last elution step with 500 mM NaCl (line V). Although lines III and IV showed the elution of little amounts of hSCOMT, the SDS-PAGE (Figure 38 (B)) also showed that line III had a lot of contaminants. On the other hand, lines IV and V had much fewer contaminants. So, line V can be considered the sample with higher ratio hSCOMT/impurities amount (more clarified sample), render this strategy as a good capture way for the isolation of hSCOMT from *K. pastoris* lysates.

However, it was also observed that a minor amount of hSCOMT protein was not retained by the gellan microspheres, but this can be a result of the level of hSCOMT protein in the lysate. Although in the gellan microspheres with nickel as a crosslinker (Table 12) the total protein concentration of the lysate was a little higher than in the gellan microspheres with magnesium as a crosslinker (Table 13), the hSCOMT content could be different, since these lysate samples may result from different fermentation runs. An alternative explanation relies on the microspheres mean diameter, this is, the smaller the microspheres, the larger is the surface contact area for the binding of hSCOMT protein [56]. This explanation is supported by WB analyses of both formulations (Figure 35 (A) and Figure 38 (A)), that showed higher hSCOMT retention (line II) with gellan microspheres with nickel as crosslinker. Thus, gellan microspheres with nickel as crosslinker seem to be more suitable for this ionic strategy.

The following assay 14 was carried out at 4 ºC and applying the stabilizers solution in order to analyse the influence of temperature and optimize the recovery of hSCOMT, preserving its bioactivity. The WB and the SDS-PAGE of the recovered supernatants from the gellan microspheres are illustrated in Figure 39 (A) and (B), respectively.

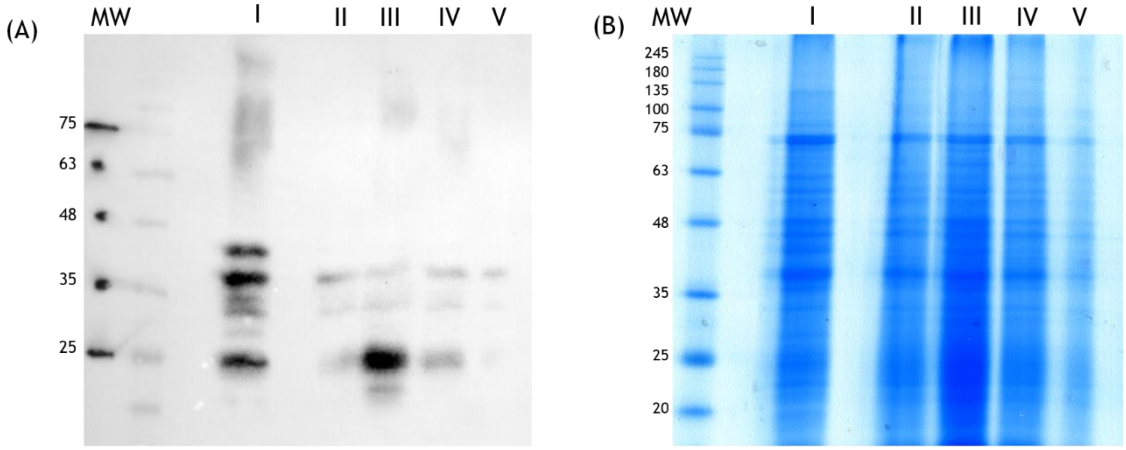

Figure 39. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 14 (35 mL of gellan microspheres with magnesium as crosslinker, through the batch method at 4 ºC). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM citrate buffer, pH 4.0, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 6.2; IV - Elution of hSCOMT lysate with 200 mM NaCl in 10 mM MES buffer, pH 6.2; V - Elution of hSCOMT lysate with 500 mM NaCl in 10 mM MES buffer, pH 6.2.

By WB analysis (Figure 39 (A)), the results are similar to the gellan microspheres with nickel as crosslinker (Figure 37 (A)). In general, the hSCOMT was almost all retained by the gellan microspheres and eluted with 100 mM NaCl at pH 6.2. These results suggested that temperature interferes with the ionic interactions because in both cases low temperatures promoted the earlier elution of hSCOMT protein [102].

By analysis of the SDS-PAGE (Figure 39 (B)), it can be observed that major contaminants were rapidly eliminated during the binding step (line II). Although some contaminants were eluted along with hSCOMT protein (line III of Figure 39 (B)) this elution step with 100 mM NaCl allowed a high hSCOMT recovery (line III of Figure 39 (A)), unlike other elution steps (lines IV and V) where some content of contaminants was eliminated (Figure 39 (A)) but it was not observed the presence of significant amounts of hSCOMT (Figure 39 (A)). Thus, since the sample containing the hSCOMT protein was clarified, this approach can be a good capture strategy for the isolation of the hSCOMT.

4.2.2.2. Affinity strategy

The last strategy applied to capture the hSCOMT from lysate consisted in using gellan microspheres with magnesium as crosslinker to explore affinity interactions between this ion and the active centre of the hSCOMT.

So, to avoid the ionic interactions of the hSCOMT with the charged groups on the gellan microspheres, the protein was put in a buffer solution at pH 5.2, which is known to be the pI of this enzyme. For the elution of protein, it was used magnesium chloride to compete with the gellan microspheres to bind to the active centre of the hSCOMT protein.

Thus, the assay 15 was made at RT, starting with the equilibration of gellan microspheres with 10 mM MES buffer, pH 5.2. After that, 6 mL of a binding solution (1500 µL of hSCOMT lysate with 4500 µL of 10 mM MES buffer, pH 5.2) was added to 35 mL of gellan microspheres for 2 hours. Then, the first elution step was carried out with 100 mM NaCl in 10 mM MES buffer, pH 5.2, the second one with 100 mM NaCl and 500 mM $MgCl_2$ in 10 mM MES buffer, pH 5.2, and finally the third elution step was made with 250 mM NaCl and 500 mM $MgCl_2$ in 10 mM MES buffer, pH 5.2. Each one of the elution steps was performed during 1 hour. The WB and SDS-PAGE of the recovered supernatants from the gellan microspheres are illustrated in Figure 40 (A) and (B), respectively.

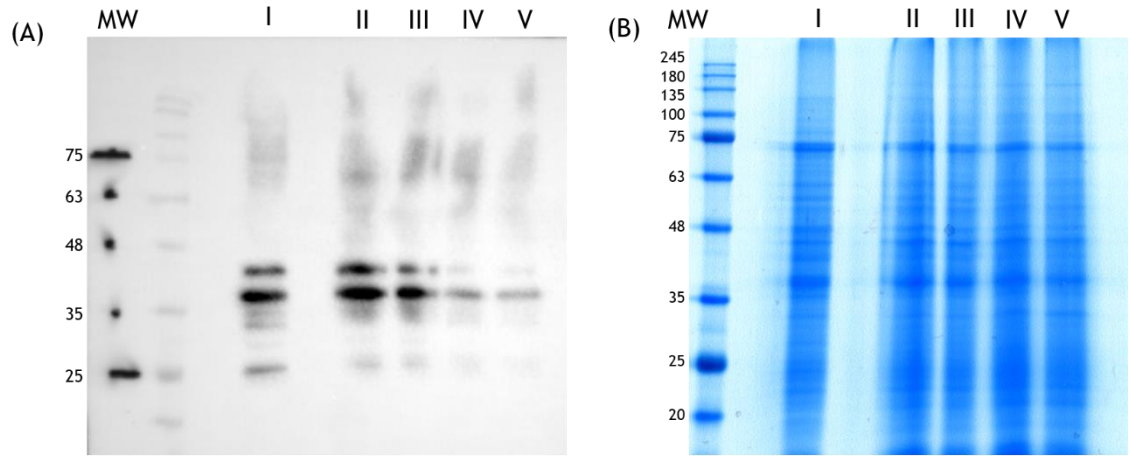

Figure 40. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 15 (35 mL of gellan microspheres with magnesium as crosslinker, through the batch method at RT). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM MES buffer, pH 5.2, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 5.2; IV - Elution of hSCOMT lysate with 100 mM NaCl and 500 mM MgCl2 in 10 mM MES buffer, pH 5.2; V - Elution of hSCOMT lysate with 250 mM NaCl and 500 mM MgCl2 in 10 mM MES buffer, pH 5.2.

By WB analysis (Figure 40 (A)), it can be observed that a high amount of hSCOMT protein did not bind to the gellan microspheres (line II) and the amount that bound to the gellan microspheres was eluted only with 100 mM NaCl (line III). It was not required the presence of a competitive agent to elute the protein. These results could be due to the lability and stability of the protein. Considering that the batch method was performed at RT, the protein could not be in a stable and correct conformation to expose its active centre for binding [80]. In the active centre, the magnesium ion plays the important structural role of orienting the substrate and the activated SAM into a reactive conformation [8]. In this line of thought, this assay was transposed to 4 ºC in order to obtain a better capture strategy.

Thus, the assay 16 was performed exactly in the same conditions but at 4 ºC. In Figure 41 (A) and (B) are illustrated the WB and SDS-PAGE of the recovered supernatants from the gellan microspheres, respectively.

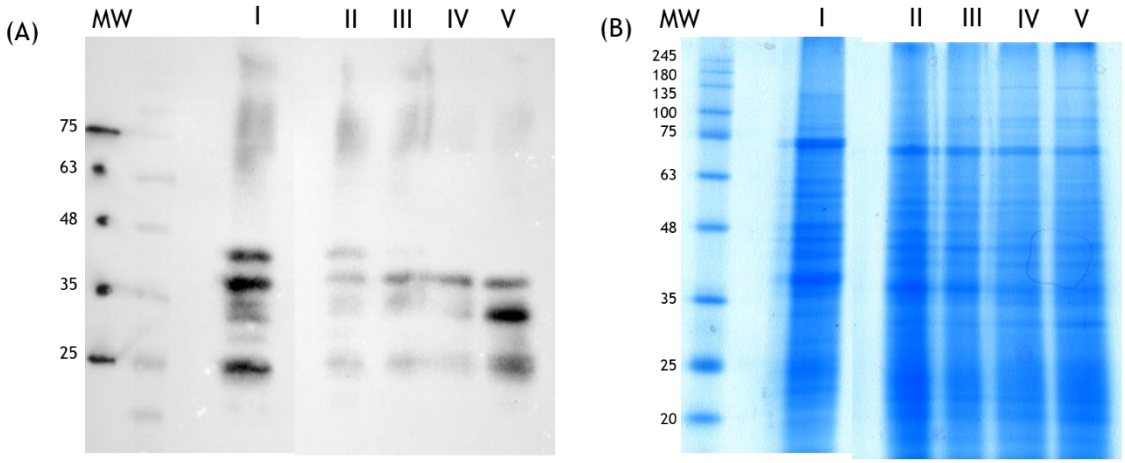

Figure 41. Western blot (A) and SDS-PAGE (B) of the recovered supernatants from assay 16 (35 mL of gellan microspheres with magnesium as crosslinker, through the batch method at 4 ºC). MW – Molecular weight; I – hSCOMT lysate; II – hSCOMT lysate in 10 mM MES buffer, pH 5.2, that did not bind to the gellan microspheres; III – Elution of hSCOMT lysate with 100 mM NaCl in 10 mM MES buffer, pH 5.2; IV - Elution of hSCOMT lysate with 100 mM NaCl and 500 mM MgCl2 in 10 mM MES buffer, pH 5.2; V - Elution of hSCOMT lysate with 250 mM NaCl and 500 mM MgCl2 in 10 mM MES buffer, pH 5.2.

By WB analysis (Figure 41 (A)), it can be observed that the hSCOMT protein was practically all retained by the gellan microspheres (line II) and it was preferentially eluted in the last elution step with 250 mM NaCl and 500 mM $MgCl_2$, at pH 5.2 (line V). In addition, the SDS-PAGE (Figure 41 (B)), showed that lines II, III and IV had a lot of contaminants, working steps of contaminants removal. These results confirm what was suggested in the previous assay, that temperature can influence the active centre of hSCOMT enzyme and consequently the interactions established with the magnesium ions on the gellan microspheres. By other words, when the hSCOMT was put in a more favourable environment (4 ºC), its stability was improved thereby promoting stronger interactions, which were only destabilized in the presence of high ionic strength and high concentration of competitive agent (line V of Figure 41 (A)).

In general, this strategy not only promotes an excellent retention degree of hSCOMT to magnesium-crosslinked gellan microspheres, but also the recovery of high hSCOMT content in a single clarified sample. Moreover, the removal of contaminants in three consecutive steps before the recovery of the target protein improve the selectivity of the affinity strategy. So, this strategy seems to possess a higher affinity recognition of hSCOMT present in complex K. *pastoris* lysates.

In comparison to the affinity strategy by using nickel-crosslinked gellan microspheres (assay 8), this one (assay 16) seems to recover practically all hSCOMT in a single step, probably resulting in better yields. However, for the hSCOMT elution are required more drastic conditions (250 mM NaCl and 500 mM $MgCl_2$), while in assay 8 hSCOMT is mostly recovered with 150 mM NaCl and 300 mM imidazole.

On the other hand, the affinity strategy with nickel-crosslinked gellan microspheres is useful only for fusion proteins to recover the target protein [103], which need to be removed for further structural and inhibition studies. The procedures applied to remove the His-tags may compromise the biological activity of the enzyme. In this way, the use of magnesium-crosslinked gellan microspheres in a capture strategy brings the advantage of no need of fusion proteins, which simplifies the steps of biotechnological procedure (regarding the production and isolation) and avoids the degradation of the target protein.

To conclude, the present work showed the versatility of the gellan gum in the formulation of microspheres for the capture of hSCOMT, not only extrapolating a cation exchanger strand due to the anionic gellan molecules, but also extrapolating different affinity strategies with the divalent ions applied in the microspheres reinforcement, thereby allowing the design of capture strategies more specific, efficient and adequate to the target molecules. The nickel ion allowed the development of a capture strategy similar to IMAC, while magnesium provided a bioaffinity approach since it was based in a biological native relationship with the target protein.

Chapter 5 – Conclusions and Future Perspectives

The increasing necessity of developing new and more effective COMT inhibitors for the improvement of PD therapy led to an advanced search in new faster and low-cost alternative procedures to acquire great quantities of recombinant protein in a highly purified form with intact biological activity. The batch method is a simple and low-cost alternative method for the capture of biomolecules and has the advantage of huge flexibility regarding the material nature of the particles. Gellan gum is an anionic microbial exopolysaccharide widely applied in food, pharmaceutical and other industries, and has the capacity of forming gels resistant to temperature and extreme acidic conditions, in presence of divalent ions. So, the present work aimed to reinforce the recent applicability of gellan gum in the capture of biomolecules through a batch method by using a new formulation of gellan microspheres to isolate or clarify the hSCOMT protein from *K. pastoris* complex lysates.

Therefore, two types of gellan microspheres were produced with two different divalent ions, nickel and magnesium, used as crosslinkers. Both formulations yielded microspheres with a consistent and uniform structure with a spherical shape. However, gellan microspheres with magnesium ions are larger that gellan microspheres with nickel ions due to its higher atomic radius, occupying more space between the gellan molecules. Then, these gellan microspheres formulations were applied in a batch method to capture the hSCOMT protein from *K. pastoris* complex lysates through two main strategies that were developed and optimized: the first one was based on the interaction between negative charges of gellan microspheres and hSCOMT positive residues (ionic strategy) and the second one was founded on the affinity of the hSCOMT to the divalent ions of gellan microspheres (affinity strategy). After optimization, all strategies showed great outputs at both RT and 4 ºC, allowing almost all hSCOMT retention and its main recovery in a single clarified sample by the elimination of contaminants in consecutive elution steps.

Regarding the ionic strategy, it was observed a similar performance of hSCOMT capture between the nickel-crosslinked and magnesium-crosslinked gellan microspheres, although at RT the nickel-crosslinked gellan microspheres retained more levels of hSCOMT. Since magnesium crosslinked gellan microspheres have a larger diameter, it can be concluded that the divalent ion used may affect the capture profile by increasing or decreasing the surface contact area.

For the capture of His-tagged proteins, in general, using gellan microspheres with nickel as crosslinker seems to be more appropriate due to the well-known affinity of histidine residues

to the nickel ions. However, the use of His-tags implicates its removal after the purification by using procedures that may compromise the biological activity of the protein. Thus, the development of an alternative strategy that involves the bioaffinity of hSCOMT to magnesium ion seemed to be a promising approach to overcome the fusion protein matter. Besides the remarkable results, the stronger interactions established also requires more drastic conditions to disfavour them. Nevertheless, it still remains a great strategy for hSCOMT capture and can be extrapolated for other proteins that hold a biological relationship to a divalent ion, not necessarily magnesium or nickel.

Regarding the main objective of this work, the gellan gum has proven to be a versatile polymer in the formulation of microspheres for the capture of hSCOMT, not only due to the cation exchanger strand that the anionic gellan molecules provide, but also due to the numerous affinity strategies that can be designed according to the divalent ions selected for the microspheres reinforcement, thereby yielding in capture strategies more specific, efficient and adequate to the target molecules.

As ongoing work, it is crucial to evaluate the enzymatic activity in order to determine the effect of several parameters used in the strategies described and the best elution and retention design for the hSCOMT isolation. After that, the conjugation of a capture strategy, like the ones explored in this work, and a final purification step by for example monolithic chromatography, will be important to achieve a complete purification of hSCOMT, extrapolating advantages and disadvantages of this strategy relatively to literature.

In future work, it will be also interesting to apply this clarifying method to other proteins, especially proteins that have bioaffinity to divalent ions.

Chapter 6 – References

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