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Licenciada em Ciências de Engenharia Química e Bioquímica

Smart Macroporous Structures for the Purification of Viral Particles

Dissertação para obtenção do Grau de Mestre em Engenharia Química e Bioquímica

Orientadores: Prof. Doutora Ana Cecília Afonso Roque, Secção de Engenharia Química e Bioquímica, Faculdade de Ciências e Tecnologias da Universidade Nova de Lisboa, e Prof. Doutora Ana Isabel Nobre Martins Aguiar de Oliveira Ricardo, Secção de Engenharia Química e Bioquímica, Faculdade de Ciências e Tecnologias da Universidade Nova de Lisboa.

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Smart Macroporous Structures for the Purification of Viral Particles

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Abstract

The increasing application of viral particles in vaccination and gene-based therapies, has led to the development of alternative and improved purification processes. Traditional purification methods include chromatographic techniques, however the chromatographic matrices used present limitations specially when aimed at the purification of large molecules. This work presents the preparation of chitosan-based monoliths using clean processes and easy functionalization techniques intending to improve Adenovirus serotype 5 (Ad5) purification.

Monoliths were prepared by blending chitosan (CHT) with glycidylmethacrylate (GMA) or poly(vinyl alcohol) (PVA), using two preparation techniques, freeze-drying and a $scCO_2$ – assisted drying process, and were subsequently functionalized with Q ligands by three different methods. In addition, monoliths blended with magnetic nanoparticles were also prepared using the same strategies to confer them a controlled magnetic response. The monoliths produced were characterized in terms of ligand immobilization yield, and evaluated for Ad5 purification. Two types of monoliths showed potential: the CHT/PVA(50:50) prepared by freeze drying and functionalized by the alternative plasma technique (M2) and the CHT/PVA(50:50) 7% monolith prepared by $scCO_2$ – assisted drying process and functionalized by the epoxyactivation technique (M1). The amount of ligand Q immobilized on the supports was monitored by titration assays, among which the CHT/PVA(50:50) 7% M2 prepared by $scCO_2$ – assisted drying process exhibited the highest immobilization yield (91%). Among the results for Ad5 purification, the CHT/PVA(50:50)M2 and the CHT/PVA(50:50)7% M1 resulted in a 40% and 14% of the viral particles, respectively. Protein-binding assays were conducted using bovine serum albumin (BSA) and lysozyme, to evaluate the anionic-exchange capacity of the supports.

The results make us believe in the potential of the produced monoliths to be applied in chromatographic techniques. However further improvements are necessary to enhance virus binding and recovery, to obtain an improved purification process.

Keywords: biopharmaceuticals, purification, monoliths, porous structure, virus particles, Q ligands

Resumo

O aumento da aplicação de vírus em terapias genéticas levou á necessidade de desenvolver processos alternativos de purificação. As técnicas tradicionais de purificação incluem a cromatografia. No entanto, os suportes tradicionalmente usados apresentam limitações quando aplicados na purificação de macromoléculas. Este trabalho de investigação propõe a preparação de monólitos de quitosano, recorrendo a processos verdes e técnicas de funcionalização simples, com o objectivo de melhorar a purificação de Adenovirus serotype 5 (Ad5).

Os monólitos foram preparados através de uma mistura de quitosano (CHT) com metacrilato de glicidilo (GMA) ou álcool polivinílico (PVA), recorrendo a duas técnicas, liofilização e secagem assistida com CO_2 supercrítico ($scCO_2$), com posterior funcionalização com ligandos Q, por três métodos distintos. Prepararam-se ainda monólitos contendo partículas magnéticas, de modo a lhes conferir capacidade de resposta a estímulos magnéticas. Os monólitos produzidos foram ainda caracterizados em termos do rendimento de imobilização do ligando e da sua eficiência na purificação de Ad5. Identificaram-se dois tipos de monólitos com maior potencial: o CHT/PVA(50:50) preparado por liofilização e funcionalizado através da técnica alternativa de plasma (M2), e o CHT/PVA(50:50)7%, preparado por secagem assistida com $scCO_2$, e funcionalizado por epoxiativação (M1). A quantidade de ligando imobilizado nos suportes, foi determinada por ensaios de titulação, tendo-se obtido maior rendimento de imobilização (91%) para o CHT/PVA(50:50)7%. Entre os resultados obtidos na purificação do Ad5, o CHT/PVA(50:50)M2 e o CHT/PVA(50:50)7% M1, apresentaram valores de recuperação de cerca de 40% e 14%, respectivamente. De modo a avaliar a capacidade de troca aniónica dos suportes, também foram realizados testes de ligação de proteínas.

Em síntese, defendemos que os resultados obtidos demonstram o potencial dos monólitos produzidos na aplicação em técnicas cromatográficas. No entanto, para atingir um processo de purificação melhorado, devem ser considerados futuros aperfeiçoamentos que favoreçam o acoplamento do vírus e a sua recuperação.

Palavras-chave: biofármacos, purificação, monólitos, estrutura porosa, partículas virais, ligandos Q

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Abbreviations

A_{280nm}	Absorbance assay at a 280 nm wavelength
Ad5	Human Adenovirus serotype 5
Ads	Adenovirus
AgCl	Silver Chloride
$AgNO_3$	Silver nitrate
AIDS	Acquired Immunodeficiency Syndrome
APS	Ammonium Persulfate
Ar	Argon
BCA	Bichinchoninic Acid solution
BSA	Bovine Serum Albumin
C	Carboxyl
CG	Chitosan/Glycidylmethacrylate monolith
CHT	Chitosan
CIM	Convective Interactive Media
CO_2	Carbon dioxide
CO_2 (3%)	CP monoliths with a 3% concentration, prepared by $scCO_2$-assisted drying process
CO_2 (7%)	CP monoliths with a 7% concentration, prepared by $scCO_2$-assisted drying process
CP	CHT/PVA(50:50) casting
CHT/GMA	Chitosan/Glycidylmethacrylate monolith
CHT/PVA(50:50)	Chitosan/Poly(vinyl alcohol) monolith with a polymeric ratio of 50/50 (% w/w)
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
El	Elution
E	Ligand used in excess during immobilization procedure
$FeCl_2$	Iron (II) chloride tetrahydrate
$FeCl_3$	Iron (III) chloride hexahydrate
FT	Flowthrough
IEC	Ion Exchange Chromatography
GMA	Glycidylmethacrylate
GQ	Glycidyltrimethylammonium chloride hydrochloride
HCl	Hydrogen Chloride
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
L	Load
LMW	Low Molecular Weight

M1	Epoxyactivation technique
M2	Plasma Alternative technique
M3	Direct Immobilization technique
Mg	Magnetic
MK	Low molecular weight protein marker
MMW	Medium Molecular Weight
MNP's	Magnetic Nanoparticles
N_2	Nitrogen
N	Native
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NE	No ligand in excess used during immobilization procedure
NH_2-Q	(2-aminoethyl)trimethylammonium chloride hydrochloride
NH_4OH	Ammonium hydroxide solution
NM	No MBA in the casting solution
PAGE	Polyacrylamide Gel Electrophoresis
P_C	Critical pressure
pI	Isoelectric point
PVA	Poly(vinyl alcohol)
Q	Quaternary amine
RNA	Ribonucleic acid
S	Sulfonated
SDS	Sodium Dodecyl Sulfate
$scCO_2$	Supercritical carbon dioxide
SEM	Scanning electron microscopy
T_C	Critical temperature
TEMED	N,N,N',N' tetramethylehtylenediamine
ToMV	Tomato Mosaic Virus
Tris-Base	Tris(hydroxymethyl)amino methane
UV	Ultraviolet light
VLP's	Virus-Like Particles
W	Wash
WM	With MBA in the casting solution

1. Introduction

1.1 Viruses and their application in gene therapy

The search for new therapeutic approaches with a higher efficiency for the treatment of human diseases is an area of great investment. One type of treatment that has been applied, with a large growth in the last decade, is gene therapy.[1] With the aim of tackling genetic diseases and slowing down the progression of tumours, gene-based therapies have been developed to make this possible through the insertion of genes into target cells. With the development of this technique, there are several on-going clinical trials for treatment of several diseases such as cancer, cardiovascular and infectious diseases, among others (Figure 1.1,A). However, the introduction of genes into cells will differ accordingly which application is targeted. For example, among the cancer treatments some strategies involve the use of tumour suppressor genes, vaccination implemented with tumours cells engineered to express immunostimulatory, vaccines composed by naked DNA, vaccination with recombinant viral vectors encoding tumour antigens and other gene-based therapies with viruses.[2] In the end, all of these strategies and other specific to each target disease have one thing in common, they are only possible while using a good delivery system to introduce the genes into the cells. Among the virus properties, the major advantage is that they own a specific system to deliver DNA to cells, which makes them very suitable to be applied in this field.[3] When implemented in therapy, viruses have the ability to enter a specific target and replicate within the cells, causing cell lysis and subsequently killing the infected tumour cells. Therefore, there are several types of vectors that can be used as genetic vehicles and can be divided into two different groups, such as viral or non-viral vectors.[4]

Non-viral vectors are vectors constituted by naked DNA with the advantage of being non-pathogenic and with an easy and economical production. The increasing use of non-viral vectors in the past years is due to the production of high levels of gene expression when applied through a direct injection to tissues. However, these vectors are not as efficient has viral vectors.[4,5] Viral vectors are vectors that take advantage of the infectious and replication system of viruses. Despite their infectivity, extensive research throughout the years led to the development of safer and more efficient viral vectors, being Retrovirus and Adenovirus, the two types of vectors more used for gene therapy. [6] Retroviruses are characterized by comprising an enveloped single-stranded positive RNA genome, which is segmented and can only replicate by inverse transcription. One example of a retrovirus well known is HIV (Human Immunodeficiency Virus) that causes AIDS.[7] A great number of clinical trials have been implemented with the aim of tackling this infectious disease, however severe adverse events were observed during these trials, indicating a necessary improvement towards the manufacturing of safer vectors and leading to a decline in the use of this kind of vector.[3,8] On the other hand, Adenoviruses are characterized by being non-enveloped virus with a non-segmented double stranded positive DNA.

With a 90-110 nm structure, adenoviral vectors can carry a larger DNA load than retroviruses, resulting in higher efficiencies of transduction and higher levels of gene expression, delivering genes to a wide variety of dividing and nondividing cells. One example of an Adenovirus is the Human Adenovirus serotype 5 (Ad5), the most studied Adenovirus for gene therapy.[9] Despite the possibility of eliciting a strong immune response, adenovirus also have the advantage of being easy to produce in culture, allowing its production in large quantities while staying stable during some essential steps as concentration and purification. All of these advantages make the Adenovirus the most commonly used vector in gene therapy (Figure 1.1, B).[2]

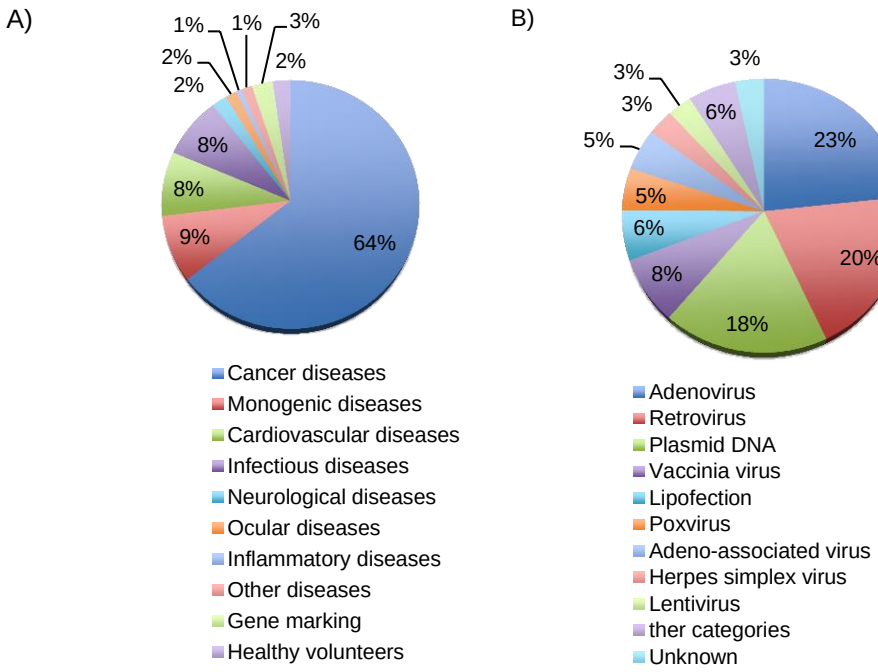

Figure 1.1 - Graphic representation of the principal target diseases aimed by gene therapy clinical trials (A) and respective types of vectors used in these clinical trials (B) during the year of 2012.[2]

As it was discussed each type of vector has its strength as well as weakness, so it is necessary their modification in the laboratory to make the vector suited for each specific application in gene therapy. Consequently, new viral vectors are produced or modified by replacing, altering or augmenting a gene to provide a missing function, replace a defective gene in the target cell, or modulate an immune response, among others. However, for a good and proper implementation of these vectors, it is required products with a high purity to optimize their application in clinical trials.[10] Within the several stages of vectors manufacturing, purification is the most important one, assuring the elimination of impurities and other species from virus solution while trying to maintain the yield.

With the implementation of these vectors in gene therapy and the increasing number of clinical trials, there is a demand for large-scale purification processes. It is necessary the development of simpler and faster purification processes that allow the purification of bigger quantities while achieving the required purity of the vectors and maintaining the infectivity for live attenuated vaccines. It is also important that these processes be both reproducible and economically viable.[2,4–7,11]

During virus purification processes, there are some features that should be taken into account. Viruses, in general, are characterized by being large particle sizes, having a low diffusion rate and a complex molecular surface. All of these factors will influence the yield and implementation of any process.

There are several purification methods that can be used to purify virus, though the traditional methods commonly implemented are centrifugation and column chromatographic techniques.[11] Centrifugation was the technique mostly used for Adenovirus purification, using a combination of density gradient centrifugation and ultracentrifugation. However this procedure could take days and can lead to co-precipitation of impurities with a density similar to the virus, making these procedures not suitable for large-scale purification.[12]

Column chromatographic techniques consist of separation processes using a column with a stationary phase that will serve as media to purify our target molecule from a complex mixture (mobile phase). Among the several columns chromatographic techniques there are some methods more used for virus purification, such as Affinity Chromatography, Anion Exchange Chromatography and Fixed-Bed Chromatography.[11,13] Though, these techniques were usually applied using resins or porous beads as supports media and despite the advantages of these kind of media[14] they are not ideal for the separation of large biomolecules such as virus, proteins or DNA, resulting in a low mass transfer. The shape of the beads will also contribute for a large void volume between the packed particles (Figure 1.2) that combined with the large size of viruses, the use of this kind of media will result in a poor purification performance with a low binding capacity. Taking into account all these drawbacks, the traditional methods are often described as being time consuming, expensive and difficult to scale up.[2,15] As an alternative, it was developed a new membrane technology with decreased mass transfer resistance, allowing the processing of higher volumes, the purification of large biomolecules and the production of therapeutic proteins through the use of a membrane as chromatographic media. Another advantage of this type of media is that the production of membranes for these methods is very simple, reducing the cost of the stationary phase and subsequently reducing the cost of downstream processes.[16] However, there are some physical properties of membranes that require improvement such as thickness, ligand density and pore size distribution for certain applications. These factors led to the development of different types of membranes, which include mixed-matrix membranes and monoliths. The respective characteristics to these several chromatography stationary phases are represented in Table 1.1.[17] However, the several advantages of using monoliths as chromatographic media, led to this being the method of choice for bioseparations of large molecules, with an increasing application for the last decade. Among the several benefits of using monoliths, it is possible to control the obtained structure by varying the conditions of the preparation methods, resulting in processes with higher yields and throughputs that when traditional methods are applied.[15,18,19]

Table 1.1 - Comparison of characteristics of current chromatography stationary phases.[17]

		Resin	Mixed-Matrix Membrane	Monolith	Membrane
Characteristic	**Flow rates**	Low	High	High	High
	Pressure drop	High	Low	Low-moderate	Low
	Dominant transport	Diffusive	Diffusive	Convection	Convection
	Binding capacities	High	Moderate	Moderate	Low
	Resolution	High	Moderate-high	Moderate	Moderate
Hardware	**Cost**	Moderate	Moderate	Inexpensive	Inexpensive

1.2 Monoliths for Purification Processes

Monoliths are characterized as single integrated units with a highly interconnected network with different sizes. They can be produced from different materials and be made with diverse shapes, selected according to the envisaged application.[20]

The first attempt of creating this new kind of supports as a “single-piece” stationary phase was during the late 1960s and 1970s.[21] However these preparations were not very successful and later approaches emerged in the late 1980s, when compressed soft gels called “continuous beds” were developed and successfully used in chromatographic separations, followed by the production of rigid macroporous polymers monoliths based in a molding process, in early 1990s.[22] These successful preparations defined the beginning of the development of this type of continuous media, being nowadays a kind of support suitable for a wide range of applications in several fields such as in biotechnology, food technology, tissue engineering and other pharmaceutical industries.[23] The development of monoliths led to the improvement of procedures such as organic synthesis, separation processes, solid-phase extraction and decontaminations.[24] They have also been used as bioreactors and have been applied as supports for a variety of applications including Hydrophobic interaction Chromatography [25], Affinity Chromatography [26], High Performance Membrane Chromatography [27], HPLC[28], Capillary Electrochromatography [29], Ion Exchange Chromatography [30] and molecular imprinting [31].

However, the major application of monoliths is in the gene therapy and vaccination fields, being the method of choice for the purification of large particles, such as viruses, proteins or plasmid DNA or VLP’s.[28,32,33,34,35] When inserted into a column and used as chromatographic media, monoliths will fill the volume of the column completely, operating as a single large particle. As membranes, monoliths also have a low resistance to mass transfer and a low back pressure, and due to their design, monolithic supports take the advantage of a convective mass transport that overcomes a diffusional flow, not being limited by the sizes of molecules, higher flow rates and shorter times are obtained.[14,16,21,22,36,37]

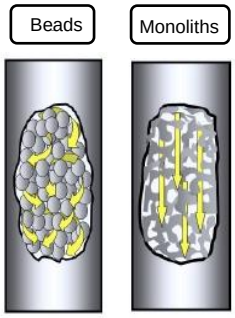

Figure 1.2 – Comparison of the flow in chromatography columns using beads and monoliths as media.[36]

It already have been reported some cases related to successful purification of virus using monoliths, such as the purification of Adenovirus (Ads), Tomato mosaic virus (ToMV) and Influenza virus, among others.[38,39,40] In both cases, the use of monoliths led to a decrease of the process duration, for example, the ToMV purification decreased from a 5-day procedure to an 8h procedure.

All of the monoliths characteristics lead to the improvement of purification processes, with a easier implementation and production, these supports result in a more economical manufacturing and a faster response to sudden demands, which is suitable for the increasing demand of viral vectors for clinical applications of gene therapy and vaccination processes.[1,6,7,13,41]

However, the performance of monoliths as supports in chromatographic processes depends of several features that have to be controlled and improved to reach the desired resolution of the purification process. Among these features, the materials, the conditions and the preparation methods selected are some of which will affect the final structure and performance of the monolith. Also it is important to each monolith to own proper characteristics, such as an inert surface, available chemical groups, a good mechanical stability and a structure with a high porosity and interconnectivity.[23,28] It is very important to find an optimal balance between surface area, porosity and pore size distribution of the monoliths, that depend of the preparation properties applied, which can be controlled to obtain specific results more suitable to each application.[42] The Table 1.2 describes the general features of chromatographic media used for large molecules separation processes.

Table 1.2 – General features of chromatographic media used for the separation of large biomolecules.[42]

Feature		Dimension
Surface area		10 – 400 m^2/cm^3
Functional Group		10 – 500 µmol/cm^3
Porosity (ε_p)	Nonporous	0
	Porous	0.25 – 0.75
Pore size	Conventional	10 – 100 nm
	Monolith	1000 – 5000 nm

Considering the material composition, monoliths can be classified into two categories: organic monoliths and silica-based monoliths.[14,43] Silica-based monoliths are generally prepared by a sol-gel process, which leads to a good network with high permeability and mechanical stability, and with a surface that can be easily modified.[44,45] This type of support has several applications, though the most successful and highly reported ones relate to HPLC and Capillary Electrochromatography processes.[29] However, nowadays the most common type of monoliths used, are the organic monoliths that are composed of polymer-based materials. These materials can be agarose, polymethacrylates, polyacrylamides, chitosan or cryogels.[7,28] In the recent past years, monoliths made with polymers and polymer blends (synthetic polymers) have received a lot of attention, in particular the chitosan-based monoliths.[25,41]

Chitosan is obtained from partial deacetylation of chitin, a biodegradable polysaccharide extracted from crustacean shells, and is characterized by owning positive attributes such as low toxicity and biological properties, being biocompatible with antimicrobial activity and anti-tumour properties. Besides being ecologically safe, this polymer also has several possibilities for structure modifications to acquire the desired properties, making this type of material a valuable polymer with multiple potentials also to be applied in biomedical, pharmaceutical and cosmetic fields.[46]

The preparation of the polymeric monoliths begins with a polymerization mixture, consisting of monovinyl monomers, a crosslinking agent, an initiator and a pore-forming solvent. Usually, the polymerization is a radical polymerization, which can occur by UV irradiation or heat.[22] In the case of synthetic polymers such as cryogels, macroporous structures prepared by cryogelation at sub-zero temperatures, it occurs a free radical polymerization where the porogen will be the ice crystals formed during the gelation process. The freezing temperature will define the size of the pores obtained, as with a higher freezing temperature, larger pores will be obtained, and smaller pores are obtained while using lower temperatures.[47]

The monomers used in the polymeric mixture are going to provide the monolith with some properties such as mechanical stability and functional groups. For example, one of the most common monomers used is glycidylmethacrylate (GMA), that can be used as blending or as a crosslinker, will introduce epoxy groups to the monolith matrix, through which further functionalization can be made.[48]

Another recent strategy for monoliths improvement is the preparation of monoliths with magnetic properties.[49,50] The blending of magnetic nanoparticles (MNP's) within the casting solutions will enhance the monoliths performance due to magnetic responsive particles.

To work as chromatographic media for several techniques, such as ion-exchange, affinity or hydrophobic chromatography, it is require the chemical interaction between the support and the target molecule we want to purify. For this purpose, it is necessary the functionalization of the monoliths through the coupling of ligands to achieve the desired chromatographic properties. Ion-exchange chromatography is the most applied technique for virus purification, in which the common ligands used for coupling include quaternary amine (Q) and diethylaminoethyl (DEAE) ligands, as strong and weak anion-exchangers respectively, and sulfonated (S) and carboxyl (C) as strong and weak cation-exchangers, respectively. However, the immobilization of each type of ligand can be implemented by different methods.[17]

Another important feature that will affect the final structure of monoliths is the preparation methods and respective conditions in which this preparation takes place. There are several methods that can be applied, being the most used freeze-drying, from which is obtained a good interconnectivity within the macroporous structure. However, conditions such as temperature, pressure and concentration of the mixture will affect the obtained structure and can be adapted to achieve the desired porous structure.[51]

All these advantages and characteristics of monoliths led to the commercial interest of several companies worldwide. Nowadays there are several types of monoliths commercialized, from compressed hydrophilic gels, silica rods to macroporous polymers that can acquire different shapes such as discs, columns or tubes.[42] Currently, four main companies invest mainly in the manufacturing of monoliths for bioseparations, among which the most marketed and used monoliths are the CIM (Convective Interactive Media) supports that are commercialized by BIA Separations.[26] The commercially available monoliths by these companies are summarized in Table 1.3, with their respective features and separation modes for which they are best suitable.

Table 1.3 - List of current commercially available monoliths for bioseparations.[23]

Product	Manufacturer	Material	Separation mode	Macro Pore Size (nm)
CIM	BIASeparations	Polymethacrylate	Ion exchange, Hydrophobic interaction, Reversed phase, Bioaffinity	0.03 – 1.5
UNO	Bio-Rad	Polyacrylate	Ion exchange	1
Chromolith	Merck	Modified Silica	Reversed phase	≥ 2
Seprasorb	Sepragen	Modified Cellulose	Ion exchange	50-300
SWIFT	Isco	Polymethacrylate	Ion exchange, Reversed	1.5

With the potential of these supports combined with the increasing demand of large-scale purification processes, it is necessary to develop the scalability of the monoliths. For the implementation of the monoliths at an industrial scale, it is essential the adaptation of their features so that a chemical and mechanical stability is achieved and the processes yield maintained. For many, scale-up is considered a limitation for monoliths development, however in the past years, some improvements have been made, and for example, some scale-up trials were accomplished while connecting columns in parallel or in series, offering a bigger volume capacity to the system. Additionally, it was also accomplished the scale-up of a disk monolith of 0.34 mL capacity to a successful 8 L radial column.[47] However, this is a current process and further development is necessary to assure the stability of these scale-up columns.

1.3 Aims of the work

This work is aimed to prepare and functionalize different types of monoliths to work as ion exchange media to improve the process of virus purification. While combining the monoliths supports produced with biopolymers materials, our strategy is to functionalize these monoliths for a better binding to the target virus within conditions that will make this a more sustainable process. Our target virus will be Adenovirus Serotype 5 (Ad5). To this aim our work is structured by different stages that complement the whole production of the monoliths and their application as chromatographic media. (Figure 1.3) The development of the monoliths started by choosing the materials from a previous work, where the monoliths were aimed for Ad5 purification, among which the chitosan (CHT) based monoliths stood out when blended with PVA or GMA, resulting in lower bindings and higher recoveries of this virus, being around 80% of the virus load recovered. These are good materials to be implemented due to their biological properties, such as being biocompatible, biodegradable and non-toxic. In addition to these polymer blends, a crosslinker, a catalyst, an initiator and a pore-form solvent will also be present in the casting solutions, forming the polymerization mixture. However, our casting solutions will also differ in some aspects such as the concentration, the presence or absence of the crosslinker or if magnetic nanoparticles (Mg) are blended in the casting solutions. We designate our monoliths as smart structures due to their ability to react to the conditions implemented during the purification process, when materials are present, such as chitosan that reacts to the pH changes, and the MNP's that are implemented to enhance the monoliths performance while an external magnetic field will be applied.

Another important feature that will affect the final structure of monoliths is the preparation methods and respective conditions in which this preparation takes place. The monoliths will be prepare through environment friendly methods, such as freeze-drying and drying assisted with $scCO_{2,}$ which will result in monoliths with different structures offering diverse benefits to each casting. Freeze-drying is a commonly used technique, in which the material is frozen and then sublimated providing the material with a good porous structure. It has advantages of obtaining dried monoliths using low temperatures with no damage to their structure. However, the use of methods assisted with supercritical fluids has been increasing in the last decades due to their many advantages when implemented. Besides being a simple method, CO_2 properties make this component a suitable supercritical fluid for the preparation of polymeric porous structures, resulting in a completely dry final product, free of solvents and with the possibility of adjusting the pore size distribution obtained in the end.

Another advantage of the biomaterials selected is that the monolith's castings will offer several possibilities for mechanical and chemical modifications. This combined with the virus charge properties, we can functionalize the monoliths to enhance the virus binding to the supports and improve our purification process. The functionalization is going to be made with quaternary amine (Q) ligands that are characterized by being strong anion exchangers, meaning that will remain fully charged over a broad pH range, resulting in a faster and easier development of separation processes. Two types of Q ligands that have different terminations are going to be immobilized by three different methods. A commonly used method for ligand immobilization consists on an Epoxyactivation of the

supports, introducing epoxy groups on the matrix where the ligand is going to be coupled, which will be designated as epoxyactivation technique. The second method implemented, the alternative plasma technique, consists of a single-step solvent free technique. Through Plasma treatment, introducing radicals in the monoliths surface for a subsequent amination, where the ligand is then going to be coupled. The last method, the Direct Immobilization Technique, consists of a single-step immobilization process where the ligands are introduced directly in the casting, no needing any further modifications after polymerization. To enhance the ligand immobilization, it were also implemented some assays were the ligand was added in excess (5 eq. to activation content) to make sure that the immobilization occurs.

Once the monoliths are ready and functionalized, they were tested as chromatographic media for virus purification with samples of an Ad5 virus. The ligand immobilized was quantified by a precipitation titration. Aside these tests, it was also implemented a proof of concept, to evaluate if the monoliths work as ionic exchange chromatographic media, using a protein mixture composed by Lysozyme and BSA by taking advantage of their charge properties among the pH medium. These tests were quantified through a BCA test and SDS-Page analysis to evaluate the partition of the proteins among the purification stages.

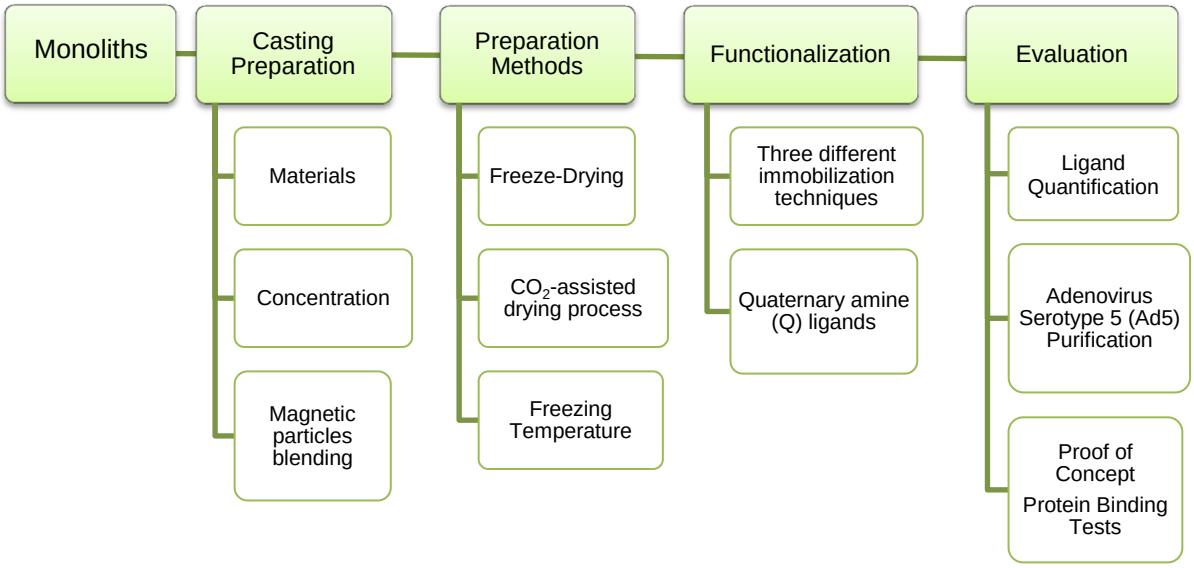

Figure 1.3 – Work scheme of this thesis.

In addition to the evaluation of the produced monoliths as chromatographic media, it is also essential to study the morphological and mechanical properties of the supports to evaluate if the properties have the desired values and assure an optimal performance. As it was mentioned before, it is important a balance between the pore size distribution and the mechanical stability of each monolith, therefore there are several methods that can been implemented to study these properties such as scanning electron microscopy (SEM) to obtain high resolution images of the porous structure, mercury intrusion porosimetry (MIP) to evaluate the porosity and average size diameter of the pores within the monolithic structure and mechanical studies to measure the strength of the support, among others.

Despite the importance of these evaluations, it was not possible to implement them throughout the duration of this work. However, in previous reported works from which we based the production of the supports, the chitosan-based monoliths were characterized and the features of the monoliths produced were studied, such as pore size distribution, biodegradability, swelling and water flux, the porosity and mechanical stability. To test the biodegradability and swelling capacity, the monoliths were studied for several weeks, among which their stability was studied by placing the supports in different pHs, among which all chitosan-based monoliths showed good swelling properties and a significant water uptake. It was also observed the supports capacity to own a pH memory and ability to respond to the medium, as it is expected to obtain in our produced smart macroporous structures. In addition, the porosity and compressive modulus of these previous monoliths were also reported. All these properties are represented in Table 1.4, and it is expected to obtained similar results on the monoliths produced in this work. In turn, the monoliths prepared by assisted drying with $scCO_2$ were not previously characterized.

Table 1.4 – Morphological and mechanical properties of chitosan-based monoliths. These results are from reported works, where Chitosan/Poly(vinyl alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA) and Chitosan/Poly(vinyl alcohol) (CHT/PVA) blended with magnetic nanoparticles (Mg) monoliths were prepared by freeze-drying.[49]

Casting Solutions	Preparation Method	$T_{Freezing}$ (ºC)	Average Pore Size Diameter (µm)	Porosity (%)	Permeability ($L/m^2.h.atm$)	Compressive Modulus (kPa)	
						Dry	Hydrated
CHT/PVA(50:50)	Freeze Drying	-80	53±5	70±5	294±15	0.41±0.5	0.2±5
CHT/GMA	Freeze Drying	-80	123±5	75±5	390±5	0.26±0.5	n.a.
CHT/PVA(50:50) Mg	Freeze Drying	-80	88±5	86±2	120±9	1.8±0.2	0.5±0.2

The thesis is organized in four main chapters. Firstly, the Introduction chapter followed by the Experimental chapter, where it will be comprised all the experimental work for the monoliths preparation such as materials, preparation methods, ligands and respective immobilization methods and protein-binding tests assays. Subsequently, the results obtained during all these stages will be discussed in the third chapter, with their comparison to some previous works done and literature results. Finally, the Conclusions chapter will include the review of our obtained results and the discussion of the future perspectives that can be applied as continuation of our work to which could result in the improvement of the virus purification process.

2 Experimental

2.1 Materials

Chitosan (75-85% deacetylated, medium molecular weight), poly(vinyl alcohol) (Mw = 89.000-98.000, 99% hydrolysed), glycidyl methacrylate, methylenebisacrylamide (purity >99%), N,N,N',N' tetramethylehtylenediamine, ammonium persulfate (purity ≥ 98%), sodium thiosulfate (purity ≥ 99%), tris(hydroxymethyl)amino methane (Tris-Base), epichlorohydrin, 1,6-hexanediamine (purity ≥ 98%), phenol, potassium cyanide and pyridine were supplied by Sigma Aldrich. Iron (III) chloride hexahydrate ($FeCl_3.6H_20$), iron (II) chloride tetrahydrate ($FeCl_2.4H_20$), bichinchoninic acid solution, copper (II) sulfate solution**,** Coomassie Brilliant Blue R, Mercaptoethanol, Bovine Serum Albumin, Lysozyme from chicken egg white, (2-aminoethyl)trimethylammonium chloride hydrochloride (purity ≥ 99%) and glycidyltrimethylammonium chloride hydrochloride were also purchased from Sigma Aldrich. Ninhydrin (purity >99%) and ammonium hydroxide solution (NH_4OH) were supplied from Fluka. Glacial acetic acid (purity≥ 99%) was purchased from Pronalab. Acetone (purity ≥ 99,5%), methanol (purity ≥ 99%), bromphenol blue sodium salt and isopropanol were purchased from Roth. Glycine was supplied by Acros. Sodium hydroxide (NaOH), ethanol, hydrogen chloride (HCl) and sodium chloride (NaCl) were purchased from Panreac. Sodium dodecyl sulphate (SDS) was supplied by BioRad.
Nitrogen, argon (Ar) and carbon dioxide (CO_2, purity≥ 99,9%) were supplied by Air Liquid. All reagents were used without any further purification.

2.2 Methods

The monoliths were prepared by the implementation of two different methodologies commonly used: freeze-drying and gel drying using supercritical CO_2 ($scCO_2$).

2.2.1 Native Monoliths Preparation by Freeze-Drying

The monoliths were first prepared by dissolving different ratios of chitosan (CHT) (50-90%) with poly(vinyl alcohol) (PVA) (50%) and glycidyl methacrylate (GMA) (10%) in acidified water (1% v/v with acetic acid) and stirred with heating (50-80°C) until the castings solutions become completely homogeneous. Then methylenebisacrylamide (MBA), the crosslinking agent (2% w/w), was added with continuous agitation for 90 min at 80°C. Afterwards, the casting solution was introduced into plastic tubes (length 3 cm, width 1 cm) with simultaneous addition of the initiator N,N,N',N' tetramethylehtylenediamine (TEMED) (23 μL) and the catalyst ammonium persulfate (APS) (42 μL) under agitation at 0°C, for 30 min. Then the tubes where frozen for 12h at -80°C and later lyophilized (Christ Alpha 1-4 Freeze Dryer). At the end, dried and slightly shrunken monoliths were obtained as can be seen on Figure 2.1.

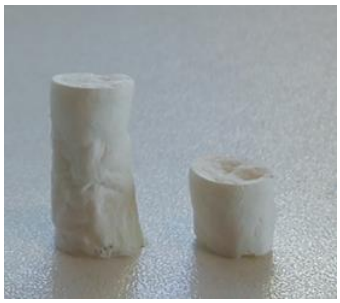

Figure 2.1 – Example of native CHT/PVA monoliths prepared by Freeze-Drying.

2.2.2 Native Monoliths Preparation by $scCO_2$ gel drying method

The monoliths were prepared by dissolving two CHT/PVA casting solutions, as described previously, in which the polymeric mixture concentrations differ (3 and 7%). However, it was also prepared monoliths with these same castings but without MBA in the mixtures.

The castings were then introduced into several steel containers (length 1 cm, width 2 cm) and were frozen for 12h at -20°C. Then it was implemented a water-acetone substitution by immersing the frozen monoliths into acetone at -20°C for 48h. Afterwards, the monoliths were dried using $scCO_2$ following a procedure already described in detail elsewhere [52,53] with few modifications.

The frozen monoliths were introduced into four steel containers, which were then introduced in the high-pressure cell. An example of a steel container can be seen on Figure 2.2. Subsequently, the vessel was closed and immersed in a visual thermostated water bath (40°C), heated by means of a controller (Hart Scientific, Model 2200) that maintains the temperature within ±0,01°C. The CO_2 was added using a Gilson 305 piston pump until the desired pressure was achieved (16 MPa) and the operation was performed in a continuous mode with a flow rate of 10 mL/min. Once the desired pressure was reached, the supercritical solution passes through a back pressure regulator (Jasco 880-81 Plus) which separates the CO_2 from the acetone. The pressure inside the system was monitored with a pressure transducer (Setra Systems Inc., Model 204) with a precision of ±100 Pa.

All the experiments were carried out for 4h. At the end, the vessel was depressurized during 4-5min and dried monoliths with no shrinkage were obtained as can be seen on Figure 2.2. The schematic representation of the installation in which the monoliths where prepared can be seen on Figure 2.3.

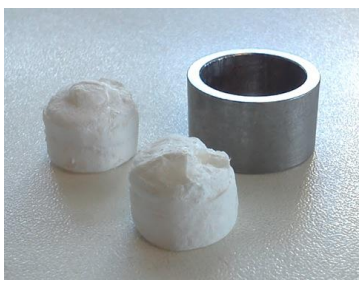

Figure 2.2 – Example of native CHT/PVA monoliths prepared by $scCO_2$ – assisted drying process.

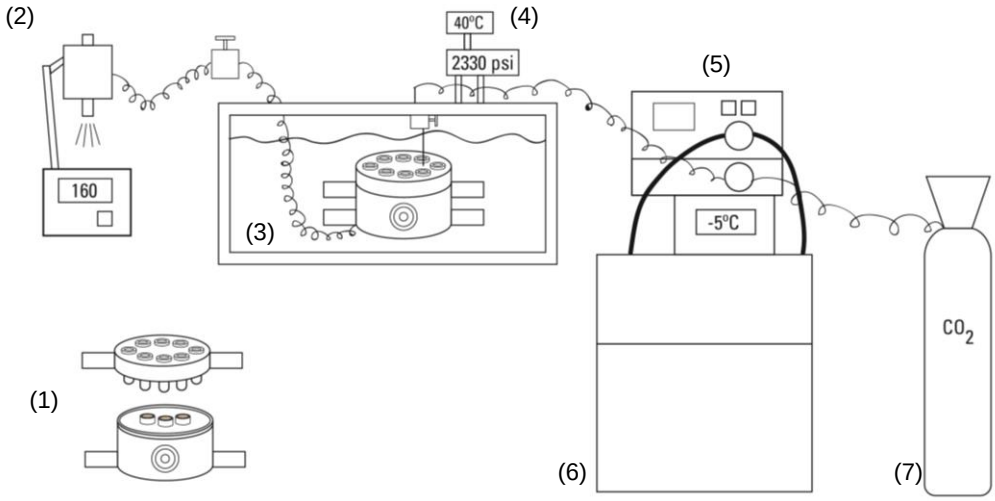

Figure 2.3 - Schematic representation of the installation used for the preparation of monoliths using $scCO_2$, consisting of: the high-pressure cell containing monoliths in the steel containers (1), a backpressure regulator (2), the thermal bath (3), the temperature and pressure controllers (4), the flow pump (5), the cryostat (6) and the CO_2 bottle (7).

2.2.3 Magnetic Monoliths Preparation

Previously to the preparation of the casting solutions and the monoliths preparation it was performed the synthesis of magnetic nanoparticles (MNP's) by co-precipitation method. An iron solution of $FeCl_3$ and $FeCl_2$ in 25 mL of distilled water was prepared containing a Fe^{3+}/Fe^{2+} molar ratio of 2. In a volumetric flask an aqueous solution of ammonium hydroxide (200 mL, 25% solution) was purged with N_2 for 15 min. Afterwards the iron solution was added drop wise with maximum agitation (2000 rpm) under an inert atmosphere, and the reaction was kept for 2h. Finally the magnetic particles were washed with distilled water using a permanent magnet and re-suspended in an accurate volume of 200 mL of distilled water. At the end, a solution of magnetic particles with a concentration of 10 mg/mL was obtained.

Once the castings solutions and the batch of magnetic particles were both prepared, a corresponding volume of MNP's ($m_{MNPs} = {}^{1}/_{3} \times m_{TotalPolymer}$) was re-suspended and added to the castings.

The magnetic castings solutions were stirred (250 rpm) during 24h at 80°C and 48h at 40°C for the CHT-PVA and CHT-GMA solutions, respectively, making sure homogeneous solutions were obtained. Finally, the casting solutions were added to the specific supports of each preparation method, freeze-drying or $scCO_2$ – assisted drying process, whose procedures were performed as mentioned before on sections 2.2.1 and 2.2.2, from which were obtained monoliths as can be seen on Figure 2.4. On Table 2.1 it is described the several monoliths developed by the two different preparation methods used.

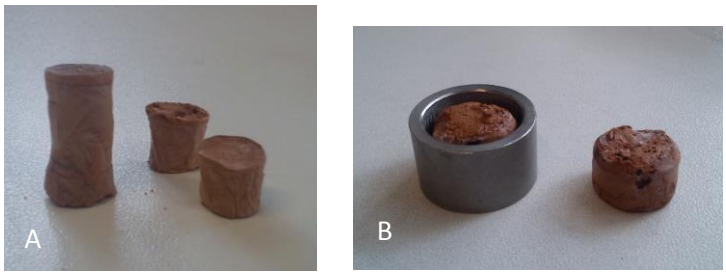

Figure 2.4 - Example of CHT-PVA monoliths blended with magnetic particles prepared by freeze-drying (A) and $scCO_2$ – assisted drying process (B).

Table 2.1 - List of the native monoliths produced. The monoliths differ in some features such as the castings, Chitosan/ Poly(vinyl alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA), CHT/PVA with magnetic particles blending (Mg), the concentration of the polymeric mixture (3% or 7%), if MBA was or wasn't added to the casting solution and if it were prepared by freeze-drying or $scCO_2$ – assisted drying process.

Casting	Preparation	$T_{Freezing}$ (ºC)
CHT/GMA	Freeze Drying	-80
CHT/PVA (50:50) 3% (With MBA)	$scCO_2$	-20
CHT/PVA (50:50) 3% (Without MBA)	$scCO_2$	-20
CHT/PVA (50:50) 7% (With MBA)	$scCO_2$	-20
CHT/PVA (50:50) 7% (Without MBA)	$scCO_2$	-20
CHT/PVA (50:50) Mg	Freeze Drying	-80
CHT/PVA (50:50) 3% Mg (With MBA)	$scCO_2$	-20
CHT/PVA (50:50) 3% Mg (Without MBA)	$scCO_2$	-20

2.3 Ligand Immobilization

The functionalization of the monoliths was performed through the immobilization of quaternary amine (Q) ligands onto the matrix of the monoliths. The monoliths structure presented several available functional groups (e.g. amine and hydroxyl groups) that can be used to carry out different functionalization techniques. Thereby the immobilization of ligands on the monoliths' surface followed three different methods, which will be designated as: the epoxyactivation technique (method 1, M1), the alternative plasma technique (method 2, M2) and the direct immobilization technique (method 3, M3). These methods have already been described in the literature.[54,55]

Afterwards the functionalized monoliths were evaluated in terms of the ligand immobilization yields and their performance on the purification of virus particles was compared.

2.3.1 Method 1 – Epoxyactivation Technique

In this method, the monoliths were epoxyactivated for further coupling with (2-aminoethyl)trimethylammonium chloride hydrochloride (NH_2-Q) ligand, by taking advantage of the free hydroxyl groups at the monolith surface.

The epoxyactivation was performed by adding 1 mL of 10 M NaOH solution (40 mL/kg of moist gel) to the monoliths and incubated for 30 min at 30°C with agitation (200 rpm). Afterwards 1 mL of epichlorohydrin (72 µL/kg of moist gel) was added and incubated again for 3 h at 36°C with the same agitation. In the end, the monoliths were washed five times with 10 mL of distilled water with agitation for 1 min. The extent of epoxyactivation was determined by adding 3 mL of 1.3 M sodium thiosulfate to the epoxy-activated monoliths followed by incubation at room temperature, for 20 min. This mixture was then neutralized with HCl (0.1 M). The amount of HCl added corresponded to the number of moles of OH^- released (10 µmoles per 100 µL added).

Prior to the immobilization of the ligand onto the epoxyactivated monoliths, a certain quantity of the ligand (5 molar excess to epoxy content) was dissolved in 5 mL distilled water and then the amine salt form of the ligand was neutralized with 1M NaOH (1 eq. to epoxy content). Afterwards, the ligand immobilization occurred overnight at 40°C with agitation (200 rpm). The complete schematic representation of the functionalization of the monoliths through this method can be seen in Figure 2.5.

After the immobilization, the monoliths went through a cleaning procedure to eliminate any remains of non-absorbed ligands. Firstly, we started by washing the monoliths with distilled water until neutral pH. Then the monoliths were introduced in a Varian column (with 3 mL capacity) and incubated with distilled water to allow swelling (2 $mL.cm^{-1}$ of support). Then, they were washed again by a procedure that consisted of three different steps:

1) Regeneration Buffer (0.1 M NaOH 30% Isopropanol) and distilled water alternated (2 mL each, 5x);
2) Elution Buffer (20 mM Tris-base, 1 M NaCl pH 8, 5 mL);
3) Binding Buffer (20 mM Tris-base, 150 mM NaCl pH 8, 10 mL).

At the end the monoliths were stored with binding buffer until tested.

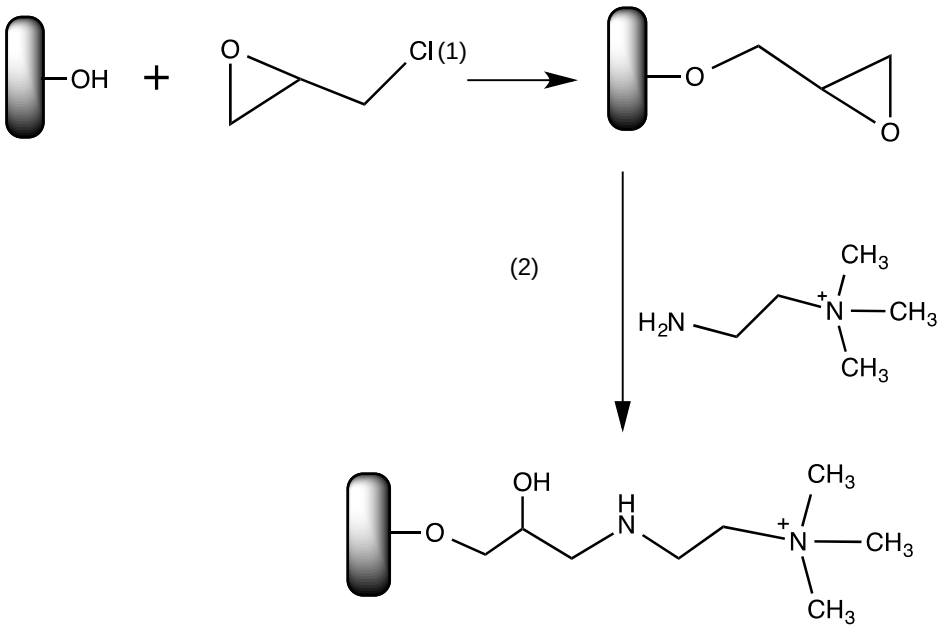

Figure 2.5 - Schematic representation of the monoliths functionalization through Epoxyactivation Technique. This method consists in the epoxyactivation the monoliths surface, with the addition of epichlorohydrin (1) epoxy groups will be introduced in the monolith's surface for subsequently coupling with the NH_2-Q ligand (2).

2.3.2 Method 2 – Alternative Plasma Technique

The alternative plasma technique (Figure 2.6) for ligand immobilization is based on the activation of the monoliths surface through Argon-plasma treatment followed by *in situ* amination for further ligand immobilization [54].

The Plasma treatment was performed according to the following procedure: the system was heated at a temperature of 150°C with agitation. Then the monoliths were inserted in the chamber and the Argon was applied for 1/2 min to inert the chamber atmosphere. Then the monoliths were activated with Argon (5 min), introducing radicals onto their surface, followed by an amination with 1,6-hexanediamine. Due to the heating of the system, which is maintained at 150°C, the gaseous 1,6-hexanediamine is vaporized to the plasma chamber that is under vacuum and amination occurs for 30 min. The extent of the amine groups was determined through the Kaiser Test. This test is based on the reaction of ninhydrin with primary amines resulting in a mixture with a characteristic dark blue colour. This was performed by adding three solutions (A - 80% crystalline phenol in ethanol (w/v)), B - 2% aqueous solution of potassium cyanide (0.001 M) in pyridine (v/v) and C - 5% ninhydrin in ethanol (w/v)) (50 µL of each one) to the aminated monoliths samples (1.5 mL).

A calibration curve was also assessed with diluted standard solutions of glycine (0-0.5 µmol/mL).

All the samples were then placed in a water bath at 100°C for 10 min. The absorbance of the samples was measured in a microplate format by adding 200 µL of each sample in each well of a 96-well transparent microplate, which was previously heated at 60°C for 20 min. The absorbance was then measured at 560 nm in a microplate reader (Tecan Infinite F200).

Then, a certain quantity of glycidyltrimethylammonium chloride hydrochloride (GQ) ligand (5 molar excess to amination extent) was dissolved in 10 mL of distilled water, neutralized by adding 1M NaOH (1eq. to amination extent) and then added to the aminated monoliths. Finally the immobilization occurred overnight at 40°C with agitation (200 rpm). The complete schematic representation of the functionalization of the monoliths through this method can be seen in Figure 2.7.

After the immobilization, the monoliths went through a clean procedure to eliminate any remains of non-absorbed ligands. The procedure was performed as already described in section 2.3.1.

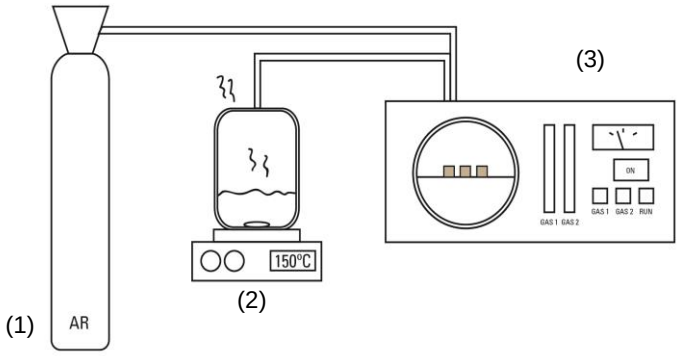

Figure 2.6 – Schematic representation of the installation for the functionalization of the monoliths through plasma treatment, consisting of: an Argon bottle (1), the flask containing 1,6-hexanediamine and respective heating (2) and the plasma installation where the monoliths are inserted for the treatment (3).

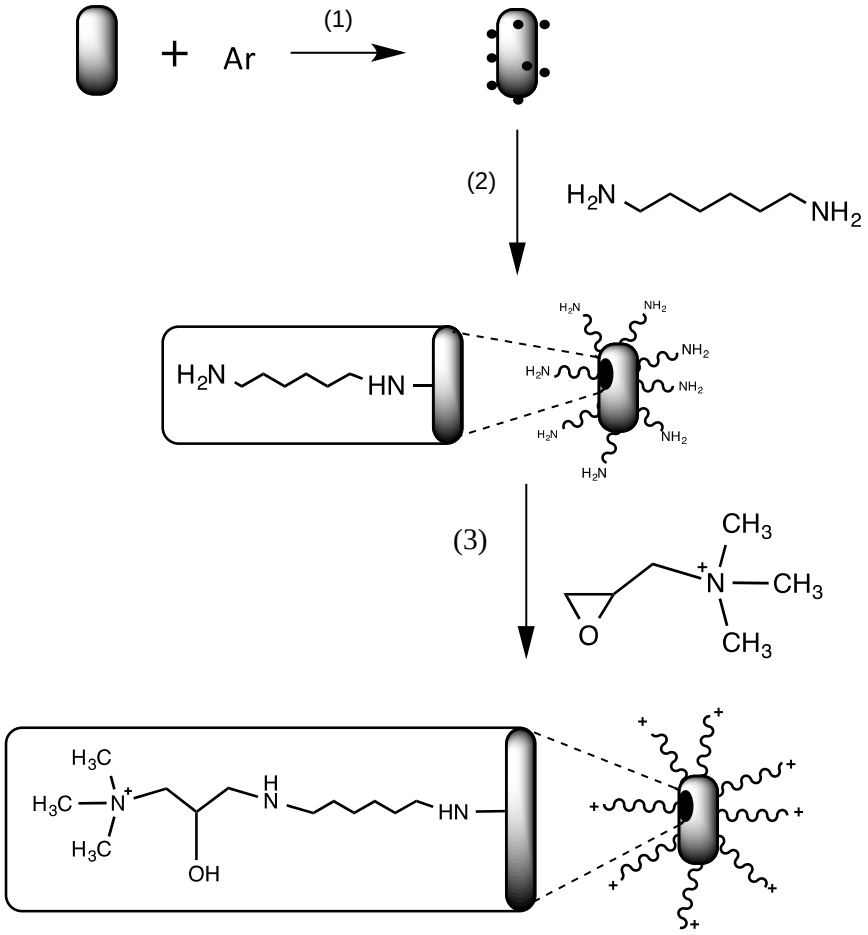

Figure 2.7– Schematic representation of the functionalization of the monoliths through Method 2, Alternative Plasma technique, which consists in applying Argon to the monoliths to introduce radicals onto the monolith's surface (1), followed by an amination with 1,6-hexanediamine for subsequent GQ ligand coupling (3).

2.3.3 Method 3 – Direct Immobilization Technique

The direct ligand immobilization technique was based on the direct addiction of the ligand to the casting solution. [55]

The monolith's casting preparation was performed as already described in section 2.2.1, with the addition of the selected ligand. The ligand GQ was added directly in the CHT/PVA casting solution with agitation at 80°C for 2h and the ligand NH_2-Q was added directly in the casting CHT/GMA with agitation at 50°C for 2h. To estimate the amount of each ligand that should be added to the castings, a Kaiser Test was performed to determine the extent of the amination of the monoliths only composed by CHT-PVA or CHT-GMA, which was performed has already mentioned in section 2.3.2.

After the direct immobilization of the ligands, it was added the crosslinking agent MBA (2% w/w) with agitation until the solutions were completely homogeneous. Afterwards the casting was introduced in plastic tubes (length 3 cm, width 1 cm) to be frozen for 12h at -80°C and later lyophilized (Christ Alpha 1-4 Freeze Dryer) until dry.

After the immobilization, the monoliths went through a clean procedure to eliminate any remains of non-absorbed ligands. The procedure was performed as already described in section 2.3.1. On Table 2.2 it is described the several functionalized monoliths developed, with the respective immobilization methods implement and the different preparation methods used.

Table 2.2 – List of the functionalized monoliths produced. The monoliths differ in some features such as the castings, Chitosan/Poly(vinyl alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA), CHT/PVA with MNP's blending (Mg), the concentration of the polymeric mixture (3% or 7%), if MBA was or wasn't added to the casting solution and if were prepared by freeze-drying or $scCO_2$ – assisted drying process. The functionalization was through different methods (M1 – Epoxyactivation technique, M2 – Alternative plasma technique, M3 – Direct immobilization technique) with two ligands (GQ and NH_2Q).

Casting	Preparation Methods	$T_{Freezing}$ (°C)	Immobilization Methods	Ligand
CHT/PVA (50:50) (No lig. excess)	Freeze Drying	-80	M1	NH_2Q
			M2	GQ
			M3	GQ
CHT/PVA (50:50) (Lig.in excess)	Freeze Drying	-80	M1	NH_2Q
			M2	GQ
			M3	GQ
CHT/GMA (No lig. excess)	Freeze Drying	-80	M1	NH_2Q
			M2	GQ
			M3	NH_2Q
CHT/GMA (Lig.in excess)	Freeze Drying	-80	M1	NH_2Q
			M2	GQ
			M3	NH_2Q
CHT/PVA (50:50) 3% (No MBA)	$scCO_2$	-20	M1	NH_2Q
			M2	GQ
CHT/PVA (50:50) 3% (With MBA)	$scCO_2$	-20	M1	NH_2Q
			M2	GQ
CHT/PVA (50:50) 7% (No MBA)	$scCO_2$	-20	M1	NH_2Q
			M2	GQ
CHT/PVA (50:50) 7% (With MBA)	$scCO_2$	-20	M1	NH_2Q
			M2	GQ
CHT/PVA Mg (50:50)	Freeze Drying	-80	M1	NH_2Q
			M2	GQ

2.4 Ligand Quantification of the functionalized monoliths

The ligands quantification was assessed through a precipitation titration, performed as described in the literature [56] with a few modifications. The chloride ion capacity of the monoliths was determined by an argentometric titration with $AgNO_3$ (0.1 M) combined with a silver ring electrode. With the addition of the titrant, the Ag^+ reacts with the Cl^- present in the solution and particles of AgCl precipitate,

resulting in a mist appearance solution. The amount of $AgNO_3$ added corresponded to the number of moles of Cl^- released (100 µmoles per 100 µL added).

2.5 Protein Binding for Anion Exchange Chromatography

In order to verify the capacity of the functionalized monoliths to act as an anion exchange chromatographic support, protein-binding assays were conducted. The monoliths used on these assays were the CHT-PVA(50:50) prepared by freeze-drying and the monoliths (7%) prepared with $scCO_2$. The functionalization of these monoliths was made with the GQ and NH_2-Q ligand, through method 1 and 2 respectively, which are strong anion exchanger and will transform our solid supports into positively charged monoliths.

The protein binding assays were performed with a mixture of proteins, consisting of Lysozyme and BSA (Bovine Albumin Serum). Each round of the assays involved regeneration, equilibration, loading, washing and elution. All the steps were performed under gravitational force. The regeneration was conducted by adding 1 mL of regeneration buffer (0.1M NaOH, 30% (v/v) isopropanol) alternated with 1 mL of distilled water, 2 times. Next, it was added 1 mL of elution buffer (20 mM Tris-base, 1 M NaCl pH8) in a total of 2 times. Afterwards, the equilibration step was carried out adding 1 mL of the binding buffer (20 mM Tris-base, 150 mM NaCl pH8), 7 times. In the last step of equilibration, the samples were collected on a 96-well microplate UV-half area (VWR) and then the absorbance was measured at 280 nm, to confirm that the $A_{280\ nm}$ reached ≤ 0.005.

Then 1 mL of the protein mixture (1 mg/mL, constituted by 0.5 mg/mL of each protein diluted in 1 mL of binding buffer) was loaded on the column and the flow-through was collected in 1.5 mL microcentrifuge tubes. Afterwards, the column was washed with 4 mL of binding buffer, and the samples obtained between the washes were collected in 1.5 mL microcentrifuge tubes. Bound protein was eluted by adding 6 mL of elution buffer (20 mM Tris-base, 1 M NaCl pH8), and the samples taken between each addition were also collected in 1.5 mL microcentrifuge tubes. After elution, the columns were well regenerated and stored at 4°C with binding buffer.

All the fractions collected (loaded, washed and eluted) were quantified by the BCA assay and SDS-PAGE analysis.

Additionally, the protein-binding assay was also attempted by a second approach. It was performed with a similar procedure to that described above but with some modifications, aiming to optimize the process and try to obtain a higher binding of the protein. Firstly, the regeneration was conducted by alternating the regeneration buffer with distilled water, 5 times, followed by elution buffer in a total of 5 times. Afterwards, the equilibration step was carried out 8 times, which samples were collected and measured at 280 nm has described above. Then 1 mL of the protein mixture (1 mg/mL) was loaded on the column and incubated overnight, stored at 4°C. The flow-through was then collected in a 1.5 mL microcentrifuge tube. Afterwards, the column was washed with 5 mL of binding buffer followed by elution, consisted by adding 5 mL of elution buffer 1 (20 mM Tris-base, 1 M NaCl pH8), followed by 5 mL of elution buffer 2 (100 mM Tris-base, 1 M NaCl pH8). All the samples between each wash and

elution were collected in 1.5 mL microcentrifuge tubes. After elution, the columns were well regenerated and stored at 4°C with binding buffer.
All the fractions collected (loaded, washed and eluted) were quantified by the BCA assay and SDS-PAGE analysis.

2.5.1 BCA Test

The quantification of total protein was assessed through the colometric BCA assay. Two calibrations curves of BSA were performed using the Binding buffer and Elution buffer. The BSA range of concentration in the calibration curve was 0.2-1 mg/mL.
To perform the assay, 25 µL of each sample was added (calibration curve and protein sample) to a 96-well microplate. Then, a solution of a BCA reagent was prepared by mixing 50 parts of reagent A (bichinchoninic acid solution) with 1 part of reagent B (copper (II) sulfate solution). A volume of 200 µL of this BCA reagent was added to the samples in each well of the microplate, and then incubated at 37°C for 30 min. At the end, the samples absorbance was measured at 560 nm and read in a microplate reader (Tecan Infinite F200).

2.5.2 SDS-Page Analysis of Protein Binding

To evaluate the partition of the proteins mixture obtained in the several steps of the protein tests and verify which protein bonded to the supports we have performed a SDS-Page analysis. An SDS-Page analysis consists of evaluating the mobility of a protein in a gel that is submitted to an electric current. The analysis was made in a 12.5% acrylamide gel.
The preparation of a 12.5% acrylamide gel combines the preparation of two different gels, a running gel and a stacking gel, that correspond to the bottom and top of the gel casing respectively. Firstly, the running gel was prepared by assemble in a tube 0.75 mL of solution I (3M Tris-HCl pH 8.8), 2.08 mL of Solution III (30% acrylamide and bis-acrylamide solution 19:1), 0.05 mL 10% SDS, 2.1 mL distilled water, 38 µL of 10% APS and 2.5 µL of 99% TEMED, which was introduced into the glass plates of the gel casing. Then, 1 mL of 2-butanol 99% was added on the top of the casing to promote a homogeneous and flat surface of the gel and polymerization occurred for 30 min. In the end, the 2-butanol was removed and the gel was washed with distilled water.
Afterwards, the stacking gel was prepared by assemble in a tube 0.45 mL of solution II (0.5 M Tris-HCl pH 6.8), 0.3 mL of Solution III (30% acrylamide and bis-acrylamide solution 19:1), 18 µL 10% SDS, 0.94 mL distilled water, 13.5 µL of 10% APS and 2 µL of 99% TEMED. Then the running gel was introduced into the gel casing together with the frame with wells and polymerization occurred again for 30 min.
Then the different samples obtained from the protein binding tests were prepared. To each volume of the samples (10 µL) it was added a volume of sample buffer (5 µL) (5 mL of 0.5 M Tris-HCl pH 6.8, 2 mL of 100% glycerol, 4 mg bromphenol blue sodium salt, 8 mL of 10% SDS, 1 mL β-mercaptoethanol and distilled water up to a final volume of 20 mL). Then, the samples were spin down and boiled in a

hot water bath (100°C) for 2 min. In conjunction with the samples, it was also prepared by the same procedure a low molecular weight protein marker (NZYTech).

In the end, all the samples were ready to be introduced into each well of the 12.5% acrylamide gel.

Then the running apparatus was assembled and the gel casings were introduced into the running module that was subsequently filled with the electrophoresis buffer (0.25 M Tris Base, 1.92 M Glycine, 0.1% SDS pH 8.3). The samples (15 µL) were then introduced into each well of the casing and the 12.5% acrylamide gel ran for 1h at 100 V and 250 mA.

In the end, the gels were stained with a Coomassie solution (1 g Coomassie Brilliant Blue R, 15 mL glacial acetic acid, 90 mL methanol and distilled water up to 200 mL) for 30 min, and subsequently treated overnight with a distained solution (75 mL glacial acetic acid, 450 mL of methanol and distilled water up to 1 L).

3 Results and Discussion

3.1 Native chitosan-based monoliths

Taking notice of all properties and benefits of using monoliths as a chromatographic media, in this work it were prepared and functionalized different types of monoliths able to work as anion exchange media and improve the virus purification process.

For the past decade, viruses have been greatly applied in the gene therapy and vaccination fields. With the aim to prevent and slow down the progression of diseases, gene therapy takes advantage of viruses infectious system to act has gene delivery systems carrying the therapeutic genes into cells. The most used gene delivery systems are viral vectors, such as Adenovirus and Retrovirus[3], which application in clinical trials has been increasing, creating a demand for the development of alternative purification techniques that allow the process of larger volumes of vectors with high purity and sterility, while being cost-effective. [27]

Biomolecules such as viruses, plasmid DNA and proteins are characterized by being large size particles with complex molecular surfaces, which limits their purification using traditional methods such as centrifugation and chromatographic techniques. Despite the many advantages of using chromatographic methods, the traditional media used, such as resins or porous beads, owned small pores that when combined with the large size molecules resulted in low mass transfers and subsequently, in long process times.[11] The need to overcome these limitations, led to the development of an alternative range of chromatographic supports with decreased mass transfer resistance, allowing the purification of higher process volumes. One of these alternative supports are monoliths, which have been applied in various types of processes, such as Affinity or Hydrophobic Chromatography, among others. However their major application has been in Ion Exchange Chromatography implemented for the purification of large biomolecules aimed at the gene-based therapies.[11,17]

Monolithic materials are characterized by having a macroporous structure with highly interconnected network of pores that can range different sizes. The large porous structure obtained in these supports offer several advantages to bioseparations. Advantages such as their design that take advantage of a predominant convective mass transport, a low back pressure and improved mass transfer, contribute for higher flow rates and shorter process times. [14,19]

However, to optimize the purification process, the supports should possess several features, such as to have an inert surface that is needed to prevent degradation by impurities [42], to have available groups on the matrix given by the materials if further modification is necessary and to be mechanically stable with a high porosity and interconnectivity. [17] To achieve a good porosity structure, it is crucial to find an optimal balance between the surface area, the porosity and the pore size distribution of the support to be suitable for the target application.[6,11,18] For example, for the purification of large molecules like Adenovirus, which have an 90-100 nm structure, the monoliths should retain a good macroporous structure with a pore average range of interest between 1000 and 5000 nm (Table 1.2). However, we have to take into account that when the monolithic structure is constituted by large pores, less surface area will be available, and consequently will result in a lower binding capacity.

Therefore it is essential to try to obtain an equilibrium between these features to achieve a high purification yield. So far, several successfully purification processes of viruses and other large biomolecules using monoliths have been reported, with recovery yields ranged from 50% to 90%. [16,39]

The strategy followed in this work was the preparation and functionalization of different chitosan-based monoliths blended with synthetic polymers to work as anion exchange media to improve Ad5 purification. To obtain the correct macroporous structure for this process, we must be aware of the several factors during monoliths production that will affect their structure and subsequently their pore size distribution. These factors include the materials to be used and their ratio, the preparation methods, the temperature, the concentration of the casting solution and the yield and method of functionalization applied to the supports. However, it is also important to improve the purification process attempting to make it a more sustainable and environmental friendly process.

The first issue when preparing monoliths is the materials selection. Firstly, we used information from a previous work[50] which consisted of testing different types of native monoliths with different castings for Ad5 purification. As the monoliths were not functionalized, the ideal supports were the ones that resulted in a lower binding (%) and a higher recovery (%) of this virus. Among the several castings tested, the better results were obtained by the chitosan-based monoliths blended with poly(vinyl alcohol) (PVA) and glycidylmethacrylate (GMA), prepared by freeze-drying, resulted in a recovery of approximately 80% of the Ad5 load sample.

Table 3.1 - Recovery values of adenovirus vectors, using chitosan-based monoliths as chromatographic media. These results are from a reported work, where Chitosan/Poly(vinyl alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA) and CHT/PVA blended with magnetic nanoparticles (Mg) monoliths were prepared by freeze-drying, resulting in an average of 80% of the virus sample. [50]

Casting Solutions	Preparation Method	$T_{Freezing}$ (ºC)	Virus Recovery (%)
CHT/PVA(50:50)	Freeze Drying	-80	79±2
CHT/GMA	Freeze Drying	-80	84
CHT/PVA(50:50) Mg	Freeze Drying	-80	81±7

As was already discussed before (see Introduction, section 1.2), chitosan-based monoliths have been receiving increasing attention in the past few years due to the benefits achieved while being implemented.[57] Chitosan (CHT) is a biopolymer obtained from chitin, a biodegradable polysaccharide extracted from crustacean shells. Besides being abundant in nature, they are also characterized by owning positive attributes such as being biocompatible, biodegradable and non-toxic. Another important advantage of using this biomaterial is the obtained structure that offers several possibilities for mechanical and chemical modifications.[46] However, the addition of other polymers to the material composition can result in supports with more suitable properties for each target application, such as mechanical stability and a more adequate porous structure. The selected monoliths casting is

constituted by polymer blends, with the addition of PVA or GMA to the chitosan. PVA is a synthetic polymer with good physical and chemical properties with many applications in the biomedical industry. Besides being a non-toxic and biocompatible material, it is also a hydrophilic polymer with a good film-forming ability.[57] GMA is a monomer commonly used due to the presence of epoxy groups on its structure. When co-polymerized with chitosan it will also increase the tensile strength of the monolith. [23,54] This is an important factor to consider since the monoliths porous structure can be highly sensitive and be damaged during experimental assays.[24,31]

During the castings preparation, the polymerization occurred by heat, in which it was also added to the polymeric mixture a crosslinking agent, MBA (N,N-methylenebisacrylamide), a catalyst (APS) and an initiator (TEMED). The addiction of the initiator and the catalyst will initiate the reticulation, opening the epoxy groups that are present and the monomer MBA will promote the network formation with a higher crosslinking of the polymers. However, monoliths with no MBA in the casting solution were also produced. The schematic representation of the chemical composition of each casting solution is in Figure 3.1 along with the final porous structure that was obtained in a previous work [54] using the same type of castings, which is expected to be obtained in the produced monoliths.

The monoliths represented in the SEM images were previously prepared by a freeze-drying method, a highly reported method [58] that is going to be applied for the virus-aimed monoliths. Lyophilisation or freeze-drying consists of a dehydration process where the water present in the material is removed by sublimation, involving a pre-congelation of the material (<0°C) followed by a drying step. During this process, the frozen material will pass through a vacuum chamber, where the temperature will be increased gradually and the surrounding pressure will be decreasing, allowing the frozen water to pass directly into the gas state without passing through the liquid state. This method has the advantage to make less damage to the materials when compared to other procedures, while operating at a lower temperature. The vacuum will result in a faster sublimation, resulting in an unchanged structure of the material. Although it is a friendly-environment process, has the drawback of being a longer process when compared to other methods. [59]

In general, freezing and lyophilisation results in monoliths with an open microstructure with high interconnectivity. As it can be observed by the SEM images in Figure 3.1, the difference of the polymers blend in each casting results in two different final porous structures, being expected to obtain more spherical pores in the CHT/PVA monoliths, while with CHT/GMA casting it is expected more larger and elongated pores due to the higher viscosity of the casting solution. Similarly to the monoliths produced, the monoliths from the SEM images were frozen at -80°C before drying, to obtain an average pore size diameter between 20 and 100 μm.[54] The porous structure is mainly defined by the solidification rate of the crystals. While implementing a lower freezing temperature, the gelation process will be faster and smaller pores will be obtained. [58]

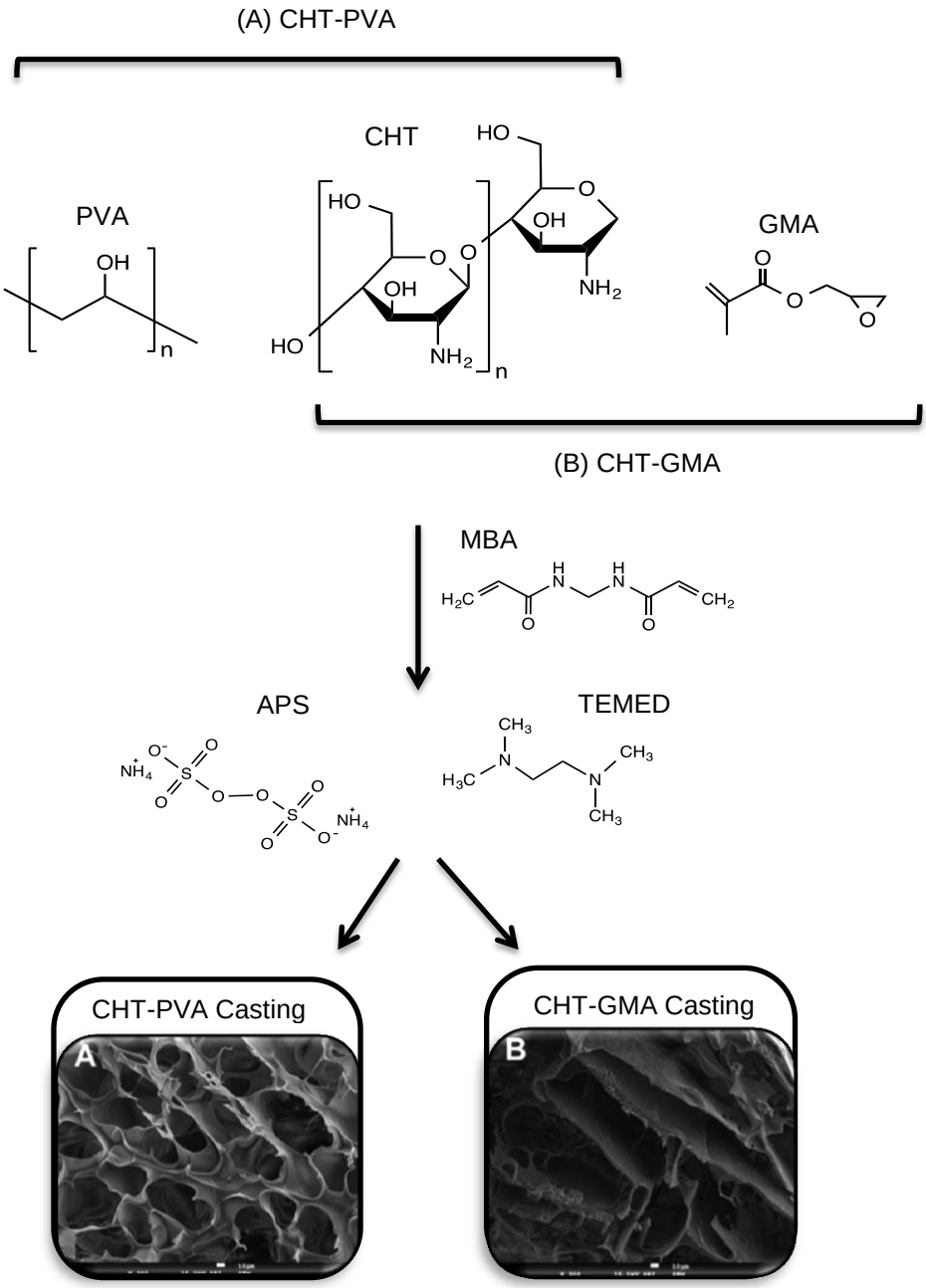

Figure 3.1 – Schematic representation of the polymerization mixture that composes each casting solution for the native chitosan-based monoliths produced. A mixture of polymers blends, Chitosan/PVA (A) or Chitosan/GMA (B), constituted each mixture with the addition of a cross-linker (MBA), an initiator (APS) and a catalyst (TEMED). As can be observed by the SEM images, the variation of the polymers components in the solution resulted in two distinctive types of macroporous structures. The images were adapted from a previous work from which we based our monolith production strategy; to leading to structures with an average pore size distribution of 53±5 μm and 123±5 μm for the native CHT/PVA monolith and CHT/GMA monolith, respectively. [54]

However, there are several other techniques that can be used for the preparation of polymeric porous structures, among which $scCO_2$ - assisted drying methods have been increasingly used in the last decades. [23,53] Due to their improvement in polymer synthesis replacing the use of organic solvents during polymerization, this greener process also improves the stability of the supports and gives the possibility of adjusting the pore size distribution to the desired application. [23,24,31,52].

Carbon dioxide has the advantage of being inexpensive, nontoxic and non-flammable. Due to its relative low critical temperature and pressure (T_C =31°C and P_C=73.8 bar) and positive properties described, it is the most common solvent used as a supercritical fluid. Combining the low viscosity of a gas and the high density of a liquid, CO_2 makes the separation of the solvent a very efficient and simple process, leaving no solvent remains in the polymer structure. [23,31,52] For the monoliths preparation, the casting solutions were frozen in specific containers at a higher freezing temperature (-20°C), followed by the drying step using CO_2. However, this solvent has a low affinity to water (that is included in the castings), making the drying process of the support impossible, being crucial to implement a water substitution to prevent the collapse of the monolith, to a solvent to which CO_2 has high affinity like acetone, that was the one implemented.[23] Another key factor of the formation of the monoliths is the depressurization time of the CO_2 from the system in the end of the process that is used to control the final porous structure obtained by ranging different times. When shorter times are implemented the monolith's structure will be "destroyed" and bigger pores will be obtained. [53]

The monoliths prepared by this method are characterized by resulting in smaller spherical structures. It has been reported the preparation of chitosan-based monoliths in which it was obtained porous structures with an average pore size between 0.5 and 20 μm and a porosity of 40%, approximately. Despite the formation of smaller pores, the average pore size is within our interest size range, thus chitosan-based monoliths for Ad5 purification, are also going to be prepared by this method [49]. The CHT/PVA monoliths were prepared using the same polymerization mixture composition previously described, except that these are going to be more diluted in the casting solution with concentrations of 3% and 7%. This way the preparation of monoliths in the same molds but with lower concentrations of casting solution will result in structures with bigger pores more suitable for viruses processing. This factor combined with the subsequently gelation process at - 20°C result in bigger pores due to the slow congelation, resulting in a larger macroporous structure suitable for larger biomolecules. At the end, a depressurization time of 4/5 min was implemented, to assure that dried monoliths were obtained with no damage. To the best of our knowledge, neither one of the monoliths produced with these diluted castings have been previously tested.

The monoliths produced were evaluated for the purification of Ad5 samples. The results will be discussed further ahead. Despite the production of monoliths with magnetic particles prepared by $scCO_2$ - assisted drying process, these monoliths were not tested for Ad5 purification.

3.2 Functionalized chitosan-based monoliths

Virus particles own several properties from which we can benefit to improve their separation and purification, such as their size and charge. [14] Taking advantage of these characteristics we can functionalize the support surface to have a good selective binding to the virus, transforming the support into an ion exchange chromatography (IEC) media. IEC is the most common technique used for the purification of viruses.[60]

The functionalization of supports such as monoliths or membranes can be made with several types of ligands, depending on the application of the support and properties of the protein that we want to

separate [61,62]. Since the aim is to purify Ad5, which is a negatively charged virus, an anion-exchange chromatography (AEC) will be performed using quaternary amine ligands (Q). This type of ligand transforms the polymeric supports into positive charged monoliths retaining the anions from the negatively charged virus. This type of ligand is characterized by being a strong anion exchanger, which designation doesn't refer to the strength with which the functional groups bind to the proteins but to the capacity of remaining fully charged over a broad pH range, having no variation in the ion exchange capacity of the support, which results in a faster and easier development of separation processes.

The required functionalization for the supports is introduced by taking advantage of the several functional groups that may be present on the monoliths structure. With the polymeric castings composed by CHT, PVA and GMA, two types of reactive groups will be present and available at their surface, hydroxyl (-OH) and amine (-NH_2) groups. Therefore, three different methodologies already reported are going to be applied to immobilize two different Q ligands to their surface. [54,52,55]

The first method, the epoxyactivation technique (M1), consists on an epoxy activation of the monoliths. Taking advantage of the hydroxyl groups available, epoxy groups will be introduced onto the monolithic matrix for further amination with the ligand (2-aminoethyl)trimethylammonium chloride (NH_2Q). The final functionalized structure of the monoliths obtained by this method is represented in Figure 3.2, which reaction scheme can be found in section 2.3.1, Figure 2.5.

The epoxy activation densities for each monolith functionalized is represented in Figure 3.3. The results obtained were significantly higher compared to the ones reported in the literature [54]**,** especially the monoliths CHT/PVA (50:50) prepared by $scCO_2$-assisted drying process, which had 3 or 4 times the epoxyactivation yield obtained by the monoliths prepared by freeze-drying. These results are a consequence of the type of porous structure obtained in each monolith. For monoliths prepared by freeze-drying, which own a large porous structure, the higher pore size distribution and porosity will result in the decrease of surface area available for immobilization, leading to lower ligand densities. In contrast, the monoliths prepared by $scCO_2$ - assisted drying process will have a smaller porous structure with a narrower pore size distribution that will increase the surface area available, and consequently enhance the immobilization yield.

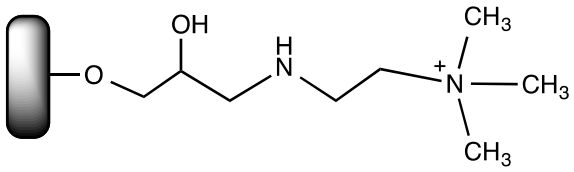

Figure 3.2 – Schematic representation of the final chemical surface of functionalized chitosan-based monolith, which immobilization was implemented using the epoxyactivation technique with NH_2-Q ligand coupling.

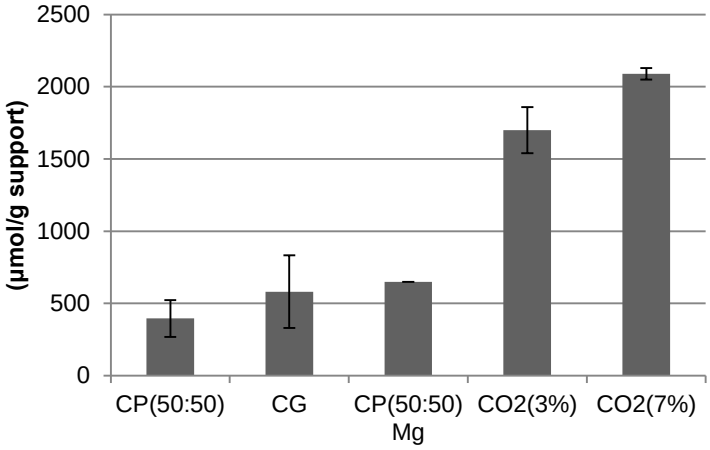

Figure 3.3 – Epoxyactivation average values, and respective standard deviation values, obtained for the several immobilizations implemented (n) on monoliths functionalized through method 1, the epoxyactivation technique. Among the functionalized monoliths are: CHT/PVA(50:50)(n=3), CG(n=3), and CHT/PVA(50:50) Mg (n=2), monoliths prepared by freeze-drying and CHT/PVA(50:50) 3% and 7% (CO_2(3%) e (7%)) (n=2 each), monolith prepared scCO_2 - assisted drying process.

The second method, the alternative plasma technique (M2), consists of introducing radicals onto the support surface, through Argon-plasma treatment for subsequent amination with 1,6-hexanediamine. The amination will introduce amine groups to the monolithic surface where glycidyltrimethylammonium chloride (GQ ligand) is going to be coupled. One advantage of using Q ligands is that they are soluble in water, not needing to add any organic solvents during the ligands immobilizations. The final functionalized structure of the monoliths obtained by this method is represented in Figure 3.4, which reaction scheme can be found in section 2.3.2, Figure 2.7.

The amination yields obtained by plasma treatment were significantly lower for all chitosan-based monoliths (Figure 3.5) when compared to reported results.[54] However, these results may be due to the large porous structure obtained within the preparation of the monoliths. With a higher pore size distribution and porosity, the surface area available for immobilization decreases, leading to lower ligand densities. Another fact that may affect the immobilization yield is the heating of the system. Since the amine has a high vapor enthalpy (ΔH_{vap}=49,3 kJ/mol), it will result in a low vapor pressure at 120 ºC, that when a insufficient heating is applied it will result in a low vaporization of the 1,6-hexanediamine, leading to a low amination efficiency and subsequently, a low ligand immobilization yield.[63]

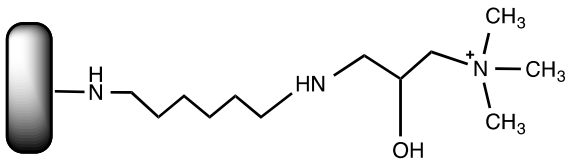

Figure 3.4 – Schematic representation of the final chemical surface of functionalized chitosan-based monolith, which immobilization was implemented using the alternative plasma technique with GQ ligand coupling.

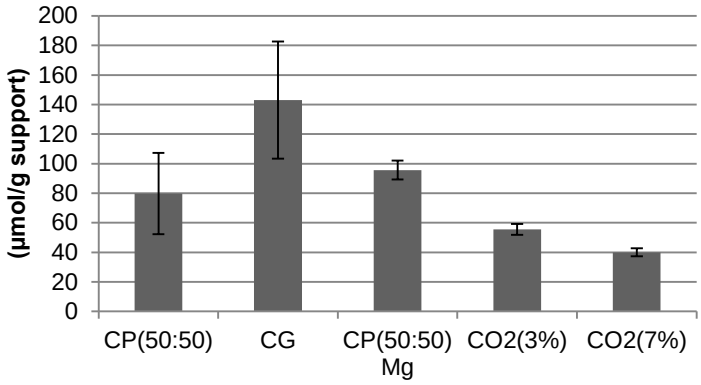

Figure 3.5 – Amination average values, and respective standard deviation values, obtained for the several immobilizations implemented (n) on monoliths functionalized through method 2, the Alternative Plasma Technique. Among the functionalized monoliths are: CHT/PVA(50:50)(n=4), CG(n=5), and CHT/PVA(50:50) Magnetic (n=2), monoliths prepared by freeze-drying and CHT/PVA(50:50) 3% and 7% (CO_2(3%) e (7%)) (n=2 each), monolith prepared by scCO_2 - assisted drying process.

The third and last method (M3) consisted of introducing the ligands directly during casting preparation and agitation. This method was based on a reported work [55] with some modifications, since the type of cross-linker used was different, as we added MBA instead of Glutaraldehyde to our castings. This method has the advantage of combining the casting preparation and immobilization into one single step, not needing any further modifications after polymerization and preparation by freeze-drying.

To estimate the amount of each ligand to be added to the castings, a Kaiser Test was performed, has already mentioned in section 2.3.2. The Table 3.2 indicates the amination values obtained with the test for the monoliths only composed by CHT/PVA or CHT/GMA; these values correspond to the quantity of ligand added to the casting solutions of the monoliths, in assays where the immobilization was implemented with no ligand in excess. Subsequently, the immobilizations were also performed with ligand added in excess, adding 5 times the values represented.

To our knowledge, this type of monoliths has never been previously tested.

Table 3.2 – Amination extent obtained through Kaiser Test, which corresponds to the quantity of ligand added during functionalization through method 3, the direct immobilization technique.

Casting	Amination extent (µmol/g support)
CHT/PVA (50:50)	279
CHT/GMA	185,4

Another approach for the monoliths production was the implementation of magnetic properties onto the supports. With the incorporation of magnetic nanoparticles in the monoliths, the separation or purification process is enhanced due to the presence of a magnetic field in some stages of the

process. This tactic has been already reported[49,50], in which resulted in a good improvement for the respective purposes of each work. The preparation of the magnetic castings solutions followed the same procedures, as well the preparation and functionalization techniques applied to monolithic structures as described, except with the addition of magnetic nanoparticles in the casting solutions.

All the functionalized monoliths produced were tested for the purification of Ad5 samples. The results will be discussed further ahead.

The ligand immobilization yields were performed only for the two polymeric monoliths that showed a better performance, CHT/PVA(50:50) prepared by freeze-drying and CHT/PVA(50:50)(7%) prepared by $scCO_2$ - assisted drying process. The ligand quantification was performed by a precipitation titration with $AgNO_3$ (0.1 M). The $AgNO_3$ is added until a sudden change of pH is observed, indicating the precipitation of Cl^- that was present in the monolithic samples. The respective titration volumes added to each monolith can be seen in Appendix 4 (Table 6.4 to Table 6.7). The following table (Table 3.3) shows the yields obtained for each immobilization (the epoxyactivation (M1) and the plasma alternative techniques (M2)), and the respective ligand densities obtained through the titration. Approximately 40% of the ligand was successfully coupled to each support, with the CHT/PVA(50:50) 7% casting achieving the highest immobilization yield, with a 91% of ligand coupled. This result is coherent with the expected porous structure, which has a higher surface area available to ligand immobilizations. Native monoliths were also tested. As expected, since the monoliths were not functionalized, no precipitate was observed during the titrations.

Table 3.3 – Immobilization yields and ligand densities for native CHT/PVA(50:50) monoliths prepared by freeze-drying and native CHT/PVA(50:50) (7%) monoliths prepared by $scCO_2$ - assisted drying process, and the respective functionalized through the two immobilization methods implemented, the epoxyactivation (M1) and the plasma alternative techniques (M2).

Casting	Ligand Immobilized	Activations yields (µmol/g support)	Immobilization yields (µmol/g support)	Ligand Immobilized (%)
CHT/PVA(50:50) N	-	-	No precipitate	-
CHT/PVA(50:50) M1	NH_2Q	530	200	37
CHT/PVA(50:50) M2	GQ	58	20	48
CHT/PVA(50:50) (7%) N	-	-	No precipitate	-
CHT/PVA(50:50) (7%) M1	NH_2Q	2850	2600	91
CHT/PVA(50:50) (7%) M2	GQ	40,50	20	51

3.3 Monolithic supports applied for Ad5 purification

Once the monoliths were prepared and functionalized, they were applied as chromatographic media for Ad5 purification.

Ad5 is one example of an Adenovirus that has been greatly applied within the gene therapy and vaccination fields, due to the high efficiency transduction and high level of expression obtained. It is essential to improve their purification to try to respond to the sudden demands created by their increasing application. Our strategy is to improve the Ad5 purification using the produced chitosan-based monoliths as supports. The monoliths were prepared and functionalized using several methodologies, which were tested to verify which type of support leads to a better purification of Ad5.

Firstly, as a control, monoliths with no functionalization were tested. Since, native monoliths are not functionalized, a full recovery of Ad5 is expected, meaning the virus does not bind to the monoliths surface. The results obtained for Ad5 recovery (%) using the monoliths produced, are represented in Figure 3.6. Two types of monoliths showed a good recovery for the viruses (100%), although this can be related to the fact that the casting solutions of these monoliths didn't contained MBA, a cross linker that promotes chain growth and network formation.[64] Unexpectedly, all the others monoliths showed a low recovery for Ad5 when compared to a previous reported results (80%), among which the CHT/PVA(50:50) (7%) demonstrated the best Ad5 recovery, of 50%. These results led to believe that the presence of MBA promoted the network formation with a high crosslinking of the polymers, resulting in the possibility of virus to be retained within the network.

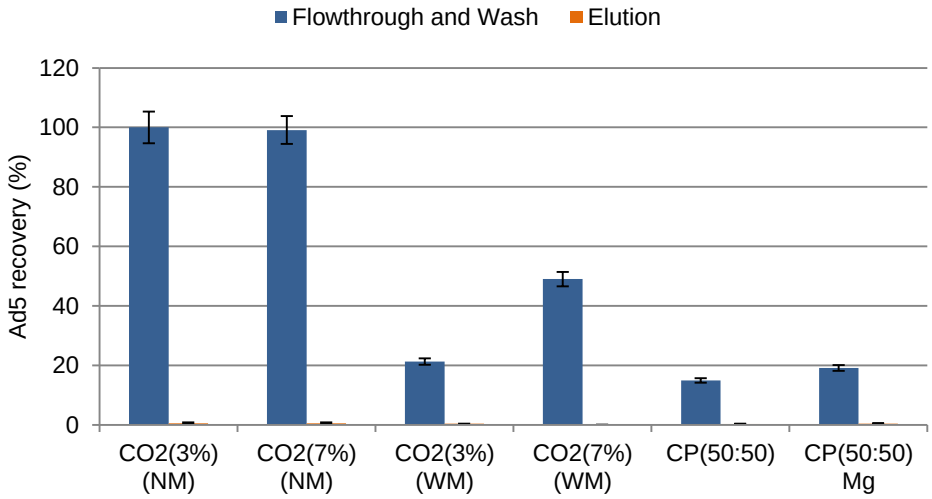

Figure 3.6 - Comparison of the results for Ad5 sample purification (1E11 TP/mL) using several types of native monoliths, expressing the percentage of virus recovered in the flowthrough and elution stages. The monoliths tested differ in some properties such as: the castings, CHT/PVA (CP) prepared by freeze-drying, CO_2 (3%) and (7%) which have a CHT/PVA casting but were prepared by $scCO_2$ - assisted drying process and the casting solutions that do not contain MBA (NM) and with MBA (WM).

Subsequently, the functionalized monoliths were tested for Ad5 purification. The results obtained for Ad5 recovery (%) using the several produced monoliths are represented in Figure 3.7, Figure 3.8 and Figure 3.9, which shows the comparison between monoliths made of CHT/PVA(50:50) casting, with

the CHT/GMA and magnetics casting (Mg), monoliths prepared by freeze-drying and prepared using $scCO_2$, respectively.

Since the monoliths are functionalized, it is expected the binding of Ad5 to the positively charged monolith, and later the unbinding when an elution buffer is applied.

During the monoliths functionalization, it is important to try to enhance the coupling of the ligand onto the monolith matrix; therefore during the immobilization process it is crucial the addition of ligand in excess (5 eq. to activation content) to make sure that the immobilization occurs. However, monoliths with no ligand in excess (1 eq. to activation content) were produced and also tested. As can be observed in Figure 3.7, the addition of the ligand is necessary to obtain an improved purification of the virus, as can be confirmed by comparing the performance of the CHT/PVA monoliths prepared by freeze-drying and functionalized by the three different methods (M1, M2, M3). The best example is the CHT/PVA(50:50) monolith, which functionalization was implemented through the alternative plasma technique with GQ ligand coupling (M2), in which the addition of ligand in excess led to an increased recovery of Ad5 in elution, from 3.7% to a 40%.

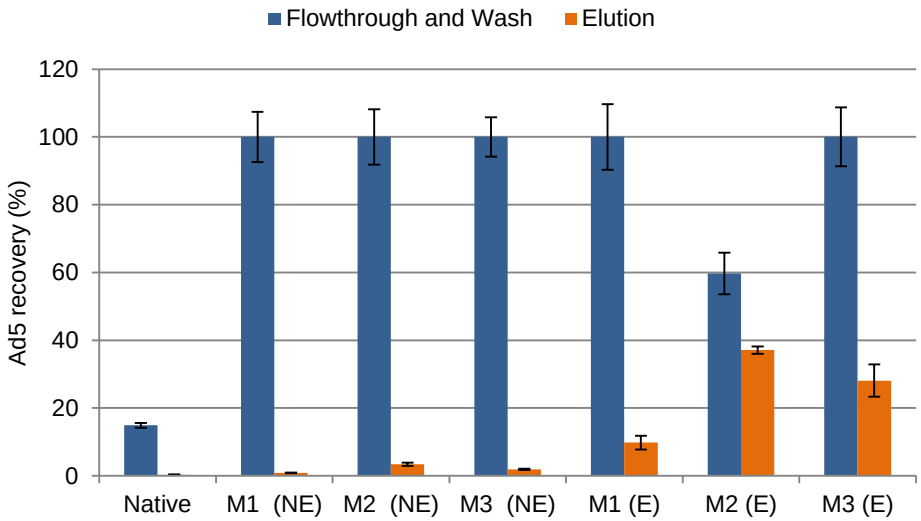

Figure 3.7 – Recovery results for Ad5 purification (1E11TP/mL) using the native and functionalized CHT/PVA(50:50) monoliths prepared by freeze-drying, expressing the percentage of virus recovered in flowthrough and elution stages. Three methods were implemented for their functionalization (M1-Epoxyativaction technique, M2-Alternative Plasma technique, M3-Direct Immobilization technique), within which the ligand immobilizations were made with no ligand in excess (NE) and with ligand in excess (E).

In addition, within the CHT/PVA monoliths prepared by $scCO_2$ - assisted drying process as can be observed in Figure 3.8, the presence of MBA in the casting also led to an increase of the % of Ad5 recovered, among which the monoliths with a 7% concentration functionalized by the epoxyactivation technique (M1) obtained a higher elution recovery of 14%. Although there was a slight recovery of Ad5 in the flowthrough, meaning that didn't bind, it was this type of monolithic casting who presented a smaller % of Ad5 during this stage.

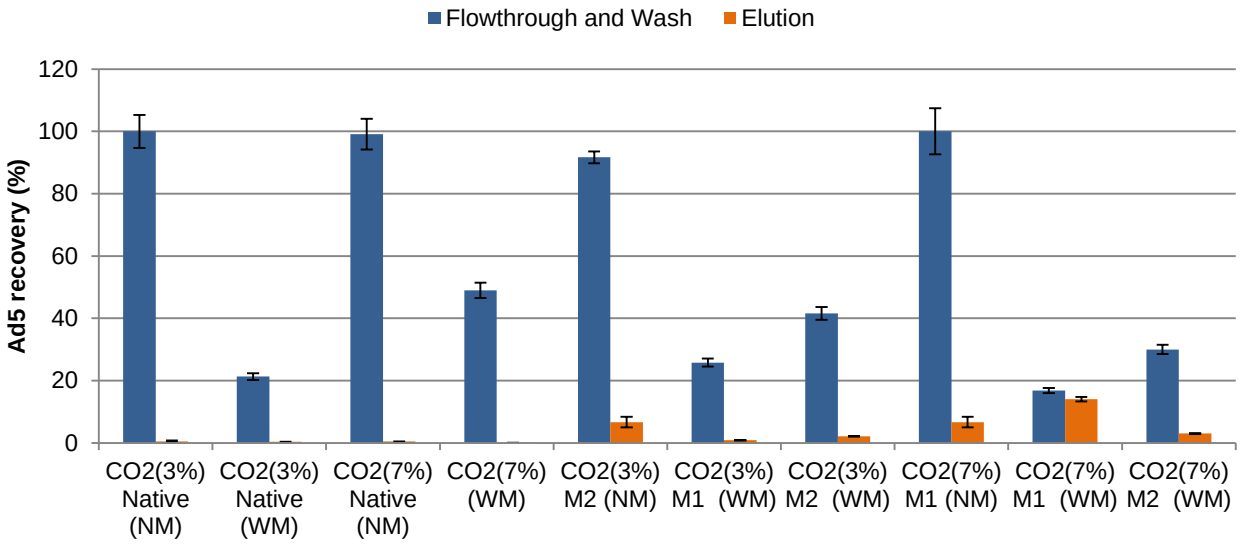

Figure 3.8 – Recovery results for Ad5 purification (1E11TP/mL) using the native and functionalized CHT/PVA(50:50) monoliths prepared by $scCO_2$ - assisted drying process, expressing the percentage of virus recovered in flowthrough and elution stages. Two methods were implemented for their functionalization (M1-Epoxyativaction technique, M2-Alternative Plasma technique), within which the casting solutions contain MBA (WM) or do not contain MBA (NM).

Then, the monoliths that showed a better performance for the Ad5 purification, from the CP(50:50) monoliths prepared by freeze-drying and $scCO_2$, were compared to native and functionalized CHT/GMA monoliths and the monoliths blended with magnetic particles (Mg) (Figure 3.9). The CHT/GMA immobilization was also implemented with no ligand in excess followed by immobilization with ligand in excess. In contrast with the previous monoliths, the CHT/GMA monolithic supports did not show an improved performance, recovering only 3.6% of the Ad5 sample. In turn, the magnetic monoliths also didn't show a good performance for Ad5 purification as expected. Despite showing some binding to the virus, it didn't elute. This could be due to the use of a weak magnetic force during the elution stage. While using the magnetic monoliths, no leaching of MNP's was observed during their application as chromatographic media.

Furthermore, the monoliths functionalized with the direct immobilization of the ligand (M3), of both castings (Figure 3.7 and Figure 3.9) did not show a suitable performance to be applied for virus purification. In addition to the low binding of virus, all the monoliths prepared showed a very stiff structure with which was not possible to obtain a good gravitational flow of the sample applied, even when higher pressure was applied.

In Appendixes, Figure 6.2, it can be observed the comparison of the Ad5 purification performance for all native and functionalized monoliths produced.

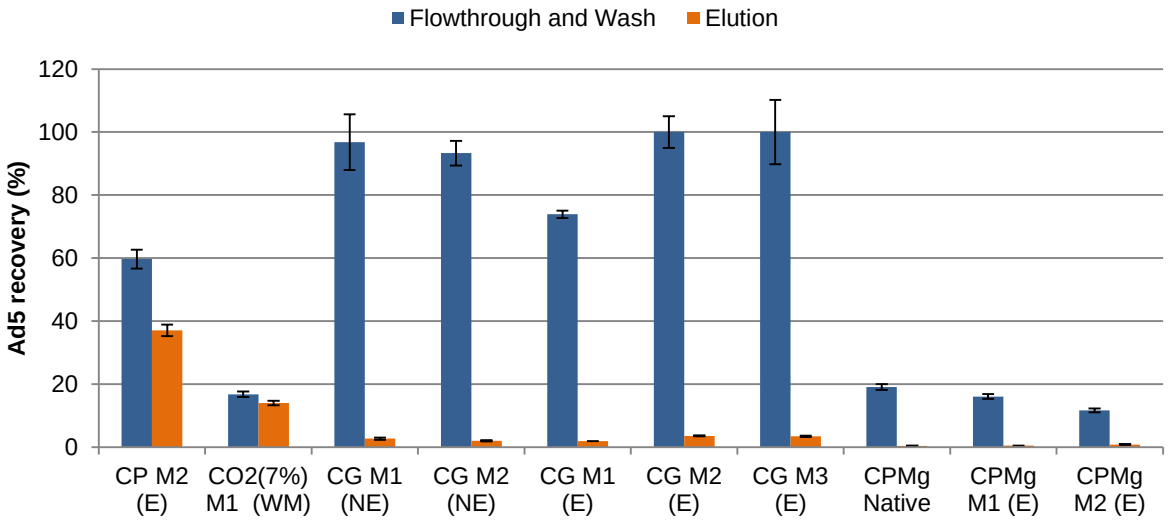

Figure 3.9 – Comparison of the monoliths that had a better performance purifying Ad5 (1E11 TP/mL) with the CHT/PVA (CP) casting prepared by freeze-drying and $scCO_2$ - assisted drying process, with the native and functionalized monoliths with a CHT-GMA casting (CG) monoliths and CP blended with magnetic particles (CPMg). The monoliths tested differ in some properties, such as being functionalized by different techniques (M1-Epoxyactivation technique, M2-Alternative Plasma technique, M3-Direct Immobilization technique), within which the immobilizations were made with no ligand in excess (NE), with ligand in excess (E).

Considering the expected structure and the immobilization yields obtained by the techniques applied (Table 3.3), the results obtained in the Ad5 purification process are consistent. The monoliths which showed a better performance for the Ad5 process purification, were the monoliths CHT/PVA(50:50) functionalized by the Alternative Plasma technique (M2) prepared by freeze-drying and CHT/PVA (50:50) monoliths with a 7% concentration functionalized by the epoxyactivation technique (M1) prepared by $scCO_2$. Monoliths prepared by freeze-drying are characterized by obtaining larger porous structure. Despite the lower surface area available that led to a lower immobilization yield through M2 (34%), the porous structure was suited for large molecules such as Ad5. In contrast, monoliths prepared by $scCO_2$ - assisted drying process, are expected to obtain a smaller porous structure, leading to a structure with a higher surface area available for ligand immobilization, which is consisting with the immobilization yield obtained through M1 (91%), resulting in a good support to be applied for virus purification.

Another fact to consider is the available groups introduced through each functionalization to the monoliths surface. Since the ligand immobilizations were not 100% effective, there will be epoxy and amine groups available and there may occur nonspecific interactions between these available groups and the impurities present in the sample to purify, which can contribute to a lower purification yield.

3.4 Protein Binding Tests

Apart from the Ad5 purification tests, it was also implemented a proof of concept to evaluate the capacity of the functionalized monoliths to work as anion exchange chromatographic media for purification processes. Among the several monoliths produced, only the two types that revealed a

higher binding to Adenovirus particles were selected for to the protein binding tests, the CHT/PVA (50:50) and the monoliths with a 7% concentration, prepared by freeze-drying and $scCO_2$ - assisted drying process, respectively. However, to analyse the properties of the magnetic monoliths, the CHT/PVA (50:50) magnetic monolith was also tested.

The protein binding assays were performed with a mixture of proteins, consisting of Lysozyme and BSA (Bovine Serum Albumin). Lysozyme is a protein obtained mostly from chicken egg white, while BSA is obtained from cows. These proteins are highly commercialized having numerous biochemical applications and several assays for their purification have already been reported. [21,30]

The proteins were selected to understand if the functionalized monoliths could behave as ligand exchangers. As they present different isoelectric points, they were used as model proteins. The isoelectric point (pI) corresponds to the pH at which a molecule or surface carries no electrical charge, and depending on the surrounding pH, the net surface charge of proteins can change. [65] Ad5 purification was performed with a pH8 medium, and the same condition was applied for the protein-binding tests. When the solution pH is higher than the isoelectric point of BSA (pI = 4.7), it will have a negative charge, contrarily at pH lower than pI the protein charge will be positive. This is an example of a good protein for an anion exchange chromatography, in which the negative charge of the protein will bind to the positive charged surface of the monoliths functionalized with Q ligands. Lysozyme (pI =11.35) in contrast with BSA, when present within a solution with a pH 8, it will be positively charged and will not bind to the positively charged surface of the monoliths. Since, our medium will have exactly a pH 8, with a mixture of these two proteins we will expect to verify the BSA binding to the support and the flow through of the lysozyme.

Despite the results of Ad5 purification indicate a better performance for a specific method for each type of casting, the monoliths tested for these assays were functionalized by the two immobilization methods previously discussed, the epoxyactivation technique with the NH_2-Q ligand and the alternative plasma technique with the GQ ligand.

Several steps, such as regeneration, equilibration, loading, wash and elution, composed the protein-binding assays. Due to the charged properties of the proteins used, it is expected that the BSA bonded during loading stage will unbind during elution due to the increased ionic strength of a buffer using NaCl. All the samples of each stage were collected and the samples absorbance was measured at 280 nm a wavelength. Additionally, a second approach to the protein-binding assay was also attempted aiming to optimize the process and try to obtain a higher binding of the protein. Among the modifications, the protein was incubated overnight at 4°C, in order to enhance the contact of the protein mixture with the positively charged support. Then, to guarantee the complete flowthrough of the lysozyme, the number of washes was increased, followed by the addition of a second elution buffer with a higher ionic strength, in elution stage, to assure the elution of the BSA that might still be bonded to the monolithic support. The graphical chromatographic assays obtained implementing the two approaches for the monoliths CHT/PVA(50:50) , can be observed in Appendix 5, Figure 6.3 to Figure 6.7.

However, to quantify the total protein bonded and eluted, it was assessed a colometric BCA assay, which samples absorbance were measured at 560 nm wavelength. With the values obtained, it was

possible to determine the percentage (%) of protein present in the flowthrough, washes and elution steps compared to the protein quantity present in load sample, which are represented in Table 3.4. Since the protein mixture was composed by BSA and lysozyme with a concentration of 1 mg/mL (0.5 mg/mL of each protein), and the loading was performed within a pH8, it was expected the binding of 50% of the protein mixture corresponding to the BSA ratio.

Initially, the native CP(50:50) monolith showed an abnormal binding. Since native monoliths are not functionalized, it is not expected any binding to proteins. Despite firstly showing some binding, when implemented the modifications related to the second approach, a lower binding was obtained for this kind of monoliths. The functionalized monoliths initially showed a lower binding than expected, however with the optimization of the process, higher binding were obtained for all chitosan-based monoliths, approximately 50% as it was expected. In Table 3.4 are also represented the binding capacities of the tested monoliths while implementing the two different approaches. As can be observed, the 2nd approach resulted in an optimization of the process, where was obtained higher protein binding capacities, with an increase of protein binding.

Table 3.4 – Protein binding results obtained through the colometric BCA assay, expressing the percentage of recovered (%) and the total protein binding capacities of using conventional and magnetic CHT/PVA (50:50) monoliths prepared by freeze-drying and CHT/PVA (50:50) (7%) monoliths prepared by $scCO_2$-assited drying process. The tests were implemented for native and functionalized monoliths using two techniques (M1-Epoxyactivation technique, M2-Alternative Plasma technique).

Casting Solutions		**Protein Binding (%)**		**Protein binding capacity (mg/g support)**	
		1st Approach	**2nd Approach**	**1st Approach**	**2nd Approach**
CHT/PVA (50:50)	Native	70	36	58	17
	M1	41	56	36	27
	M2	0	39	0	21
CHT/PVA (50:50) Mg	Native	36	42	29	43
	M1	-	56	-	33
	M2	0	54	0	45
CHT/PVA (50:50) (7%)	Native	-	50	-	17
	M1	-	39	-	24
	M2	-	57	-	26

For a further evaluation of the partition of the proteins mixture obtained in the several steps of the protein tests, it was performed an SDS-Page analysis, which consists of evaluating the mobility of the proteins in a 12.5% acrylamide gel that is submitted to an electric current. However, to accomplish this assessment it is necessary the usage of a staining solution to highlight the lines and a low molecular weight protein marker (Mk) to compare the obtained protein bands. This analysis was made using a Coomassie solution and the samples of the Load (L), Flowthrough (FT), Wash (W) and Elution (El) stages previously obtained in the protein-binding tests.

Initially, this analysis was implemented for the conventional CHT/PVA (50:50) monoliths and for the CHT/PVA (50:50) magnetic (Mg) monoliths, prepared by freeze-drying. In **Figure 3.10** are represented the gels obtained using both native and functionalized monoliths with GQ ligand, functionalized by the

alternative plasma technique, of each type of monolith. Two main lines can be observed in the gels, the top line corresponding to the BSA (MW= 66,5 kDa) and the lower line to the lysozyme (MW = 14,3 kDa). Through this analysis it is possible to see the partition of both proteins, as can be observed (A and B); both proteins are present in the flowthrough and first washes. This was as expected since the supports were not functionalized. However, comparing the two types of casting, we can conclude the CHT/PVA (50:50) Mg had a better performance since it can be observed more highlighted lines of both proteins in the different stages. These results are no in concordance to the ones obtained by BCA test, however colometric assays have associated errors that may influence the results.

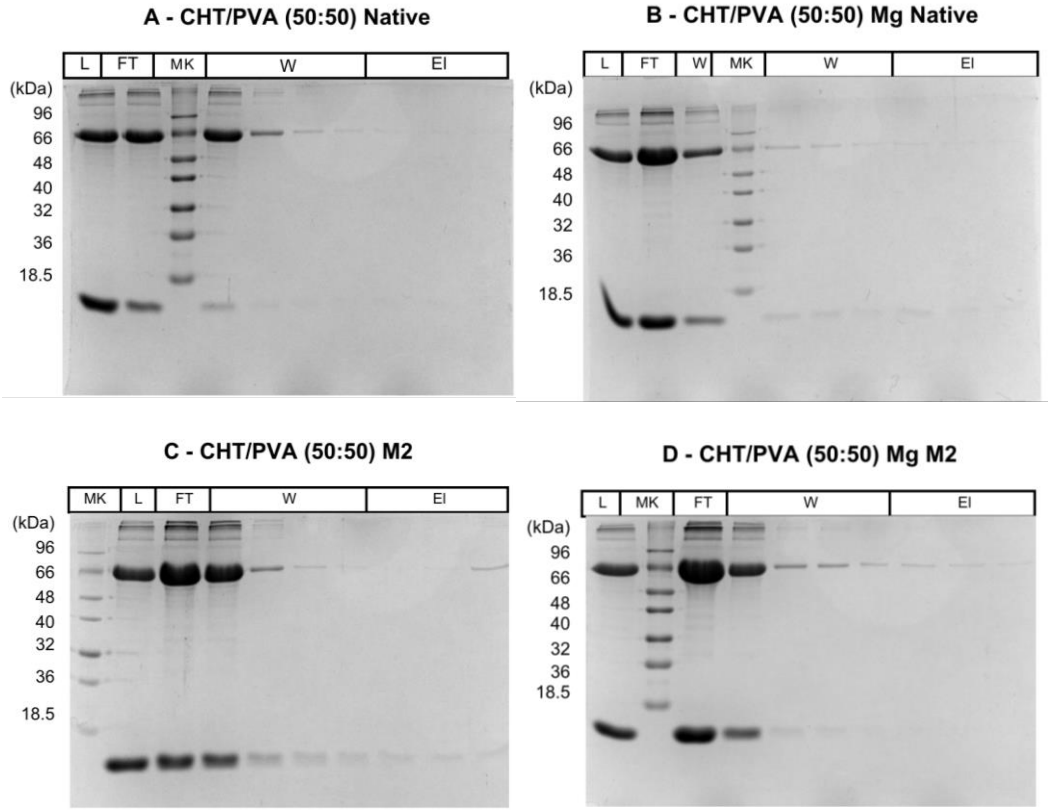

Figure 3.10 – Staining gels obtained by SDS-Page analysis of the protein binding results using as chromatographic media: conventional and magnetic CHT/PVA (50:50) monoliths (A and B) and functionalized conventional and magnetic CHT/PVA (50:50) monoliths (C and D), by the alternative plasma technique (M2). The protein mixture was composed of BSA (MW= 66,5 kDa) and lysozyme (MW = 14,3 kDa) and the staining was made with a Coomassie solution. The samples tested corresponded to the Load (L), Flowthrough (FT), Wash (W) and Elution (El) stages and were compared to a low molecular weight protein marker (Mk).

In the gels obtained for the functionalized monoliths (C and D), it can be observed some binding of the BSA to the supports since there is a more highlighted wash lines of lysozyme. This is expected due to the positively charged Q ligand present in the monolith surface and the negatively charge of the BSA at pH 8. Despite the evident flowthrough of BSA in both gels, it is visible that some protein did bind to supports, since there is elution of this protein. In return, lysozyme is positively charged at pH 8 and should not be retained by the Q ligand, however it can be observed in both gels a slightly binding to the CHT/PVA (50:50) monolith.

Then, the same analysis was implemented for the samples collected during the 2nd approach assay, in which, in addition to the conventional and to the CHT/PVA (50:50) Mg monoliths, it was also tested the native and functionalized CHT/PVA (50:50) 7% monoliths prepared by $scCO_2$-assisted drying process. However, the gels obtained from these results didn't differ much from the previous tested monoliths as expected. All the chitosan-based native monoliths showed a better performance, resulting in a higher flowthrough of the proteins during washing step. These results support the results obtained by the BCA test, where when applied the 2nd approach for the protein binding tests, a lower percentage of protein mixture bound to the monoliths.

For the functionalized monoliths, the results were also similar to the previous assay, continuing to show a slightly binding to the lysozyme. However, they also show BSA binding to the positively charged support, among which the two functionalized monoliths prepared by $scCO_2$ showed a slightly higher BSA elution. These results confirm the BCA test for this kind of monoliths, showing a higher protein binding percentage.

Despite the BSA binding to the monoliths indicate their potential application as anion-exchange chromatography media, further modifications are necessary to obtain a more suitable and optimized process.

4 Conclusions

Comparing the results obtained in the Ad5 purification with the protein-binding tests, it is visible a consistency between the monoliths performance. Among the several types of monoliths produced and tested, both preparation methods are adequate to prepare monoliths with the necessary large porous structure for virus purification. When native monoliths were tested for Ad5 purification, the CHT/PVA (50:50) prepared by $scCO_2$ – assisted drying process exhibited the performance: the monoliths prepared without crosslinker (no MBA added to the casting solution) showed a 100% flowthrough of the virus, while the crosslinked monoliths, presented 50% of virus in the flowthrough. The addition of MBA (crosslinking agent) promotes a porous structure with smaller average pore size diameter that may lead to the entrapment of the virus within the monolithic network. Then, the monoliths functionalization was implemented by three different methods, among which the epoxyactivation technique showed to be more suitable for virus purification since higher ligand immobilization yields were obtained, and 91% of the ligand was bonded to the supports prepared by $scCO_2$ – assisted drying process. This result can be explained considering the structure of monoliths prepared by this method; as the matrix exhibit smaller average pore size a higher surface area is available for ligand immobilizations, increasing the support performance in chromatographic applications.

After the immobilizations, the functionalized monoliths were also tested for Ad5 purification. Comparing the results obtained for the functionalized monoliths, despite not having high yield performances, two types of monoliths stand out, the CHT/PVA (50:50) monoliths with a 7% concentration functionalized by the epoxyactivation technique (M1) and prepared by $scCO_2$ and the CHT/PVA (50:50) alternative plasma technique prepared by freeze-drying. However, contrary to what was expected with the higher ligand immobilization yield achieved, the CHT/PVA (50:50) 7% monolith resulted in a 17% flowthrough of the virus and a subsequently 14% elution of the bonded viral particles. Since the immobilization yield was high, a higher binding of the virus was expected, however it was not observed the complete elution of the virus what leads us to consider that some improvements in this stage of the process have to be implemented, such as the increase of the ionic strength of the buffer used. In turn, in the CHT/PVA (50:50) monoliths functionalized using the alternative plasma technique and prepared by freeze-drying, it was verified that only 34% of the immobilized ligand actually was bonded to the monoliths. Despite the lower value, this immobilization yield achieved is consistent to the monolith structure expected. As was previously discussed, monoliths prepared by this technique own a larger porous structure and consequently a lower surface area is available for immobilization, explaining the lower binding of the ligand. However, it was obtained a higher Ad5 binding to the monolithic support, despite the 60% in flowthrough, 40% of the virus bonded and eluted.

On the other hand, the monoliths functionalized by the direct immobilization of the ligand (M3) did not show a suitable performance to be applied for virus purification. When used as chromatographic supports, low bindings of the virus were achieved and due to their stiff structure no gravitational flow could be obtained.

As the immobilization of the ligand was not 100% effective, some of the chemical groups, as epoxy and amine groups, introduced to the support during functionalization steps can stay available, to establish nonspecific interactions with the impurities present in the Ad5 sample that was purified. These interactions will also contribute for the decrease of the purification yield.
Subsequently, protein-binding tests were also performed. Despite, the initial non-specific binding that occurred to non-functionalized monoliths, after optimization of the process, the binding to the supports decreased. In turn, for the functionalized monoliths, it was verified the anionic-exchange capacity of the supports, binding approximately 50% of the protein mixture.

The results obtained in this work led us to consider that in addition to the environmental advantages of the implemented strategies used to prepare these smart porous monolithic supports, the combined methods proposed, once optimized will allow the tuning of morphological properties and chromatographic performance of the monoliths for bioseparations purposes. Besides these chitosan-based monoliths being ecologically safe, they have a low cost production with no pressure needed during their implementation. However, further improvement is necessary to try to enhance the virus binding and recovery to obtain an improved purification process. To make further considerations about the monoliths performance it is also essential to evaluate the mechanical and morphological properties of the monoliths produced, to assure that the expected results were obtained and that these supports are suitable to be applied for virus purification, which was not possible to conduct during the duration of this work.
Another factor to consider for the improvement of this process is the blending of magnetic particles in the CHT/PVA (50:50) 7% monolith. Despite the preparation of native CHT/PVA (50:50) 3% monoliths, they were not tested and with the reasonable results for the 7% casting, the introduction of magnetic properties can enhance the performance as a chromatographic support. Also, it may be important the tuning of the quantity of the MBA to be added, in order to obtain a better flow.
In addition, during Plasma treatment, the power of the amination can also be increased to try to enhance the amination of the monoliths. Also, further testing of the monoliths under controlled pressure and flow is also a future goal for testing these types of supports. Since scale-up is an important factor for the development of alternative purification processes, it is necessary to optimize features such as flow-rate, flow of feed solution and the continuous activation of the supports.

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6 Appendixes

6.1 Appendix 1

Table 6.1 - Epoxyactivation values obtained during several assays with the two methods implemented for ligand immobilization (M1- the epoxyactivation technique, M2 - the Alternative Plasma technique), for different types of monoliths. The monoliths were prepared with different castings such as: Chitosan/Poy(vynil alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA) and CHT/PVA with MNP's blending (Mg), which were prepared by freeze-drying; and CHT/PVA casting with two different concentration of polymeric mixture (3% or 7%) were prepared by $scCO_2$ – assisted drying process. These results are represented in Figure 3.3.

Method 1- Epoxyactivation (µmol/g support)					
Castings	**Preparation Method**	**Exp.1**	**Exp.3**	**Exp.3**	**Average ± Std.Dev**
CP(50:50)	Freeze-Drying	225	435	530	400 ± 127
CG	Freeze-Drying	345	470	930	582 ± 252
CP(50:50) Mg	Freeze-Drying	650	-	-	650 ± 0
CP(50:50) 3%	$scCO_2$	1540	1860	-	1700 ± 160
CP(50:50) 7%	$scCO_2$	2130	2050	-	2090 ± 40

Table 6.2 - Amination values obtained during several assays with the two methods implemented for ligand immobilization (M1- the epoxyactivation technique, M2 - the Alternative Plasma technique), for different types of monoliths. The monoliths were prepared with different castings such as: Chitosan/Poy(vynil alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA) and CHT/PVA with MNP's blending (Mg), which were prepared by freeze-drying; and CHT/PVA casting with two different concentration of polymeric mixture (3% or 7%) were prepared by $scCO_2$ – assisted drying process. These results are represented in IFigure 3.5

Method 2 - Plasma Activation NH_2 (µmol/g support)						
Castings	**Preparation Method**	**Exp.1**	**Exp.2**	**Exp.3**	**Exp.4**	**Average ± Std.Dev**
CP(50:50)	Freeze-Drying	120,47	89,4	57,07	52,37	80 ± 27
CG	Freeze-Drying	148,47	92,09	188,58	-	143 ± 40
CP(50:50) Mg	Freeze-Drying	120,47	89,4	-	-	95 ± 6
CP(50:50) 3%	$scCO_2$	51,91	59,32	-	-	55 ± 4
CP(50:50) 7%	$scCO_2$	36,42	40,5	43,24	-	40 ± 3

6.2 Appendix 2

Table 6.3 – List of the native and functionalized monoliths tested for Ad5 sample purification. The monoliths differ in some features such as the castings, Chitosan/Poy(vynil alcohol) (CHT/PVA), Chitosan/Glycidylmethacrylate (CHT/GMA), CHT/PVA with MNP's blending (Mg), the concentration of the polymeric mixture (3% or 7%), if MBA was (WM) or wasn't added (NM) to the casting solution, if ligand was added in excess (Ex) during immobilizations or not (NE), and if were prepared by freeze-drying or $scCO_2$ – assisted drying process. The functionalization was made through different methods (M1 – Epoxyactivation technique, M2 – Alternative plasma technique, M3 – Direct immobilization technique) with two ligands (GQ and NH_2Q). These results are represented in Figure 3.6 to Figure 3.9.

Casting Solutions	Preparation Methods	$T_{Freezing}$ (°C)	Immobilization Methods	% Ad5 Recovered (avergade±SEM)	
				Flow-through and wash	Elution
CHT/PVA (50:50) 3% (NM) Native	$scCO_2$	-20	-	107.0±5.3	0.5±0.3
CHT/PVA (50:50) 3% (NM)	$scCO_2$	-20	M1	-	-
			M2	91.7±1.9	6.7±1.7
CHT/PVA (50:50) 3% (WM) Native	$scCO_2$	-20	-	21.3±1.1	0.41±0.1
CHT/PVA (50:50)) 3% (WM)	$scCO_2$	-20	M1	25.8±1.3	0.86±0.1
			M2	41.6±2.1	2.12±0.1
CHT/PVA (50:50) 7% (NM) Native	$scCO_2$	-20	-	99.1±4.7	0.5±0.3
CHT/PVA (50:50) 7% (NM)	$scCO_2$	-20	M1	108.1±7.4	6.7±1.7
			M2	-	-
CHT/PVA (50:50) 7% (WM) Native	$scCO_2$	-20	-	49±2.5	0.10±0.1
CHT/PVA (50:50) 7% (WM)	$scCO_2$	-20	M1	16.8±0.8	14.06±0.7
			M2	30±1.5	3.03±0.2
CHT/PVA (50:50) Native	Freeze Drying	-80	-	14.9±0.7	0.34±0.1
CHT/PVA (50:50) (NE)	Freeze Drying	-80	M1	131.4±7.4	0.9±0.1
			M2	108.7±8.2	3.4±0.5
			M3	104.8±5.8	1.9±0.2
CHT/PVA (50:50) (Ex)	Freeze Drying	-80	M1	100.7±9.7	9.8±2.0
			M2	59.7±6.1	37.1±1.1

Casting Solutions	Preparation Methods	$T_{Freezing}$ (°C)	Immobilization Methods	% Ad5 Recovered (avergade±SEM)	
				Flow-through and wash	Elution
CHT/GMA (NE)	Freeze-Drying	-80	M1	96.8±8.8	2.7±0.4
			M2	93.3±3.9	2.0±0.2
			M3	-	-
CHT/GMA (Ex)	Freeze-Drying	-80	M1	73.9±1.17	1.9±0.0
			M2	103.1±1.7	3.6±0.9
			M3	116.9±10.2	3.5±0.2
CHT/PVA Mg Native	Freeze Drying	-80	-	19.1±1.0	0.46±0.1
CHT/PVA Mg (Lig.excess)	Freeze Drying	-80	M1	16.1±0.9	0.49±0.1
			M2	11.7±0.6	0.85±0.1

6.3 Appendix 3

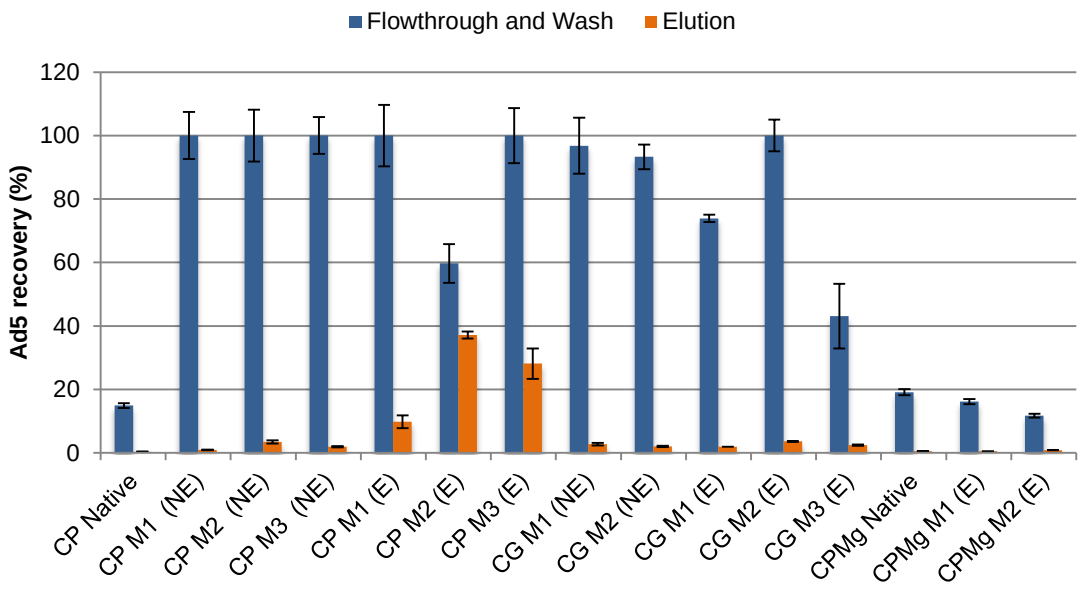

Figure 6.1 – Comparison of the results for Ad5 purification (1E11 TP/mL) using several types of monoliths prepared by Freeze-drying, expressing the percentage of virus recovered in flowthrough and elution stages. The monoliths differ in some properties, such as being functionalized by different techniques (M1-Epoxyativaction technique, M2-Alternative Plasma technique, M3-Direct Immobilization technique), within which were made with no ligand in excess (NE), with ligand in excess (Ex), and casting contain without MBA (NM) and with MBA (WM).

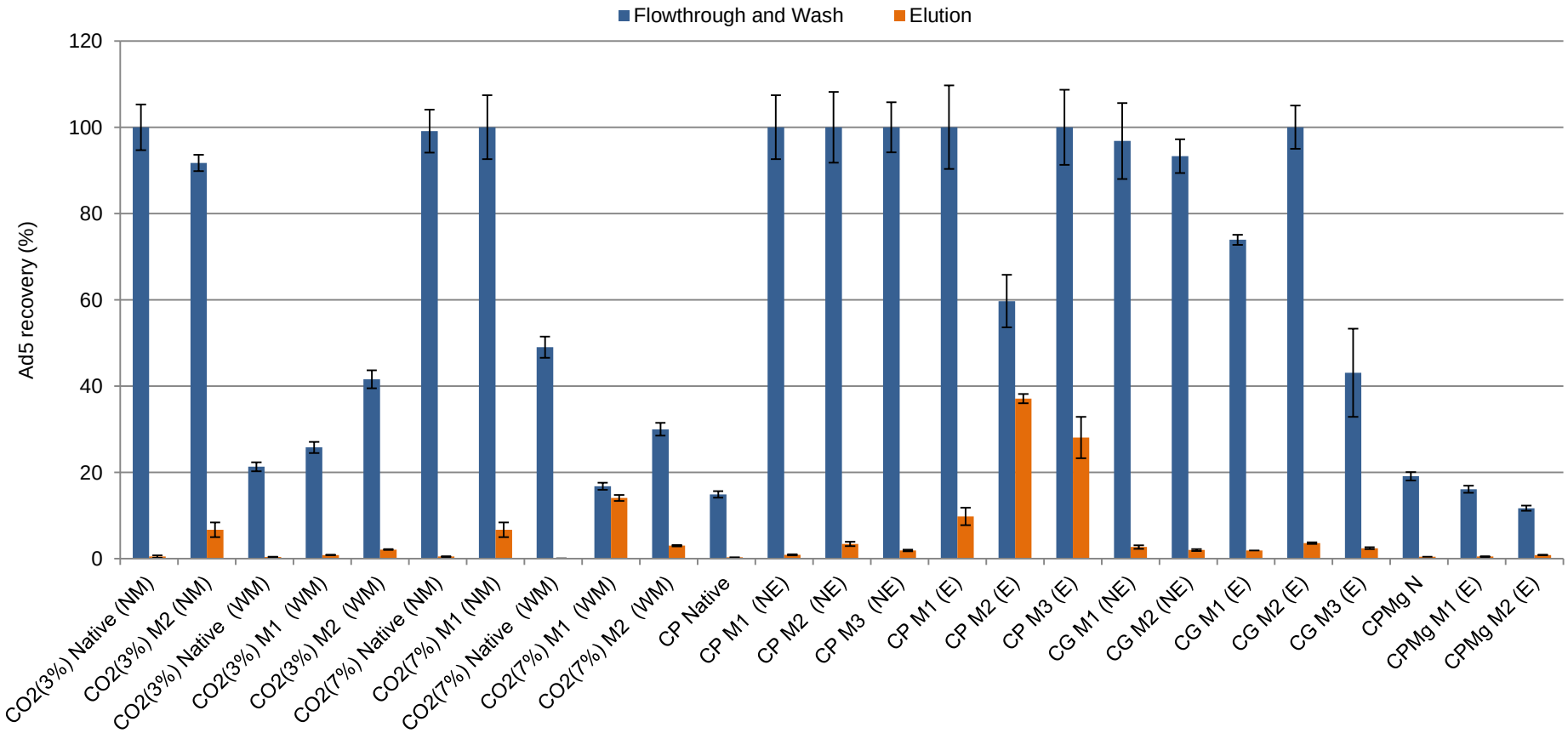

Figure 6.2 - Comparison of the results for Ad5 purification (1E11 TP/mL) using the native and functionalized monoliths produced with the CHT/PVA and CHT/GMA casting, expressing the percentage of (%) of virus recovered in flowthrough and elution stages. The monoliths were prepared by two techniques, freeze-drying (CP and CG) and $scCO_2$ – assisted drying process (CO2), and differ in some properties, such as being functionalized with different techniques (M1-Epoxyativaction technique, M2-Alternative Plasma technique, M3-Direct Immobilization technique), within which immobilizations were made with no ligand in excess (NE), with ligand in excess (E), and the casting solutions without MBA (NM) and with MBA (W).

6.4 Appendix 4

Table 6.4 –Titration curve values obtained with the precipitation titration with $AgNO_3$ (0.1 M) to the CHT/PVA (50:50) monolith functionalized using the epoxyactivation technique (M1) prepared by freeze-drying. With the addition of AgNO3 it was observed a sudden change of potential, indicating that approximately 200µmoles of ligand was immobilized.

CHT/PVA(50:50) M1		
0.1 M $AgNO_3$ V_{added} (µL)	**E (mV)/100**	**dE/dV**
0	7	
20	7	0
80	6,9	-0,00167
120	6,8	-0,0025
170	6,7	-0,002
200	6,5	-0,0067
240	6,2	-0,0075
290	6,1	-0,002
330	5,95	-0,0038
370	5,85	-0,0025
410	5,8	-0,0013
450	5,8	0
500	5,8	0
550	5,75	-0,001
600	5,75	0

Table 6.5 – Titration curve values obtained with the precipitation titration with $AgNO_3$ (0.1 M) to the CHT/PVA (50:50) monolith functionalized using the plasma alternative technique (M2) prepared by freeze-drying. With the addition of AgNO3 it was observed a sudden change of potential, indicating that approximately 20µmoles of ligand was immobilized.

CHT/PVA(50:50) M2		
0.1 M $AgNO_3$ V_{added} (µL)	**E (mV)/100**	**dE/dV**
0	7	0
20	6,7	-0,015
80	6,5	-0,0033
120	6,35	-0,0038
170	6,2	-0,003
220	6,1	-0,002
270	6,05	-0,001
320	6	-0,001
370	5,95	-0,001
420	5,95	0
480	5,9	-0,00083
520	5,9	0
560	5,9	0
670	5,9	0
720	5,9	0
780	5,9	0

Table 6.6 – Titration curve values obtained with the precipitation titration with $AgNO_3$ (0.1 M) to the CHT/PVA (50:50) 7% monolith functionalized using the epoxyactivation technique (M1) prepared by $scCO_2$ – assisted drying process. With the addition of AgNO3 it was observed change of potential, indicating that approximately 2600µmoles of ligand was immobilized.

CHT/PVA(50:50) 7% M1		
0.1 M $AgNO_3$ V_{added} (µL)	**E (mV)/100**	**dE/dV**
0	7	0
100	7	0
200	7	0
300	7	0
400	7	0
700	7	0
900	7	0
1100	6,9	-0,0005
1200	6,9	0
1400	6,85	-0,00025
1540	6,85	0
1700	6,8	-0,000313
1900	6,8	0
2100	6,8	0
2300	6,7	-0,0005
2400	6,6	-0,001
2500	6,5	-0,001
2600	6,2	-0,003
2700	5,85	-0,0035
2800	5,65	-0,002
2900	5,65	0
3000	5,6	-0,0005
3100	5,6	0
3200	5,6	0
3400	5,5	-0,0005
3500	5,5	0

Table 6.7 – Titration curve values obtained with the precipitation titration with $AgNO_3$ (0.1 M) to the CHT/PVA (50:50) 7% monolith functionalized using the plasma alternative technique (M2) prepared by $scCO_2$ – assisted drying process With the addition of AgNO3 it was observed a change of potential, indicating that approximately 20µmoles of ligand was immobilized.

CHT/PVA(50:50) 7% M2		
0.1 M $AgNO_3$ V_{added} (µL)	**E (mV)/100**	**dE/dV**
0	7	0
20	6,6	-0,02
80	6,5	-0,00167
120	6,35	-0,00375
160	6,15	-0,005
220	6,05	-0,00167
260	6	-0,00125
320	5,9	-0,00167
380	5,9	0
420	5,85	-0,00125
460	5,85	0
520	5,8	-0,00083
560	5,8	0
660	5,8	0
780	5,8	0
880	5,8	0

6.5 Appendix 5

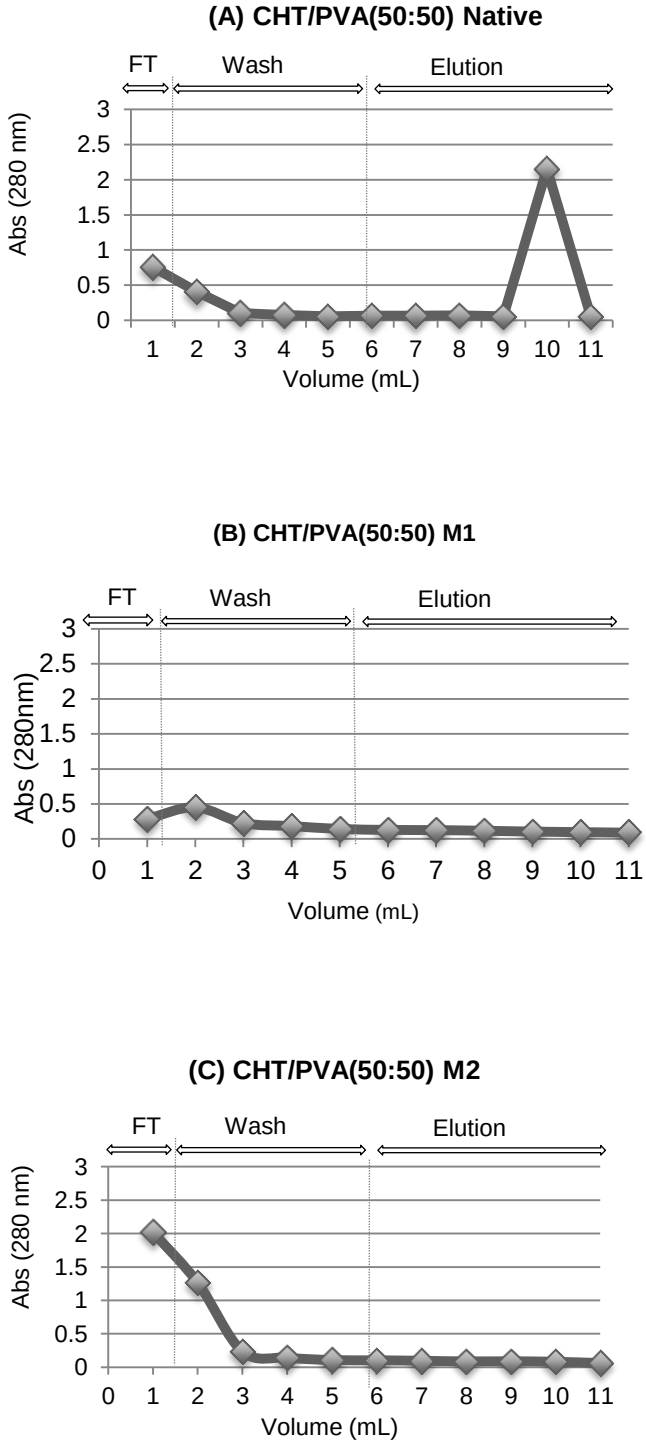

Figure 6.3 – Graphical representation of chromatographic performance for native (A) and functionalized CHT/PVA (50:50) monoliths functionalized by epoxyactivation technique (M1) (B) and the plasma alternative technique (M2) (C) prepared by freeze-drying. The chromatographic procedure represented consists of the loading, washes and elution stages of the protein-binding tests, which absorbance was measured at 280 nm. These tests were implemented applying the 1st approach of the assay (See sections 2.5 and 3.4).

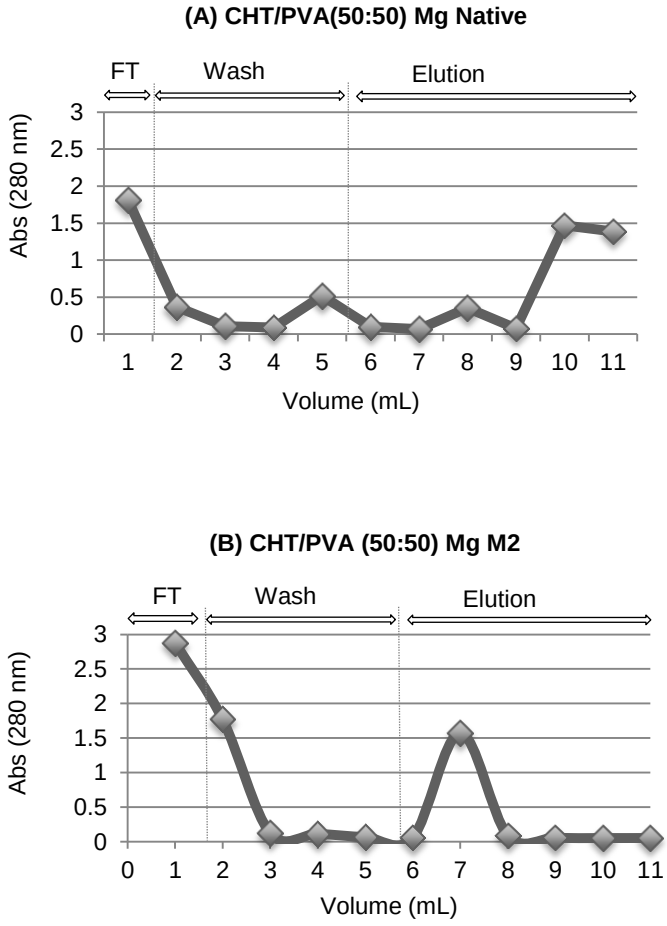

Figure 6.4 - Graphical representation of chromatographic performance for native (A) and functionalized CHT/PVA (50:50) magnetic monoliths functionalized by the plasma alternative technique (M2) (B) prepared by freeze-drying. The chromatographic procedure represented consists of the loading, washes and elution stages of the protein-binding tests, which absorbance was measured at 280 nm. These tests were implemented applying the 1st approach of the assay (See sections 2.5 and 3.4).

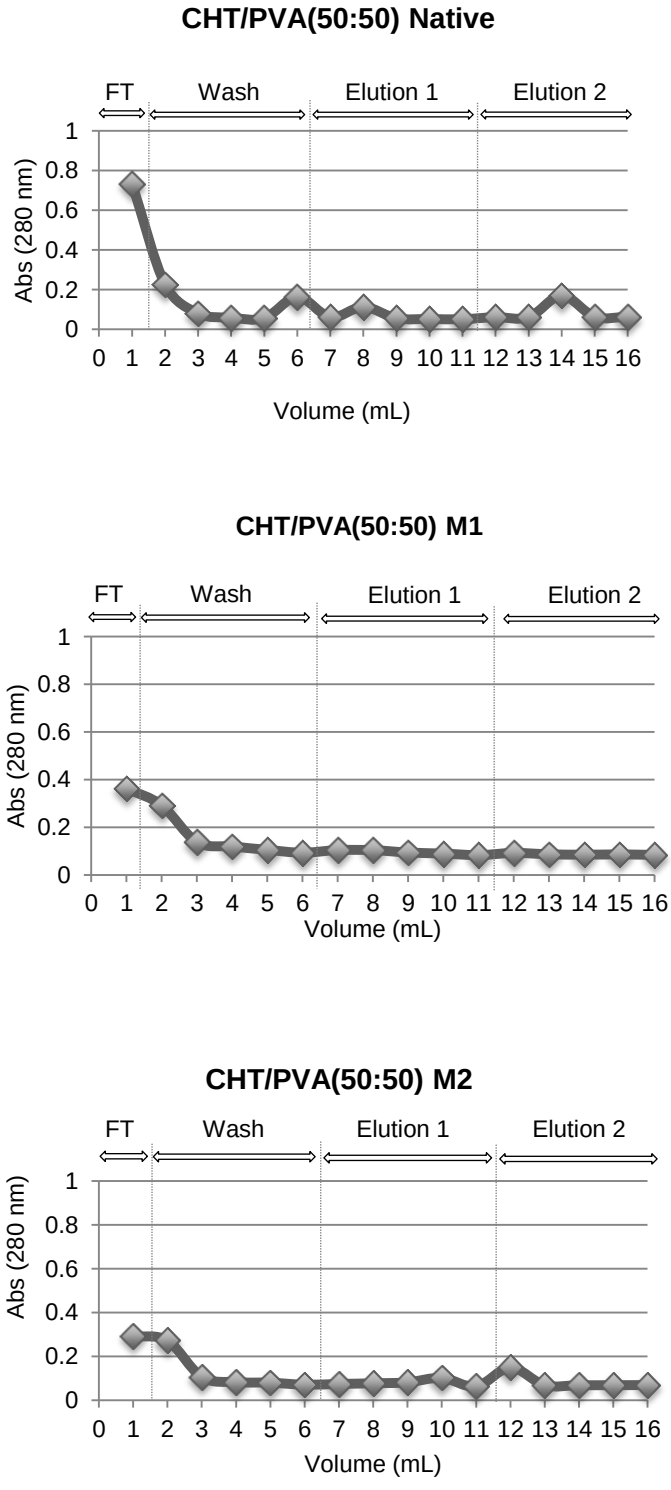

Figure 6.5 – Graphical representation of chromatographic performance for native (A) and functionalized CHT/PVA (50:50) monoliths functionalized by epoxyactivation technique (M1) (B) and the plasma alternative technique (M2) (C) prepared by freeze-drying. The chromatographic procedure represented consists of the loading, washes and elution stages of the protein-binding tests, which absorbance was measured at 280 nm. These tests were implemented applying the 2nd approach of the assay (See sections 2.5 and 3.4).

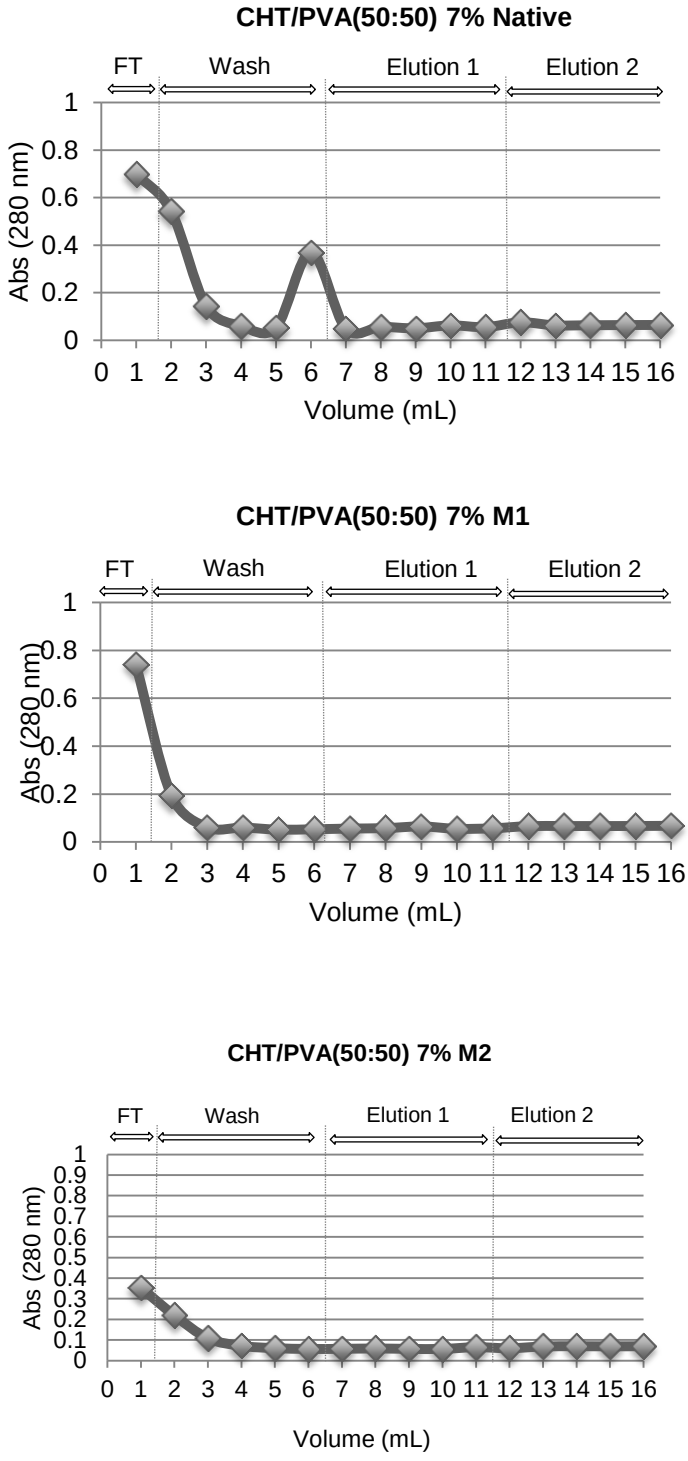

Figure 6.6 – Graphical representation of chromatographic performance for native (A) and functionalized CHT/PVA (50:50) 7% monoliths functionalized by epoxyactivation technique (M1) (B) and the plasma alternative technique (M2) (C) prepared by $scCO_2$ – assisted drying process. The chromatographic procedure represented consists of the loading, washes and elution stages of the protein-binding tests, which absorbance was measured at 280 nm. These tests were implemented applying the 2nd approach of the assay (See sections 2.5 and 3.4).

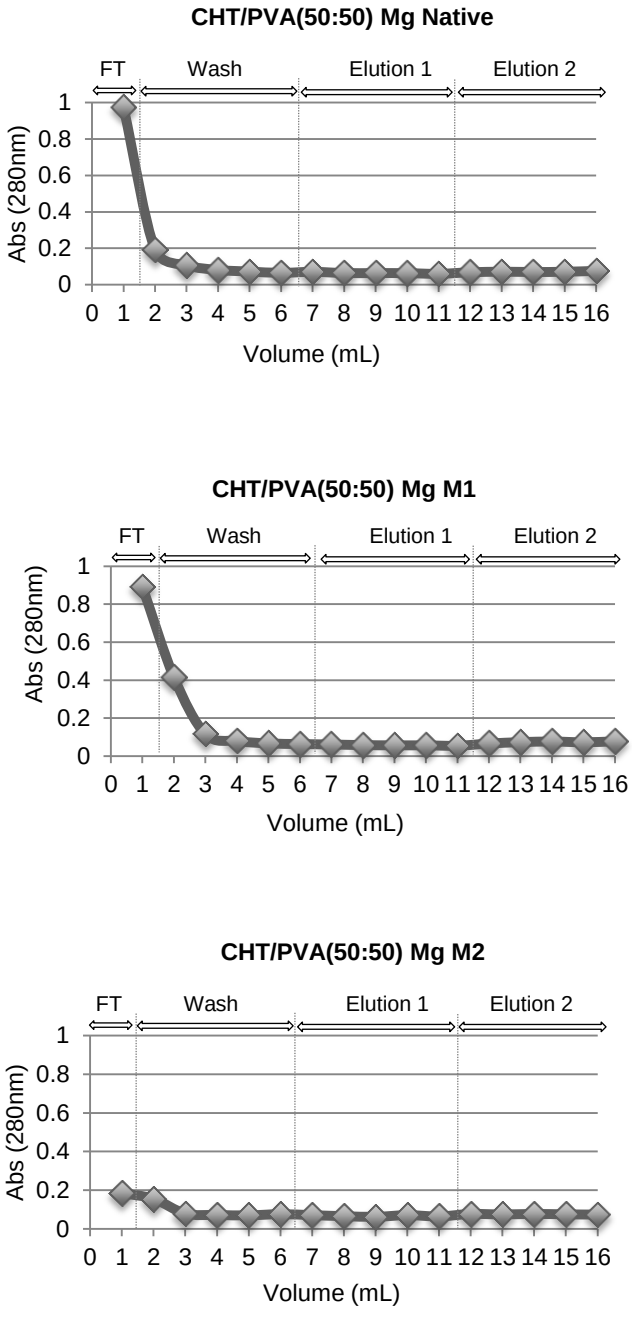

Figure 6.7 – Graphical representation of chromatographic performance for native (A) and functionalized CHT/PVA (50:50) magnetic monoliths functionalized by epoxyactivation technique (M1) (B) and the plasma alternative technique (M2) (C) prepared by freeze-drying. The chromatographic procedure represented consists of the loading, washes and elution stages of the protein-binding tests, which absorbance was measured at 280 nm. These tests were implemented applying the 2nd approach of the assay (See sections 2.5 and 3.4).