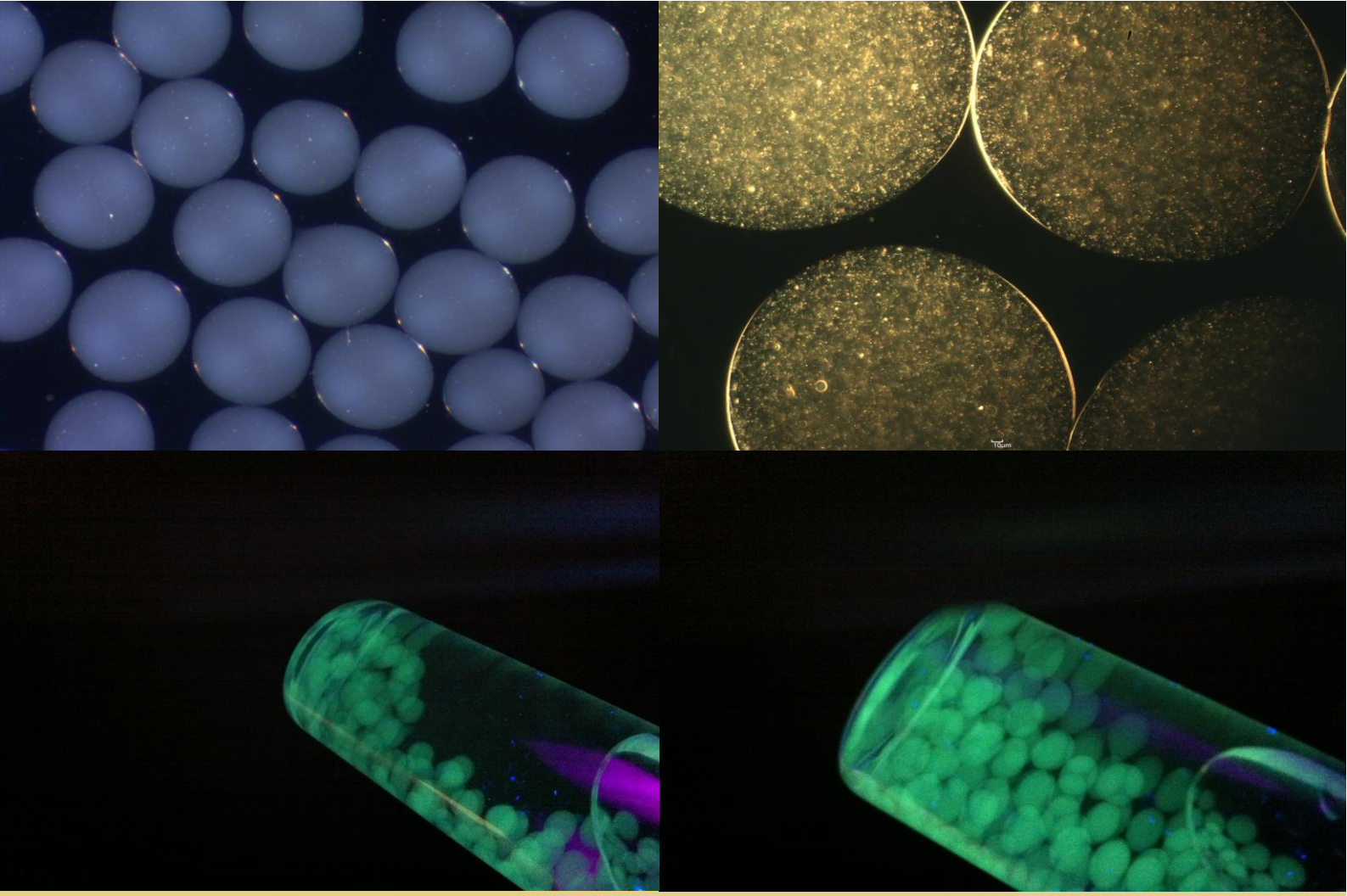

Francisco Tiago Almeida Catalão

Modification of Natural-based Polymers for Biomedical Applications

Master Degree Thesis in Biomedical Engineering, oriented by Doctor Jorge F. J. Coelho, by Doctor Arménio C. Serra and by Ph.D. student Joana C. Silva, submitted to the Faculty of Science and Technology of the University of Coimbra

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ABSTRACT

Currently, the use of natural-based polymers in biomedical applications has increased the demand for investigation in this field. Although this family of polymers already presents some great qualities and properties for biomedical use, there is a rising necessity to input some modifications in their structure in order to enhance their characteristic and widen their application spectrum.

The objective of this work is to test some chemical modifications in two polysaccharides of highly biomedical interest, namely dextran and sodium alginate, in order to provide them some extra properties which allow their use in biomedical applications like the encapsulation of cells or bioactive agents. The modifications attempted were: the input of azide and alkyne groups through hydrolysable carbonate esters, periodate oxidations and functionalizations with a fluorescent compound (3-amino-7-diethylaminocoumarin) and a provider of methacrylate groups (2-aminoethyl methacrylate).

In this report are described all the modifications carried out in these polymers in terms of chemical aspects, procedures results.

At the end of this work, were obtained successful results for the periodate oxidations and the fluorescence and methacrylate functionalizations. However for the modification of the polymers with azide and alkyne groups is a process that requires further study and development.

RESUMO

Actualmente, o uso de polímeros naturais em aplicações biomédicas provocou um aumento na exigência de mais investigação nesta área. Embora este tipo de polímeros já disponha de algumas qualidades excelentes para a área da biomédica, verifica-se uma necessidade crescente de realizar modificações na sua estrutura por forma a potenciar as suas características e alargar o seu espectro de utilização.

O objectivo deste trabalho é testar algumas modificações químicas em dois polissacarídeos de elevado interesse biomédico, nomeadamente o dextrano e o alginato de sódio, com o intuito de os dotar de novas propriedades que permitam a sua aplicação em aplicações do foro biomédico, como encapsulamento de células ou de agentes bioactivos. As modificações realizadas foram as seguintes: a introdução de grupos azida e alcino através de ésteres de carbonato hidrolisáveis, oxidações por periodato de sódio e funcionalizações com um produto fluorescente (3-amino-7-dietilaminocumarina) e com um doador de grupos metacrilato (2-aminoetil metacrilato).

Neste relatório encontram-se descritas todas as modificações realizadas nestes polímeros no que respeita a aspectos químicos, procedimentos e resultados.

No final do trabalho, foram constatadas modificações bem sucedidas no que respeita às oxidações por periodato de sódio e às funcionalizações com fluorescência e grupos metacrilato. No entanto, o processo de modificação dos polímeros com grupos azida e alcino ainda carece de estudo mais aprofundado e alterações.

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INTRODUCTION

Nowadays the demand for polymeric solutions in the biomedical field has an enormous impact in polymer science. Some examples of this kind of applications are the drug delivery systems, microcapsules, multilayer films, hollow capsules, nanoparticles, scaffolds for tissue engineering, and many others [1].

Amongst the different polymeric materials used for these devices, the focus of this work sets on the natural based ones.

Natural-based polymers, or polymers of natural origin like polysaccharides or proteins, were traditionally used as sources of wound dressings and suture threads either in their natural form or chemically modified. Despite being rather difficult to process, exhibit poorly reproducible properties, the possibility of being immunogenic and difficulty on the storage due to their sensibility to micro-organisms and humidity, they are commonly regarded as suitable therapeutic devices [2].

These polymers hold many properties that make them extremely suitable for this kind of use. They are extremely biocompatible, biodegradable, renewable, nontoxic and affordable at reasonable values. However, these applications demand natural polymers with some slight chemical modifications that need to be well-defined in order to allow the fine tuning of the properties required for the materials [3, 4].

Often the structural and functional properties of natural-based polymers are superior to the synthetic ones [5]. Nonetheless, we can increase their value by inducing chemical modifications to their structure [6]. With this in mind, we established the objective of this project the testing of several chemical modifications to this type of polymers in order to increase their interest and their utility in biomedical applications.

In Nature, there's a wide range of natural polymers derived from several renewable sources [5]. To narrow our field of interest we focused in two specific polysaccharides, dextran and sodium alginate. The diversity, abundance and considerable low cost, allows them to play a prominent role in many industrial and biomedical uses [6].

This work was intended to be a support to an ongoing Ph.D. work, developed by Joana C. Silva, on micro-encapsulation of Langerhans's islets for controlled delivery of insulin for the treatment of diabetes. The first approach was to take advantage of "click-chemistry" to serve the purpose of developing encapsulation methods for the cells. The first step of this pathway is the functionalization of natural-based polymers (dextran and sodium alginate) with azide and alkyne groups and then carried on functionalization reactions by "click-chemistry" processes.

Besides the modifications aiming the encapsulation of cells or bioactive compounds, there is also the intention of modifying dextran and sodium alginate conferring them distinct features like fluorescence or functional groups that can facilitate its use in the desired applications (cell encapsulation). For this purposes the first stage was to realize a periodate oxidation of these polymers in order to functionalize them with aldehyde groups and then take advantage these functional groups to input fluorescence and methacrylate groups. The fluorescence is given by a fluorescent compound which has an amine group in its constitution (3-amino-7diethylamino coumarin), and is intended to be used to evaluate the use of this type of modified polymers in future encapsulations. The methacrylate groups are donated by a compound that also as an amine group in its constitution (2-aminoethyl methacrylate) and are intended to be used by Michael addition in applications of cell adhesion or encapsulation.

This report describes several chemical modifications performed in this two natural-based polymers as well as the respective outcome of each procedure. In order to assess their viability for future work in this area.

CHAPTER I

BASIC CONCEPTS

1.1. Polysaccharides

Polysaccharides are naturally occurring carbohydrates. These macromolecules have two main functions in life: to be energy sources and to be extra-cellular and structural elements. This kind of polymers has various sources; they can be of algal origin, plant origin, microbial and animal origin [7, 8].

The basic constitution of these macromolecules is either one small repeating unit or two alternating units linked together by glycosidic bonds. Their structures are often linear, but it's possible the formation of six or more different units of sugars connected in a branch chain which holds specific information about the recognition of the polysaccharide in comparison with others [7].

The general formula of a polysaccharide is $C_x(H_2O)_y$ being x a large number between 200 and 2500. If we consider that often the polymer backbone repeating units are six-carbon monosaccharides, we can assume $(C_6H_{10}O_5)_n$, where n is a number between 40 and 3000, also as a representation of the general formula [8].

In nature, the most abundant polysaccharides are starch and cellulose, both consisting in repeating D-glucose units. Another way of referring to these polymers is to use the term glycan. Glycans differ from each other in the repeating unit (monosaccharide), in the length of the chain, in the types of linking units and in the degree of branching [7].

There are two main types of polysaccharides: homopolysaccharides and heteropolysaccharides. In figure 1 are shown some examples of these two types of polysaccharides [9].

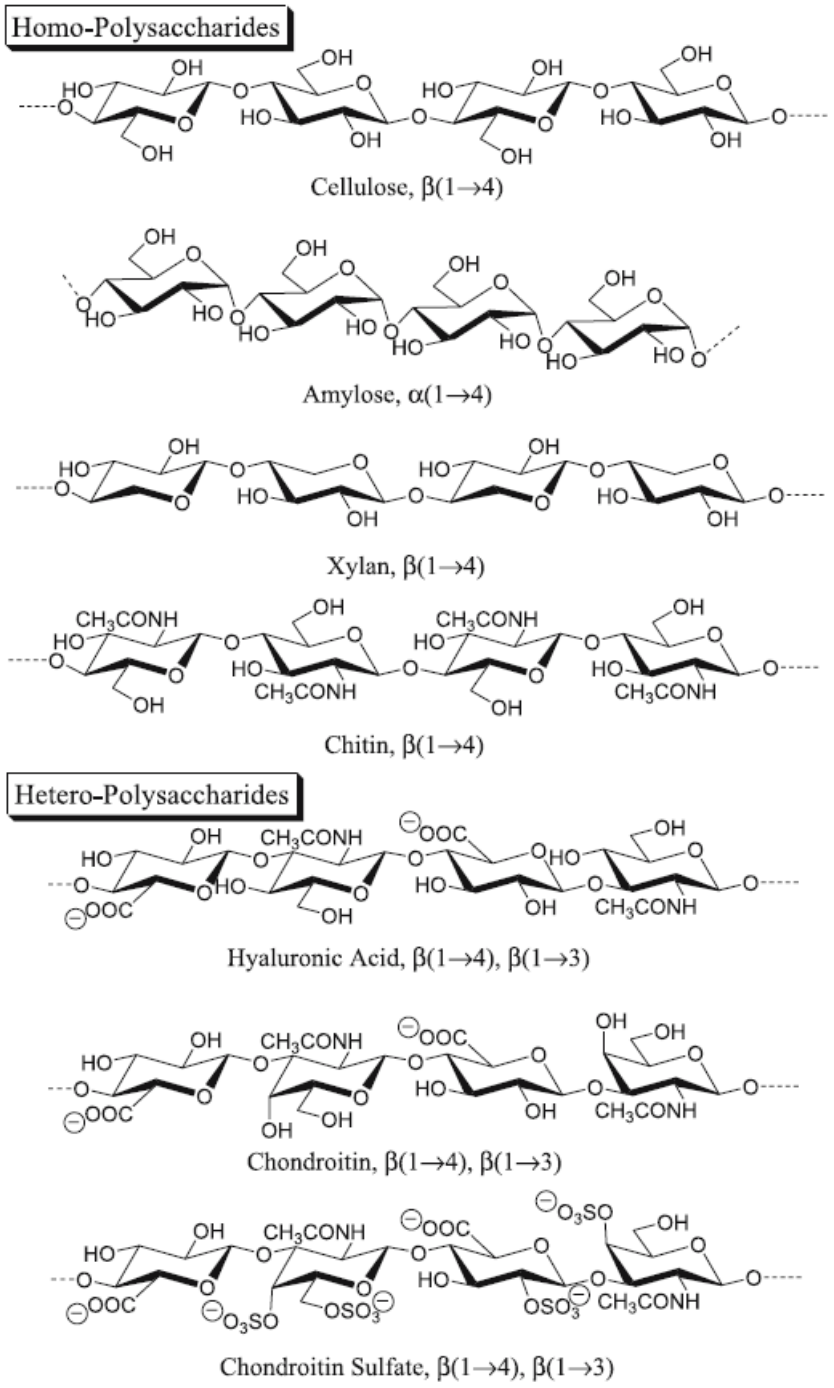

Figure 1 - Examples of homo and heteropolysaccharides.

Homopolysaccharides are made up of one type of repeating unit, or monomer; while heteropolysaccharides have two or more types of monomers. In terms of use, the majority of homopolysaccharides are used as sources of energy for biological processes, as is the case of starch and glycogens; heteropolysaccharides are responsible for providing structural and extracellular support, such as chitin and cellulose in animal exoskeletons and plant cell walls respectively, they are main constituents of the extracellular matrix too [7, 9].

The molecular weight of natural occurring polysaccharides has a wide range, due to the natural process of polymerization of monomeric units catalyzed by enzymes [7]. However, every day there are improvements to the enzymatic polymerization process of polysaccharides, making it possible to obtain more controlled and specific polymers [9, 10].

When we want to classify polysaccharides we have to take in account several aspects: the monosaccharides present, the sequences of linkages between them, the anomeric configuration of linkages, the ring size (furanose or pyranose), the absolute configuration (D- or L-), and the presence of other substituents like nucleic acids or proteins which have great influence in the specificity of the polysaccharide [7, 11].

The physicochemical properties of this kind of polymers are greatly influenced by the linkage mode of the monosaccharides within the polysaccharide and also by certain structural characteristics and intermolecular associations. A good example of this is the difference between amylose and cellulose. Both are homopolymers composed by glycosidic residues. However their linkage mode is different which makes amylose water-soluble and digestible whereas cellulose is neither water-soluble nor digestible [12].

The arrangements between the interunit glycosidic bonds are in order to satisfy the *intra*- and *inter*- molecular forces. The regularity and degree of stiffness of the chains affects the rate of fermentation, yielding polysaccharides more flexible or rigid than others [7].

In terms of hydrophobicity and hydrophilicity, the large number of hydroxyl groups is responsible for the hydrophilic character of these polymers, but they are also responsible for the presence of apolar surfaces depending on the epimeric structure, the monomer ring conformation and the stereochemistry of the glycosidic bonds. This factor explains why even when there are many hydroxyl groups present in the polysaccharide, it has a hydrophobic character, or at least a less hydrophilic one, because the interaction of the hydroxyl groups occurs amongst themselves, or with the hydroxyl groups of a neighboring polysaccharide, instead of occurring with water molecules [7].

There have been many advances in the use of Polysaccharides due to the ability of the scientific community to unravel the complexity and the specific characteristics of this kind of polymers in nature. The knowledge acquired about these products is essential to make practical applications like model and shape the various biological, physical and chemical inter-relations taking place, and also to create new and more developed characterization tools [13].

The main areas that evolved the use of polysaccharide-based substances were the health and cosmetic industry, food and feed production, and cellulose derived industries (paper, wood products and other cellulose derivatives like textiles) [13].

To the applications shown on table 1 is essential to add the various structures that can be obtained with these sugar-based synthetic polymers, including linear, branched, comb-like polymers, dendrimers and cross-linked hydrogels. These structures are synthesized according to the application that the polymer will be used, for example hydrogels swell significantly in water, they are biocompatible and hydrophilic, so they can be used as matrices for drug delivery systems and contact lenses amongst other biomedical and tissue engineering applications [7].

Table 1 - Applications that involve the use of several polysaccharides [13].

Polysaccharides	Applications
Starch and starch derivatives, cellulose and cellulose derivatives, chitosan, dextran, hyaluronan, pectin, carrageenan	Drug delivery systems
Chitosan, alginates, bacterial cellulose, hyaluronan	Wound healing
Cellulose, hyaluronan, chitosan, alginates	Tissue engineering scaffolds and implants
Heparin, chitosan	Bioactive compounds as anti-microbial, anti-clotting of blood, drugs and vaccines
Hyaluronan, chitosan, aloe	Skin hydration, anti-aging agents, skin protection (antibacterial agents)
Bacterial polysaccharides	Vaccines

In the two sections below will be addressed the two polysaccharides used in this work, dextran and sodium alginate.

1.2. Dextran

Dextran is the term used to name a big class of α-D-glucans, containing anhydro-D-glucopyranose. The composition of this kind of polymers is 95% α-1,6-glucopyranosidic linkages and 5% 1,3-linkages which are branching points for attachment of side chains. The side chains have 1 or 2 glucose residues in length in about 85% of the polymers; the remaining 15% have an average length of 33 glucose residues. The branches (or side chains) are usually attached to the main chains by different α-1,2-, α-1,3-, and α-1,4-glycosidic bonds [7, 8, 14].

Figure 2 shows the structure of Dextran, it is visible the α-1,6- linked glucose main chain as well as the branching points in 2-, 3- and 4-positions [8, 15].

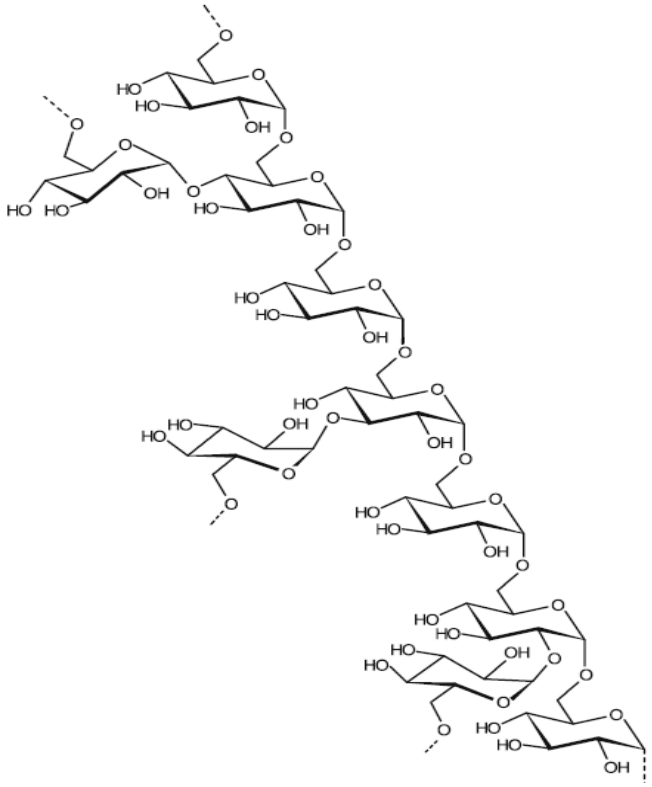

Figure 2 - Part of the Dextran chemical structure.

1.2.1. Origin

Dextran is a microbiological product of a large number of bacteria of family *Lactobacillaceae*, especially from *Leuconostoc mesenteroides*, *Leuconostoc dextranicum* and *Streptobacterium dextranicum* [7, 16].

The products of these bacteria have different chemical composition, structures and properties, like degree of branching, relative quantity of particular type of glycosidic links, molecular weight, solubility, optical activity and physiological action.(Natural) The most commonly used dextran for industrial and investigative purposes is the Leuconostoc mesenteroides NRRL B-512 [16].

Native-dextran, as it is called the product of the microbiological synthesis, is a polymer of high molecular weight. However it is possible to synthesize low molecular weight dextran in sucrose mediums or other mediums containing anhydro-D-glucopyranose units by fermentation. Another way of obtaining dextran with lower molecular weight is to undergo a partial depolymerization of native-dextran by acid hydrolysis and subsequent fractionation (clinical dextran) [7]. It can also be produced enzymatically, using cell-free culture supernatants which contain dextransucrase [15].

1.2.2. Structure

As shown in figure 2 and described above, the structure of dextran is mainly α-1,6-linkages between glucose units with branching points, with the degree and nature of branching being dependent on the bacterial strain used to produce the polymer [17].

There are several methods to assess if the structure is really the one described, optical rotation, infrared spectroscopy, periodate-oxidation reactions and methylation analysis combined with a capillary gas-liquid chromatography/mass spectrometry (GLC-MS), being this last one the most effective [15, 18].

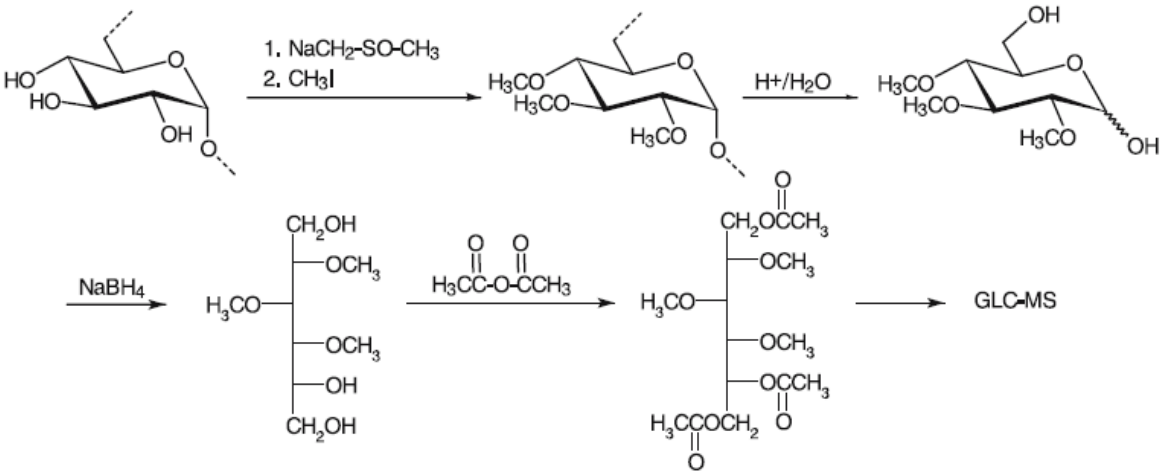

Figure 3 - Dextran structure determination by methylation analysis with subsequent GLC-MS.

In figure 3 is portrayed the method of methylation analysis to determine the structure of dextran demonstrated in a α-1,6-linked glucose unit.

In order to completely understand the structure of this polymer were performed studies using degradative enzymes, thin-layer chromatography, HPLC (high-performance liquid chromatography) and ^{13}C NMR (Carbon-13 nuclear magnetic resonance) [15]. To evaluate the length of the side chains it is used a method of sequential alkaline degradation [18].

In figure 4 is shown a ^{13}C NMR spectrum of Dextran. Along with figure 4, is presented table 2 that shows the chemical shifts for this polymer [15].

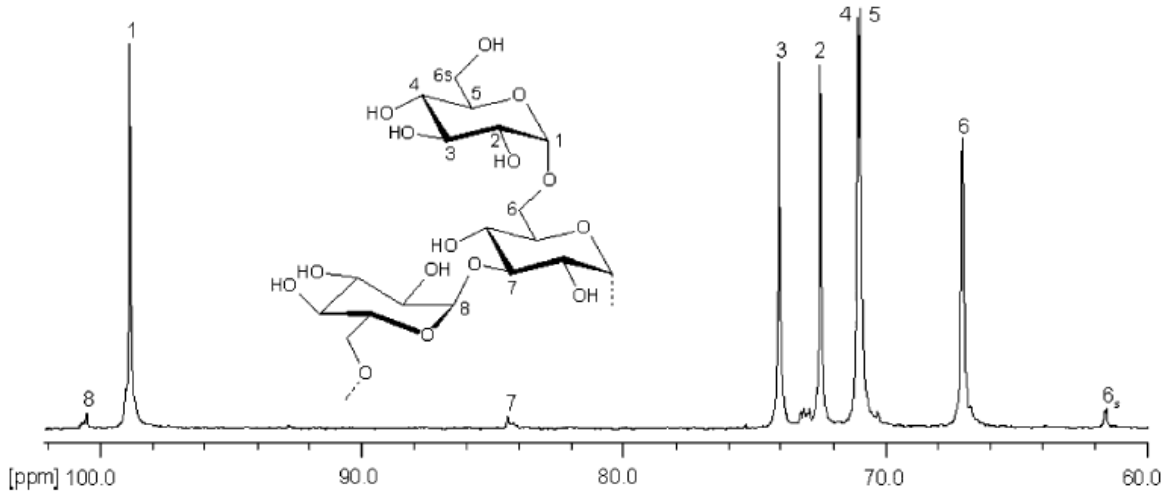

Figure 4 - ^{13}C NMR spectrum of Dextran (Mw 60000 g mol-1) in DMSO-d6.

Table 2 – Chemical shifts for Dextran in DMSO-d_6 [15].

Position	1	2	3	4	5	6	6'
^{1}H NMR	4.70-4.69	3.19-3.28	3.43-3.47	3.19-3.28	3.63-3.65	3.73-3.77	3.55-3.59
^{13}C NMR	98.9	72.5	74.1	71.0	71.1	67.1	-

1.2.3. Physico-Chemical characteristics and properties

Native-dextran is normally of high molecular weight (ranging from 10^7 to 10^8 g mol^{-1}) associated with high polydispersity as result of the increase in branching density. However the most interesting dextrans for most of the applications are fractions with more defined molecular weight [7, 15].

In low concentration aqueous solutions, dextran molecules have a random coil behavior (Newtonian behaviour) [19]. Nonetheless if the increase of concentration reaches an overlap value the chains get entangled amongst themselves forming a more compact geometry like a transient network (non-Newtonian behaviour) [20, 21].

Native-dextran is normally amorphous but in a mixture of water/polyethylene glycol with temperatures between 120 and 200ºC the formation of small crystals can occur [15].

The different bacterial strains from which dextran is produced give it different properties, due to the structural diversity. For the most common, the one with the α-1,6- glycosidic bonds, the chains have a certain mobility that allow it to be soluble in water, dimethyl sulfoxide (DMSO), dimethylacetamide/lithium chloride (DMA/LiCl), formamide, ethylene glycol, glycerol, aqueous urea (6 M), aqueous glycine (2 M) and 4-methylmorpholine-4-oxide. The kinetics of dissolution is influenced by the molecular weight of the polymer [15, 19, 22].

Low molecular weight dextran solutions are not very stable, when stored there can be the occurrence of precipitates, if the solution is highly concentrated (50-60%) there can even be the formation of a hydrogel caused by sol-gel conversion. The viscosity of the polymer in solution is directly related to the concentration of the solution, as well as to the molecular weight of the polymer [15].

Physiologically, this polymer is harmless due to its biocompatibility, biodegradability and non-immunogenic or non-antigenic character. The degradation of this polymer in the human body is done by the action of dextranases (α-1-glycosidases) present in the liver, spleen, kidney and lower part of the gastrointestinal tract [2, 15].

1.3. Sodium Alginate

Alginate is a natural anionic polysaccharide found in seaweed, mainly composed of (1-4)-linked β-D-mannuronic acid and α-L-guluronic acid repeating units. In its constitution it has a number of blocks or regions of sequential units of mannuronic acid, guluronic acid or a combination of both. The origin of the alginate is responsible for the amount and distribution of the blocks along the polymer [2, 23, 24].

1.3.1. Origin

Aside from the seaweeds, mentioned before, alginates are produced by the microorganism *Azotobacter vinelandii* and certain *Pseudomonas* strains*. Laminaria hyperborea*, *Macrocystis pyrifera* and *Ascophyllum nodosum* are the main sources from which most of the commercially available alginates are isolated from. There are other minor sources like *Laminaria digitate*, *Laminaria japonica*, *Eclonia maxima*, *Lesonia negrescens* and *Sargassum sp.* [25, 26].

The natural occurrence of alginic acid in the form of mixed salts of Na^{+}, Mg^{2+}, and Ca^{2+} is the main constituent of the cell wall of brown macro algae [25].

The process of extraction and purification of alginate from brown algae has five main steps [25]:

1. Extraction of the soluble alginic acid with 0.1-0.2 N mineral acid, along with mechanical treatment to facilitate the diffusion of the material out of the algal mass. This step removes other salts and polymers;
2. Neutralization with sodium hydroxide in order to obtain sodium alginate;
3. Precipitation by adding $CaCl_2$ or ethanol;

4. Bleaching with H_2O_2 and $NaClO_2$, repeated precipitation with ethanol or acetone and treatment with activated carbon or polyvinylpyrrolidone to remove polyphenols (contaminants);
5. Ultrasonification to obtain polymers with different chain length.

The harvesting of algae for alginate producing is done in cold and temperate waters like in the North of Europe, South American west coast, Southern Australia, Japan and China; being the total worldwide production of this polymers approximately 30000 Mtons per year [24, 25].

1.3.2. Structure

As mentioned before, alginates are linear unbranched polymers made of both mannuronic and guluronic acid residues. The occurrence of the monomers has three different ways: blocks of only mannuronic acid units (M blocks), blocks of only guluronic units (G blocks) and blocks of both units in an alternating sequence (MG blocks) [23]. In figure 5, it is visible the structure of the monomers of an alginate as well as part of an alginate structure with the various monomer arrangements possible [24].

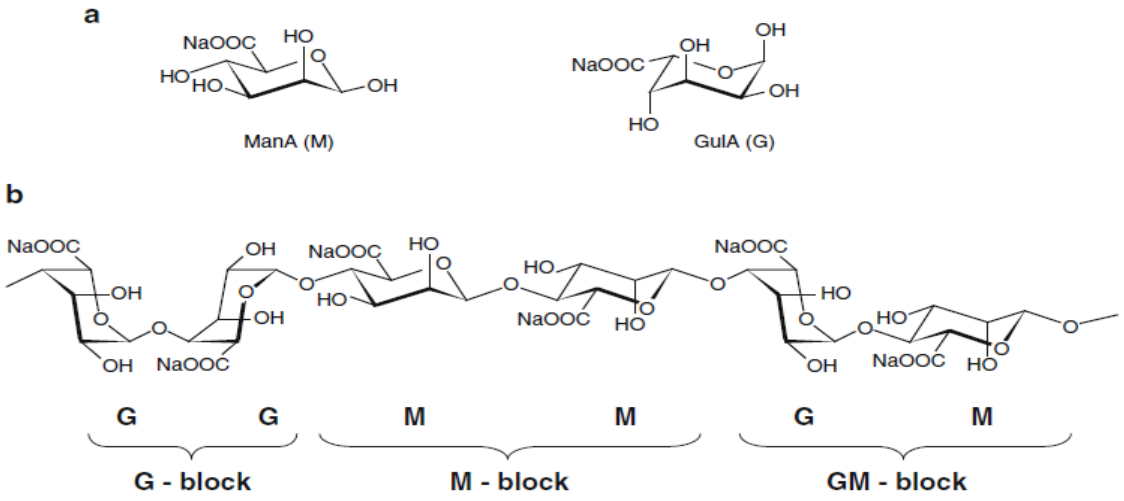

Figure 5 -.- Part of the structure of Sodium Alginate. In a it is shown the conformations of the β-D-mannuronic acid (M) sodium salt and the α-L-guluronic acid (G) sodium salt. In b it is visible the block composition of the polymer.

In figure 5 it is also visible that the ring conformation for mannuronic acid is $^{4}C_{1}$ and for guluronic acid is $^{1}C_{4}$ [24].

The confirmation of the structure and composition of the polymer can be made by high resolution ^{1}H NMR and ^{13}C NMR. Figure 6 shows an example of a ^{1}H NMR spectrum of an alginate isolated from *Laminaria hyperborea* [24].

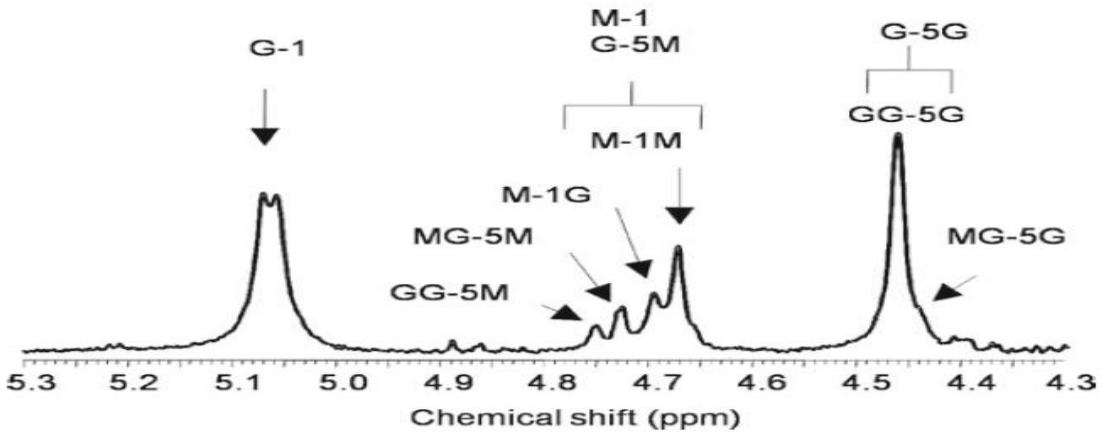

Figure 6 -^{1}H NMR spectrum of an alginate isolated from Laminaria hyperborea

The various points marked in figure 6 represent the following segments of the polymer [24]:

- M-1M and M-1G represent the anomeric proton of an M residue neighboring another M residue or a G residue, respectively;
- MG-5M , GG-5M , and MG-5G refer to the H-5 proton of the central G residue in an MGM, GGM, or MGG triad, respectively;
- G-1 refers to the anomeric proton of G residues and GG-5G refers to the anomeric proton of G residues in G-blocks.

There can be certain variations in the composition of this polymer, for instance alginates extracted from *Azotobacter vinelandii* initially present homopolymeric M-blocks and some of them are later converted into epimer C-5 G-blocks by the action of an extracellular enzyme, mannuronan C-5 epimerase. This epimerization process is also used to modify the composition of algal alginates; this

process is inhibited by the presence of O-acetyl groups which are also present in bacterial alginates [25].

The ratio between M and G blocks differs according to the specie from which the polymer was extracted. This fact has an influence in the polymer properties, because of the particular shapes of the monomeric units and their modes of linkage which create different geometries for all of this it is possible to say that alginates don't have a regular repeating unit [24, 25].

1.3.3. Physico-chemical characteristics and properties

One of the most interesting characteristics of alginates is their ion binding properties [24]. These polymers can form gels resulting of the interaction between the carboxylic groups present in their backbone and some divalent cations [26, 27]. The affinity order for alginate's different blocks is described in table 3 [24, 25].

Table 3 - Affinity order between alginate blocks and some divalent cations.

G-blocks	$Ba^{2+} > Sr^{2+} > Ca^{2+} >> Mg^{2+}$
M-blocks	$Ba^{2+} > Sr^{2+} \approx Ca^{2+} \approx Mg^{2+}$
MG-blocks	$Ba^{2+} \approx Sr^{2+} \approx Ca^{2+} \approx Mg^{2+}$

The carboxylic groups when in contact with those ions form an "egg-box"-shaped structure thus forming the hydrogel [23]. This process is portrayed in figure 7, where we can see the formation of the gel by the "egg-box"-model as well as the Ca^{2+}-dependent epimerization process [26].

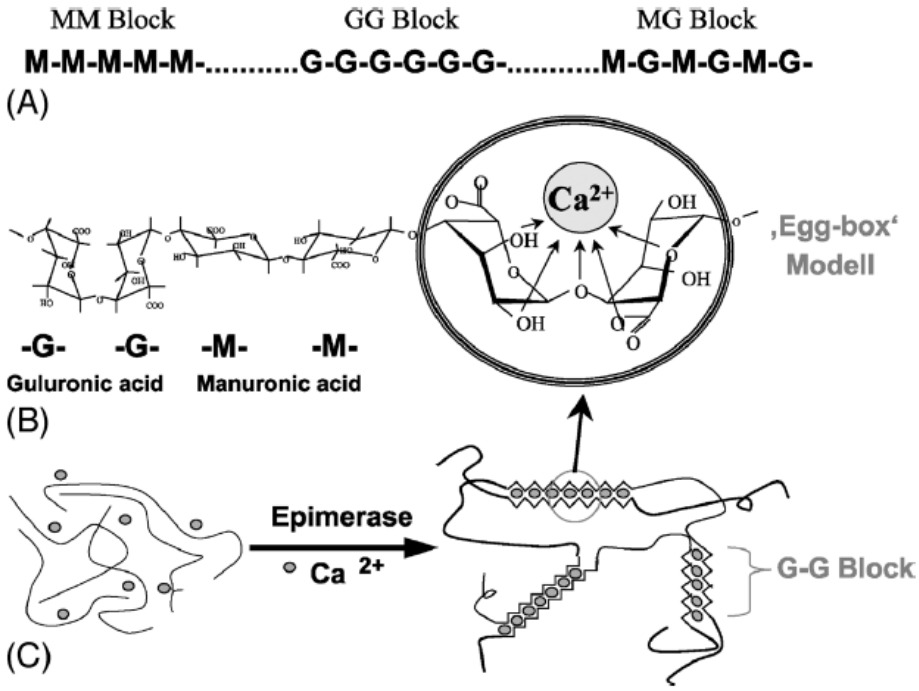

Figure 7 - Structure of Azotobacter alginate. (A) Block structure; (B) and (C) forming a hard gel.

After the formation of the hydrogel, there is a way of disrupting it. The use of a chelating agent (like sodium citrate) is an effective way of carrying out this task in a way that it captures the cations that hold this network together [23].

Another way of taking advantage of these ion binding properties is the fact that when a sodium alginate solution is poured drop-wise into a $CaCl_2$ solution it takes the shape of spherical beads of Ca-alginate. These beads have very high interest for biomedical applications [25].

Concerning the molecular weight, this kind of polymers is generally polydisperse, whether they have natural or bacterial origin or even if they were enzymatically tailored. The reasons for this to happen are: the production of polysaccharides is not gene-encoded but it has an enzymatic dependence and the extraction causes substantial depolymerization. Most of the commercially available alginates present a value of 200000 g mol^{-1} for the weight-average molecular weight, although there are samples that can present values as high as 400000 to 500000 g mol^{-1}. The polydispersity index (ratio between weight-average and

number-average molecular weights) for most of the alginate samples is between 1.5 and 3, being 1 the index value for a perfectly monodisperse sample, but there can be samples with index values climbing up to 6 [24].

In terms of solubility, it must be taken in account that the pH of the aqueous solution has a great importance, influencing greatly the solubility of the polymer in water [28]. In solution, alginate is highly viscous. This viscosity is due to the extended conformation of the alginate molecule which confers it a large hydrodynamic volume. Factors like ionic strength of the solution and molecular weight and composition of the alginates (ratio of M and G blocks) are responsible for variations in viscosity [26].

Alginates are biocompatible, biodegradable, mucoadhesive, hemocompatible, non-toxic, non-immunogenic and reasonably priced. These characteristics along with all the others mentioned before make it a very interesting polymer for biomedical applications [29-31].

1.4. Chemical modifications

As described in the sections above, polysaccharides have a wide range of molecular weights and varying chemical compositions which contribute to their diversity in structures and properties [8]. They also have a large number of reactive groups (i.e. hydroxyl, amino, and carboxyl) that can be chemically modified to obtain interesting derivatives in the applications point of view [32].

After the chemical modification, polysaccharides present different characteristics or properties, so this amphiphilic behaviour presented post modification opens a wider spectrum of possibilities in applications for these polymers (for example rheology modifiers, emulsion stabilizers, surface modifiers for liposomes and nanoparticles used as drug delivery systems) [8, 11].

In this section will be addressed some chemical modifications that can be performed in polysaccharides, namely in dextran and sodium alginate.

1.4.1. Modification of dextran and sodium alginate with alkyne and azide groups through hydrolysable carbonate esters

This modification comprises the connection of alkyne or azide groups to the dextran backbone resulting in a hydrolysable carbonate ester [33].

To achieve the intended objective for this process it's necessary to perform two reaction steps:

1. Activation of propargyl alcohol or 3-azidopropanol with carbonyl diimidazole (CDI);
2. Grafting of the activated products onto the dextran backbone chain.

In the first step the activation process involves the conversion of propargyl alcohol and 3-azidopropanol (alcohols) into propargyl carbonylimidazole and 3-azidopropyl carbonylimidazole (carbamates), respectively. The products of the first step serve as reagents for the second step, in which the same type of conversion takes place to graft propargyl carbonylimidazole and 3-azidopropyl carbonylimidazole into the dextran backbone, forming a carbonate ester between the backbone of the polymer and the pending modified moieties [33].

Figure 8 shows the scheme of the reaction of the synthesis of dextran propargyl carbonate (dex-C≡C) and dextran azidopropyl carbonate (dex-N_3). Represented by number (1) and (2) are the events referring to the first reaction step; numbers (3) and (4) show the second step events [33].

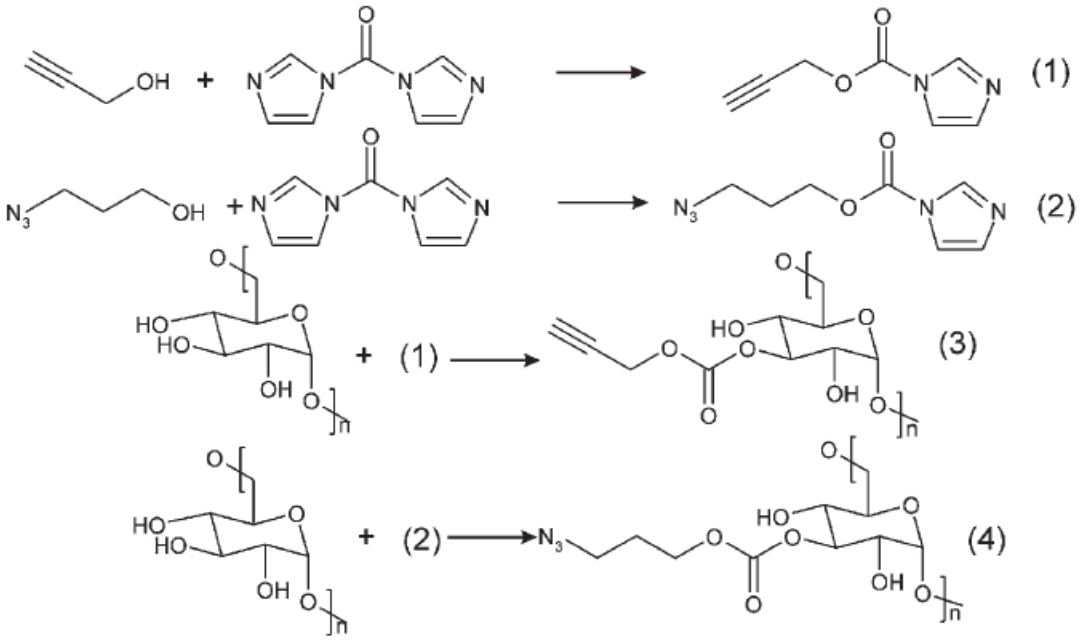

Figure 8 - Reaction scheme of the synthesis of dextran propargyl carbonate (3) and dextran azidopropyl carbonate (4).

This modification has a great deal of interest because it opens the door for the use of “click-chemistry” to obtain multilayered polymers (cross-linked) that can be degraded by hydrolysis [1].

1.4.2. Modification of Dextran with CDI and functionalization with alkyne and azide groups

In this modification is applied the same principle of the one before. However there are some changes in the reaction order.

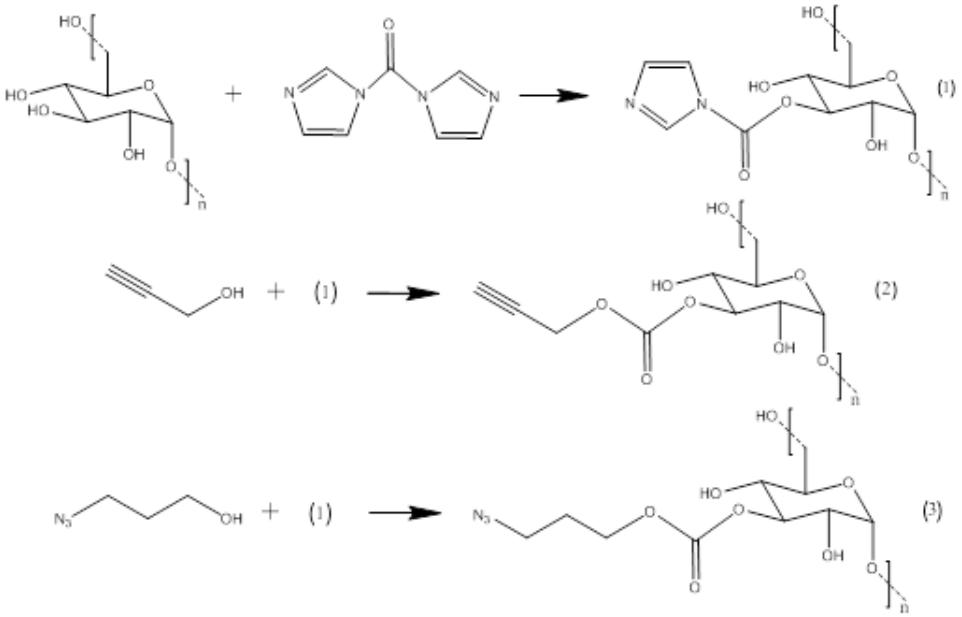

Figure 9 - Schematics for the reaction of synthesis of dex-C≡C (2) and dex-N_3 (3).

According to figure 9, the first step of the process is the grafting of the CDI into the dextran chain, resulting in the formation of the carbamate group in the linkage. The second step is the functionalization of the product obtained in the first step with the propargyl alcohol and the 3-azidopropanol, forming dex-C≡C (2) and dex-N_3 (3), respectively, with the appearance of the carbonate ester group in the linkages [33].

The purpose of this modification is to try an alternative route to the one described above and to observe if the products obtained have the same properties and applications.

1.4.3. Periodate oxidation of polysaccharides

Nowadays the periodate oxidation is seen as a way of introducing dialdehydes into polysaccharides and glycoproteins. However initially it was considered a great technique of structure determination for complex carbohydrates. Still with the appearance of novel and more efficient analysis techniques this use was abandoned [34].

This modification consists in the cleavage of the carbon-carbon bond in compounds that have an oxygen bearing carbon each as an hydroxyl or as a carbonyl group [35]. It is of high interest because it enables the polysaccharide structures with aldehyde functionalities, which makes them very reactive towards *N*-nucleophiles, like amines, hydrazines or carbazates [16].

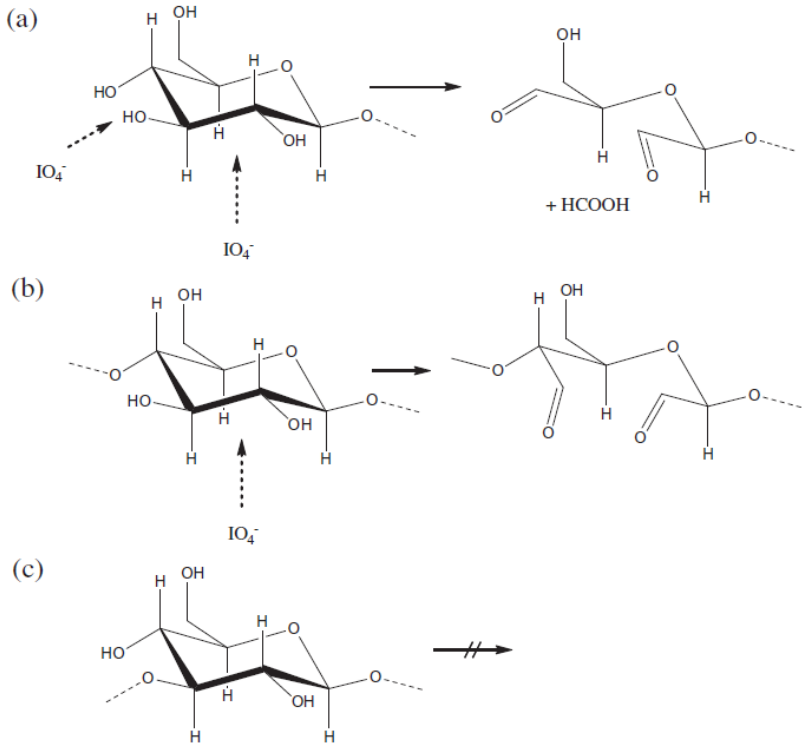

Figure 10 - Main products (dialdehydes) obtained for (1→2)-linked, (1→3)-linked, (1→4)-linked, (1→6)-linked, and terminal (non-reducing) hexose residues of β-D-glucose.

According to figure 10, (a) represents terminal (non-reducing end) residues that undergo double oxidation between C2 and C3 and between C3 and C4, respectively, with the release of C3 as formaldehyde; (b) are (1→4)-linked residues where cleavage occurs between C2 and C3, and (c) stands for (1→3)-linked residues (non-terminal), which are resistant to oxidation [34].

It is important to know the oxidation pattern obtained because it has great influence in the final properties of the polymer. For most simple glycans it is reasonable to say that oxidation is almost random, however that fact can't be assured because there can always occur some deviation due to possibilities for faster oxidation of reducing and non-reducing ends. The oxidation of a residue in the vicinity of another oxidized residue isn't a major factor, unless we're dealing with very high degrees of oxidation [34].

In terms of properties it is known that periodate oxidation of polysaccharides creates a liability in the hydrolytic stability of the polymers. This susceptibility along with the increase in flexibility of the chains due to the ring opening followed by the glycol cleavage can be used to tailor biomaterials with enhanced degradability and suitability for in vivo applications [34, 35].

1.4.4. Periodate oxidation of dextran

In the periodate oxidation of dextran the periodate ion attacks the α-1,6 units breaking the C-C bond and yielding aldehyde groups , regularly the attack occurs between carbons C_3-C_4 or C_3-C_2. This process is developed in aqueous medium with no need for a catalyst [16, 36].

During the oxidation process, the C_3-C_4 cleavage is favoured when compared to the C_3-C_2 in a proportion of 7.5:1. There is also the possibility of a second oxidation because C_3 aldehyde group has a vicinal hydroxyl group, which is susceptible to further attack, expelling this carbon as formic acid [37]. This second attack is due to the high reactivity of originated aldehyde groups, which are

very susceptible to attacks from neighbouring hydroxyl groups, thus forming hemiacetals. As result of this modification it is also observed a decrease in the average molecular weight and an increase in polydispersity [16, 38].

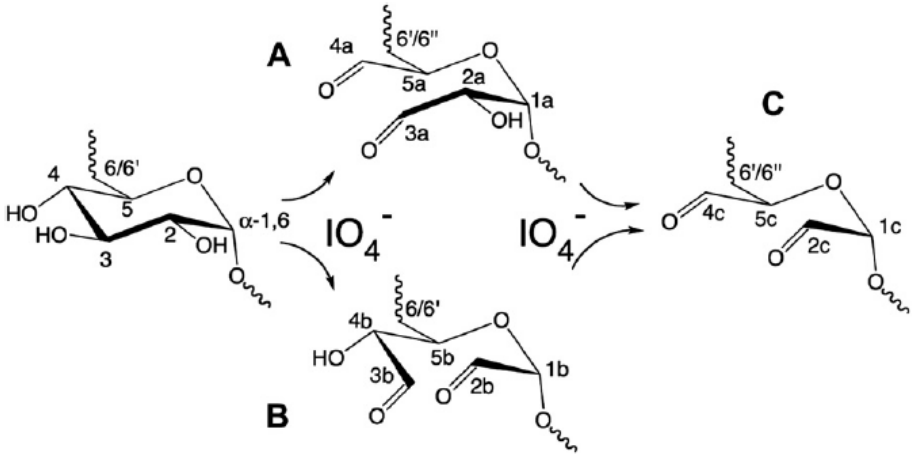

Figure 11 - Three possible periodate oxidations for α-1,6 glucose residues of dextran. (A) Attack at C_3-C_4; (B) C_3-C_2 and (C) double oxidation.

In figure 11 are portrayed the three possible outcomes of the periodate oxidation process of dextran. Figure 12 shows the possible hemiacetal groups formation that take place after the oxidation.

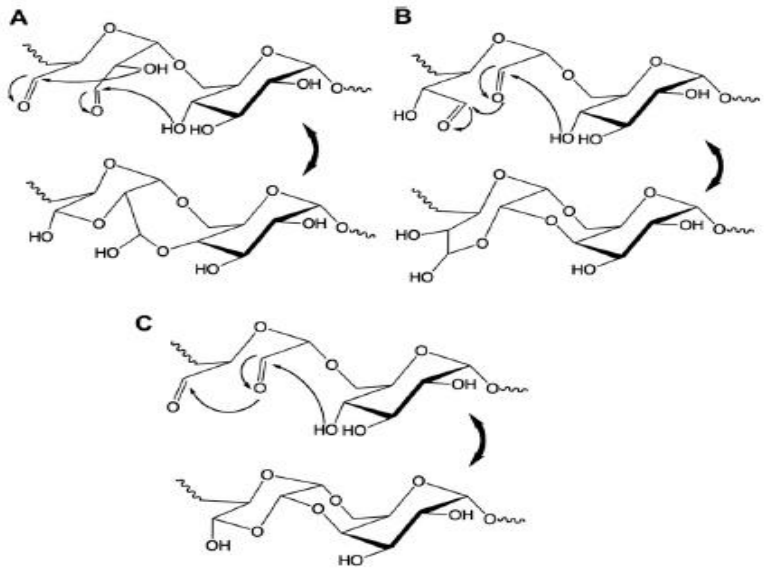

Figure 12 - The possible hemiacetal formations for the three possibilities of oxidation.

1.4.5. Periodate oxidation of Sodium Alginate

The periodate oxidation of sodium alginate in dilute solutions occurs randomly, in a way that in one chain only one monomeric unit is oxidized at a certain time, and immediately the protection of the vicinity of that unit takes place, before the occurrence of another oxidative attack on the chain [35].

The process of oxidation by periodate ions involves and extensive cleavage of the alginate chains that can occur by two ways [35]:

- The fast one (not mediated by hydroxyl free-radical) due to the cleavage of infrequent and unusual monomers, which break the main chains;
- The slow one (due to low concentrations of hydroxyl radicals) attributed to the appearance of free-radicals during the oxidation resultant of the oxidation of phenolic impurities present in alginate.

To oxidize alginate, periodate ions cleave the C-C bond of the *cis*-diol group in the uronate residues, yielding aldehyde groups and conformational chances to the polymer chain (figure 13) that, as mentioned before, significantly influence the backbone scission. As result of this process there is a decrease in molecular weight of alginate dependent on pH and temperature of the medium. However the periodate oxidation of alginate has no effect on the ion binding properties of the polymer, making it suitable for many biomedical applications [39].

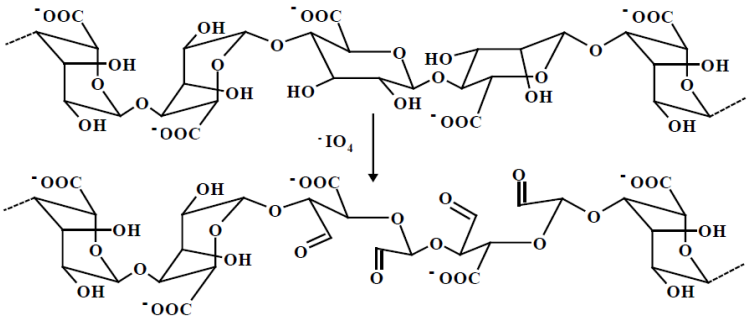

Figure 13 - Outcome of the periodate oxidation of sodium alginate.

1.4.6. Nucleophilic addition and elimination with imine reduction by Sodium cyanoborohydride

This modification consists in taking advantage of the affinity between aldehydes and N-nucleophiles more specifically amines to confer the oxidized alginate with specific properties interesting for future application [16].

The reaction mechanism is constituted by two steps [40-43]:

- Nucleophilic addition between and aldehyde and an amine group, resulting in and intermediate carbinolamine, which then dehydrates yielding a substituted imine;
- Imine reduction with sodium cyanoborohydride, yielding a secondary amine group.

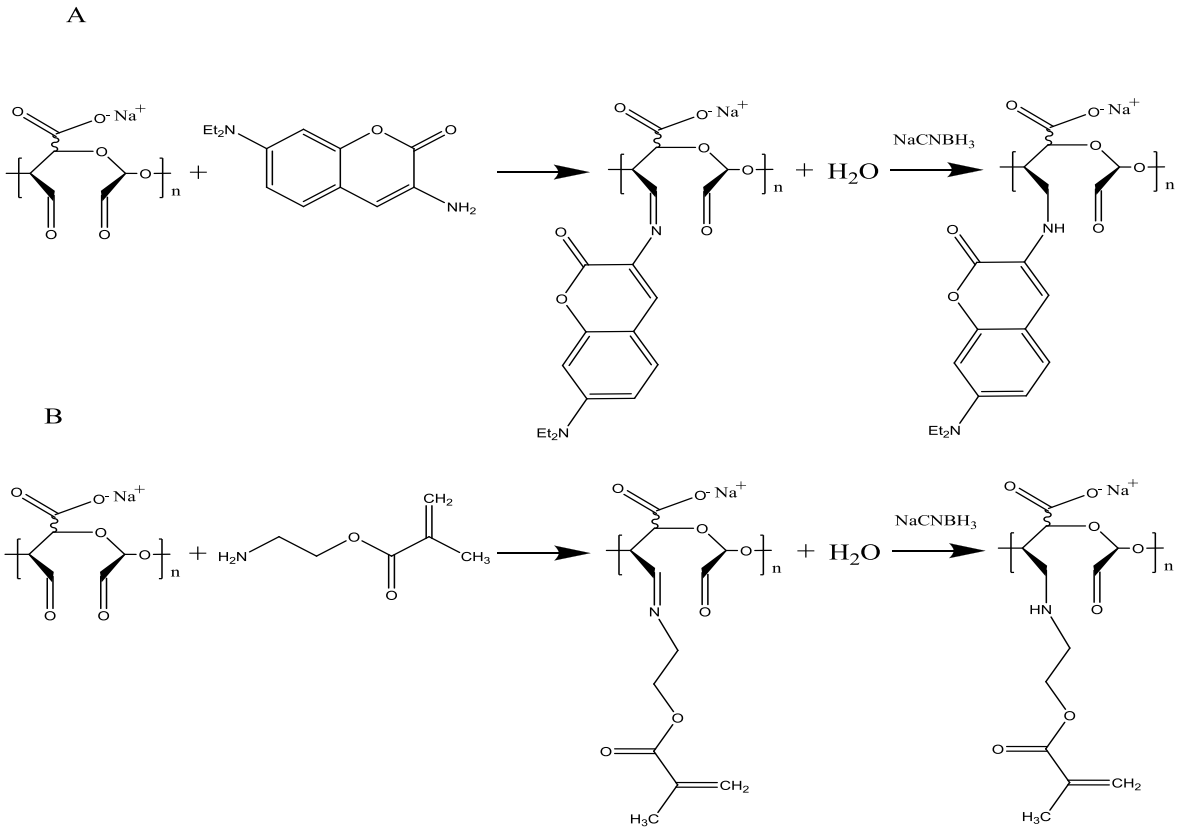

Figure 14 - Scheme of the reaction of nucleophilic addition and the reduction by Sodium cyanoborohydride.

In figure 14 is showed the reaction mechanism described before with two different sources of amine groups: in A it´s 3-amino-7-Diethylaminocoumarin, a fluorescent product [44]; and in B is 2-aminoethyl methacrylate, a product that besides having a part as an amine provider has interesting properties as a functionalization way (acrylate groups).

CHAPTER II

EXPERIMENTAL WORK

2.1. Materials

Dextran (from *Leuconostoc mesenteroides*; M_w~10200 Da and M_w~70000 Da determined by size exclusion chromatography; Sigma-Aldrich), Sodium Alginate (M_w~100-200 kDa; 65-70% guluronic acid and 25-35% mannuronic acid; Fluka BioChemika), carbonyl diimidazole (CDI), dimethylsulfoxide (DMSO), sodium periodate (≥99%; Sigma-Aldrich), ethanol (≥95%; Panreac), phosphate buffer solution (Sigma-Aldrich), sodium cyanoborohydride (≥90%; Tokyo Chemical Industry Co., LTD.), methanol (≥95%; Panreac), ethylene glycol (≥99%; Sigma-Aldrich), 2-propanol (≥99% Fisher Scientific UK Limited), 2-aminoethyl methacrylate (≥90%; ACROS Organics), Calcium Chloride (≥99%; Sigma-Aldrich) and distilled water were used as received in the chemical modifications.

Propargyl carbonylimidazole, 3-azidopropanol and 3-azidopropyl carbonylimidazole were synthesized according to literature [33]. They were used in the functionalization of dextran with alkyne and azide groups.

3-amino-7-Diethylaminocoumarin was synthesized as described in the literature and used as source of amine groups for nucleophilic addition[44].

Deuterium oxide (D_2O) (Eurisotop; +1%TMS), deuterated dimethyl sulfoxide (d_6DMSO) (Eurisotop; +1%TMS) and deuterated chloroform ($CDCl_3$) (Eurisotop; +1%TMS) were used as received in the chemical analysis as solvents for ^{1}H NMR samples.

2.2. Characterization techniques

In this section will be explained the basic principles of the techniques used to characterize the products obtained throughout the experimental work.

2.2.1. Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

The ^{1}H NMR spectroscopy is a technique that facilitates the understanding of the chemical behaviour of a given sample when exposed to a powerful magnetic field and irradiated with radiofrequency radiation [45].

When a magnetic field is applied on the sample, the nuclei of non-null nuclear spin tend to align and to acquire the orientation of the same or the opposite direction of the applied field. At this point, the irradiation of the nuclei with radiofrequency radiation causes their spins to transit to a higher level of energy. The absorbed and emitted energy is then quantified and represented in a spectrum [45, 46].

This spectrum data allows the characterization of the chemical products both in a quantitative and a qualitative way.

Proton Nuclear Magnetic Resonance (1H NMR) spectra were obtained at 25ºC in a Varian Unity 600 MHz Spectrometer using a 3 mm broadband NMR probe, in Deuterated Tetrahydrofuran (THF-d8). Tetramethylsilane (TMS) was used as internal reference.

2.2.2. Fourier Transform Infrared Spectroscopy (FTIR)

This analytical technique is very useful for the identification and characterization of the functional groups present in a given sample. This identification is based on the detection of the vibrations caused by the interaction between the atoms of the sample and the incident infra-red (IR) radiation [45].

With the incidence of IR radiation on the sample, it is obtained a spectrum that shows the fraction of radiation absorbed in a particular energy range, compared to a reference spectrum of energy absorbance recorded previously with no samples. Each functional group exhibits a specific vibration frequency so in the spectrum this specific vibration corresponds to a distinct energy peak, allowing the identification of the group [45].

Fourier Transform Infrared (FTIR) spectra were carried out in a FTIR-4200 spectrophotometer by *Jasco* recorded at a wavelength comprised between 550 and 4000 cm-1 and with 4 cm-1 resolution. Attenuated Total Reflection (ATR) mode was used.

2.2.3. Ultraviolet-Visible spectrophotometry and fluorescence spectroscopy

This technique was used to obtain absorption spectra from fluorescent compounds. It consists in the incidence of a beam of light with wavelength ranging between 200 and 800 nm in a sample which causes molecules to undergo electronic transitions from ground to excited state. The spectrophotometer registers the amount of energy (photons) absorbed during this excitation process for the range of wavelengths [47].

Fluorescence spectroscopy is a complementary technique to Ultraviolet-visible spectrophotometry. The basic principle behind this technique is to use a beam of light to excite the molecules of a certain sample from ground state, to the excited electronic state and then register the loss of energy in the return to the ground state as light emission [47].

The devices used were:

- Shimadzu UV-2100 spectrophotometer (UV-visible spectrophotometry);
- Perkin Elmer Fluorescence Spectrometer LS45.

2.3. Procedures

This section consists in detailing every experimental procedure realized during this investigation work.

2.3.1. Synthesis of Propargyl Carbonylimidazole (PA-Cl)

This procedure was carried out as it was described in the literature [33].In a dry round bottomed flask were charged 14.59 g (90 mmol) CDI and 100 ml dichloromethane yielding a turbid suspension, followed by the addition of 2.89 ml (50 mmol) propargyl alcohol under vigorous stirring yielding a clear solution upon dissolution of the propargyl alcohol. The reaction time was 1 hour at room temperature.

The mixture was then extracted three times with 17.5 ml water. The organic layer was dried over sodium sulfate. The sodium sulfate was then filtered and the resultant liquid was evaporated by rotary evaporation yielding the final product as a dry powder.

2.3.2. Synthesis of 3-azidopropanol

The synthesis of this compound was carried out according to literature [48]. An aqueous solution of 3-Chloropropanol (14.96 ml; 16.95 g; 0.179 mol), sodium azide (23.278 g; 0.358 mol) and tetrabutylammoniumhydrogen sulfate (0.5 g) was prepared and stirred at 80°C for 24h followed by overnight stirring at room temperature.

The resulting solution was then three times extracted with 50 ml ether and dried with sodium sulfate. Ether was evaporated by rotary evaporation and the remaining liquid was purified by vacuum distillation yielding the final product.

2.3.3. Synthesis of 3-azidopropyl carbonylimidazole (AP-Cl)

In a dry round bottomed flask were charged 9.12 g (0.0562 mol) CDI and 100 ml ethyl acetate resulting in a turbid suspension. Under a vigorous stirring, 3.48 ml (0.0375 mol, 3.79 g) 3-azidopropanol was added dropwise as the dissolution takes place the solution becomes clear. The reaction time was 2 hours at room temperature [33].

The solution was then extracted three times with 100 ml of distilled water. The organic layer was dried with sodium sulfate and subsequently filtered. The solvent was evaporated by rotary evaporation and the final product was obtained as a liquid.

2.3.4. Synthesis of dextran-propargylcarbonate (dex-C≡C)

In a dry round bottom flask was charged 20 ml of anhydrous DMSO in which was dissolved 1 g dextran (corresponding to 6.167 mmol glucopyranose repeating units). To this solution were added 280 mg (1.86 mmol) PA-CI and the mixture was stirred overnight at 50ºC under nitrogen atmosphere.

The resulting mixture was then poured into dialysis bags (M_w cut off 3.5 kDa) and dialysed against distilled water for 5 days. The mixture was then lyophilized yielding a white fluffy powder as final product.

2.3.5. Synthesis of dextran-azidopropylcarbonate (dex-N_3)

1 g of dextran (corresponding to 6.167 mmol glucopyranose repeating units) was dissolved in 20 ml anhydrous DMSO, in a round bottom flask.

To this solution were added 1.204 g (6.16 mmol) AP-CI and the reaction was stirred overnight at 50°C under a nitrogen atmosphere.

Subsequently the reaction mixture was put in dialysis bags (Mw cut off 3.5 kDa) and dialysed against pure water for 5 days. After lyophilization the final product was obtained as a white fluffy powder.

This procedure was also carried out in sodium alginate with the same steps and specifications.

2.3.6. Synthesis of CDI modified dextran

This synthesis is very similar to the procedures of synthesis of dex-C≡C and dex-N_3. However there was an alteration in the order of events, being first the activation of dextran with CDI and then the functionalization with alkyne and azide groups.

In a round bottom flask was charged 1 g of dextran (corresponding to 6.167 mmol glucopyranose repeating units) and it was dissolved in 20 ml DMSO. To the mixture were added 200 mg CDI and the reaction was stirred overnight at 40ºC under nitrogen atmosphere.

The mixture is then precipitated with 2-propanol and immediately filtered and subsequently dried under vacuum yielding the final product as a white hard powder.

The step of functionalization requires the dissolution of the sample in DMSO and then the addition to the solution of 3-azidopropanol or propargyl alcohol as sources of azide and alkyne groups respectively. This step follows the procedure explained before in sections 2.3.4 and 2.3.5.

2.3.7. Periodate oxidation of dextran

A solution of Dextran (M_w~70000 Da) with 12.5% concentration (1 g Dextran/8 ml distilled water) was prepared in a round bottom flask. To this solution were added 3 ml of a sodium periodate solution with 177.33 mg/ml concentration in order to obtain approximately 27% degree of oxidation.

The reaction time was 24 hours under magnetic stirring, ambient temperature and in the dark.

Neutralization of the mixture was made by adding 1 ml of ethylene glycol for 15 minutes. Then it was dialysed against water for 3 days and subsequently lyophilized yielding a white “fluffy” product.

2.3.8. Periodate oxidation of sodium alginate

To a solution of sodium alginate (1g; 40 ml distilled water) was added 1.08 g of sodium periodate. The mixture was magnetically stirred for 24 hours in the dark.

The reaction was then neutralized with 2 ml ethylene glycol for 15 minutes.

To start the purification process 500 mg of sodium chloride and 60 ml ethanol were added in order to obtain a precipitate. The resulting polymer was dissolved in 20 ml distilled water again and re-precipitated by addition of 40 ml ethanol. This step was repeated three times. The precipitates were pump filtered and dried at room temperature under vacuum.

2.3.9. Synthesis of 3-amino-7-Diethylaminocoumarin

In a round bottom flask with magnetic stirring were poured 5 ml of HCl 37.4% and slowly added $SnCl_2$ (1.6 g; 7.12 mmol) until it became a colorless solution.

3-nitro-7-diethylaminocoumarin (Coumarin-NO_2) was very slowly added in small portions for a period of 30 minutes. The reaction occurred for 4 hours at room temperature.

The solution (yellowish) is placed in an ice bath in order to cool down up to 3 to 5ºC, and then an aqueous solution of sodium hydroxide (5 M) was slowly added to obtain alkaline pH. With this addition the colour of the solution changed, yielding a darker (reddish) colour and the formation of a precipitated.

The extraction was achieved by adding diethyl ether (2x25 ml). Then the organic and aqueous phases were separated (the organic phase was yellow and the other one had the precipitated).

The organic phase was washed with 50 ml distilled water, dried with sodium sulfate and the solvent was evaporated in the rotary evaporator, yielding a pasty residue which was then triturated with hexane to obtain a solid compound.

2.3.10. Nucleophilic addition between oxidized dextran and oxidized sodium alginate and 3-amino-7-diethylaminocoumarin

500 mg of oxidized dextran were dissolved in 10 ml phosphate buffer saline (PBS). To that mixture was added 1 mg of sodium cyanoborohydride ($NaCNBH_3$).

Separately, 2 mg of 7-diethylamino-3-amino coumarin were dissolved in 1 ml ethanol. This solution was then added dropwise to the first one in order for the two of them to mix slowly. The reaction was stirred for 24 hours in the dark and with ambient temperature.

The solution was then poured into dialysis bags (M_w cut off 3.5kDa) and dialysed against water for 3 days. At last the liquid was lyophilized to obtain a yellow “fluffy” product.

The same procedure was applied to oxidized alginate changing some quantities: 200 mg oxidized alginate; 10 ml PBS; 3 mg $NaCNBH_3$ and 2.64 mg 3-amino-7-diethylaminocoumarin dissolved in 1 ml methanol.

2.3.11. Synthesis of Alginate/Modified oxidized alginate beads

A solution of sodium alginate and oxidized alginate with concentration 1% (80 mg sodium alginate; 20mg oxidized alginate; 10 ml distilled water) was prepared. This solution was stirred at approximately 250 rpm. 200 ml of a Calcium Chloride solution with 0.05 M of concentration were previously prepared also.

In a beaker cup were poured 50ml of the second solution, subsequently 2.5 ml of the first solution were added dropwise with the help of a needle, yielding beads with the size of the droplets. This procedure is portrayed in figure 15.

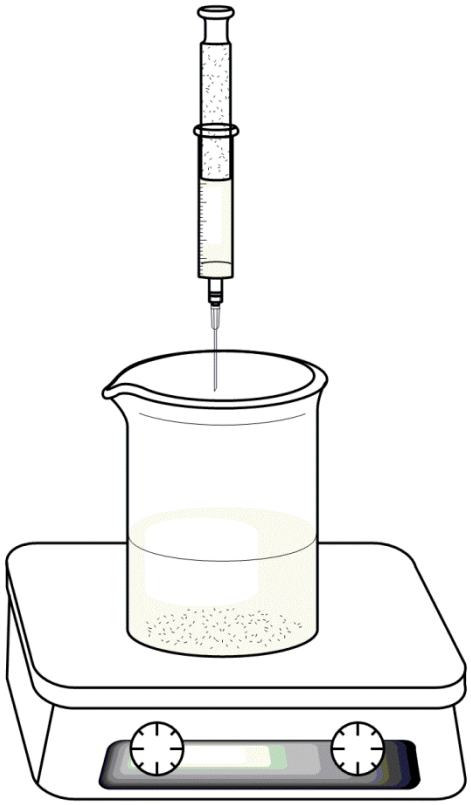

Figure 15 - Schematics of the synthesis of the alginate/modified oxidized alginate beads.

2.3.12. Nucleophilic addition between oxidized sodium alginate and 2-aminoethyl methacrylate

The procedure used to obtain this product cannot be specified due to protection of intellectual property.

CHAPTER III

RESULTS AND DISCUSSION

In this chapter will be described and discussed all the results obtained throughout this work. For better understanding it is crucial to divide the experimental work in three parts.

The first part consisted in the attempt of modification of dextran with PA-Cl and AP-Cl in order to functionalize dextran with alkyne and azide groups, based on the information present in literature [33]. The modification using AP-Cl was also carried out in sodium alginate. In this phase of the experimental work was also tried a modification of dextran directly with CDI and after a functionalization with propargyl alcohol and 3-azidopropanol.

The second concerned the periodate oxidation of dextran and sodium alginate with the objective taking advantage of the reactive characteristics of the aldehyde groups present in the structure of these polymers repeating units after this procedure [16, 34-36, 49].

The use of the polymers obtained in the second part of the work leaded to the third phase which consisted in the use of 3-amino-7-diethylaminocoumarin (fluorescent) as sources of amine groups for nucleophilic addition and reductive amination (reduction of imine group to a secondary amine by sodium cyanoborohydride) with oxidized dextran and oxidized sodium alginate in order to try to synthesize a fluorescent probe of interest for encapsulation purposes. In this phase was also carried a procedure between oxidized sodium alginate and 2-aminoethyl methacrylate as a source of amine groups for the same type of reaction to obtain a functionalized product with methacrylate reactive groups.

3.1. Modifications with alkyne and azide groups

The procedures realized in this part of the work were based on the literature with some alterations on the scale of the reactions and the quantities of the reagents used in some of the processes (see appendix A) [33].

The first products to be synthesized were the 3-azidopropranol (key ingredient in the synthesis of AP-Cl), PA-Cl and AP-Cl because of their importance in the modification process. These three products were synthesized with success as expected and as it is described in sections 2.3.1, 2.3.2 and 2.2.3. The ^{1}H NMR results of these reactions is shown in appendix B.

The following step was to take advantage of the imidazole group of PA-Cl and AP-Cl to graft these products into the dextran and sodium alginate backbones. With dextran were tried the two modifications, with azide and alkyne groups, as to alginate only the one, with the azide groups.

After the ^{1}H NMR analysis of the modified polymers it was very difficult to evaluate the success of the reactions because the characteristic peaks for the azide and alkyne groups were not visible as expected. However it was possible to notice the presence of imidazole characteristic peaks in some spectra.

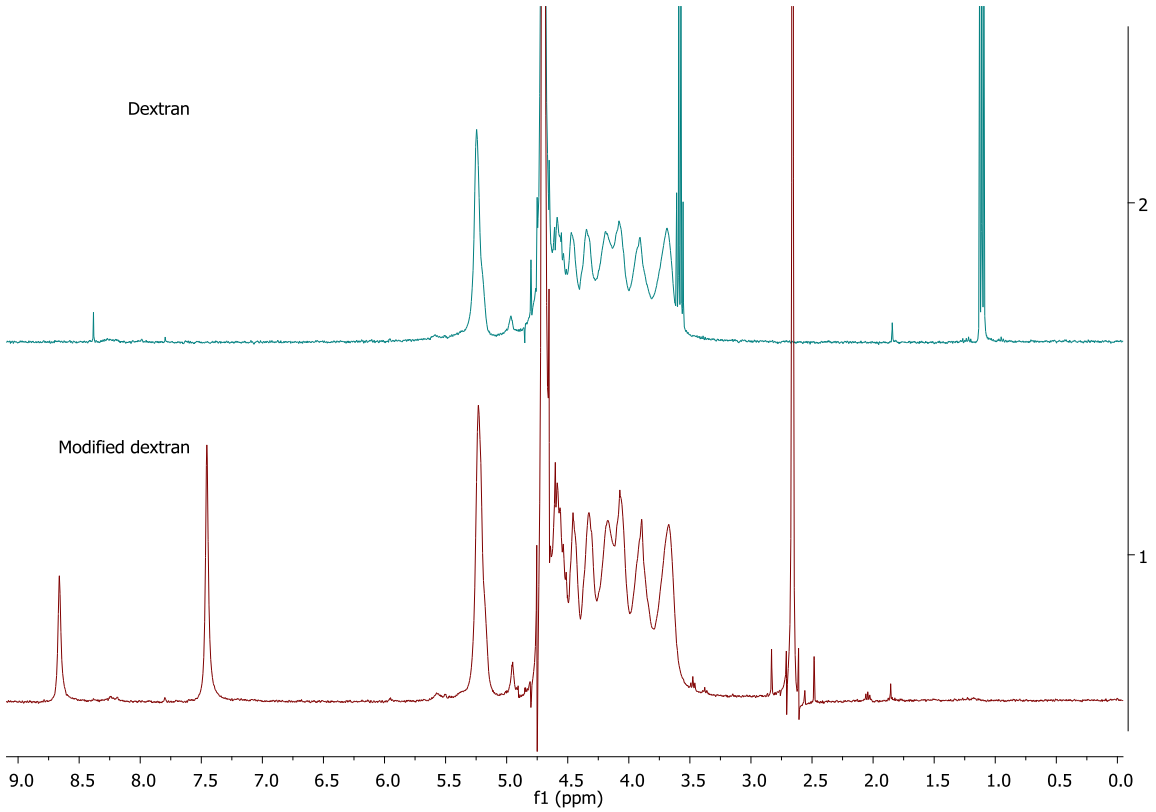

Figure 16 – ^{1}HNMR spectra of dextran (M_w~10200) and dextran after the attempt of modification with AP-Cl.

This result is portrayed in figure 16 were the imidazole peaks (δ = 7.45 ppm and 8.66 ppm) are clearly visible. The appearance of this imidazole groups in the structure of the modified polymer gave place for a rethinking of the modification process, because the dialysis, during the synthesis of this compounds, should be responsible for the elimination of any remains of imidazole. The appearance of these signals can be an evidence of the attachment of imidazole to the polymer.

This fact repeated itself in the modification of sodium alginate as we can see in figure 17.

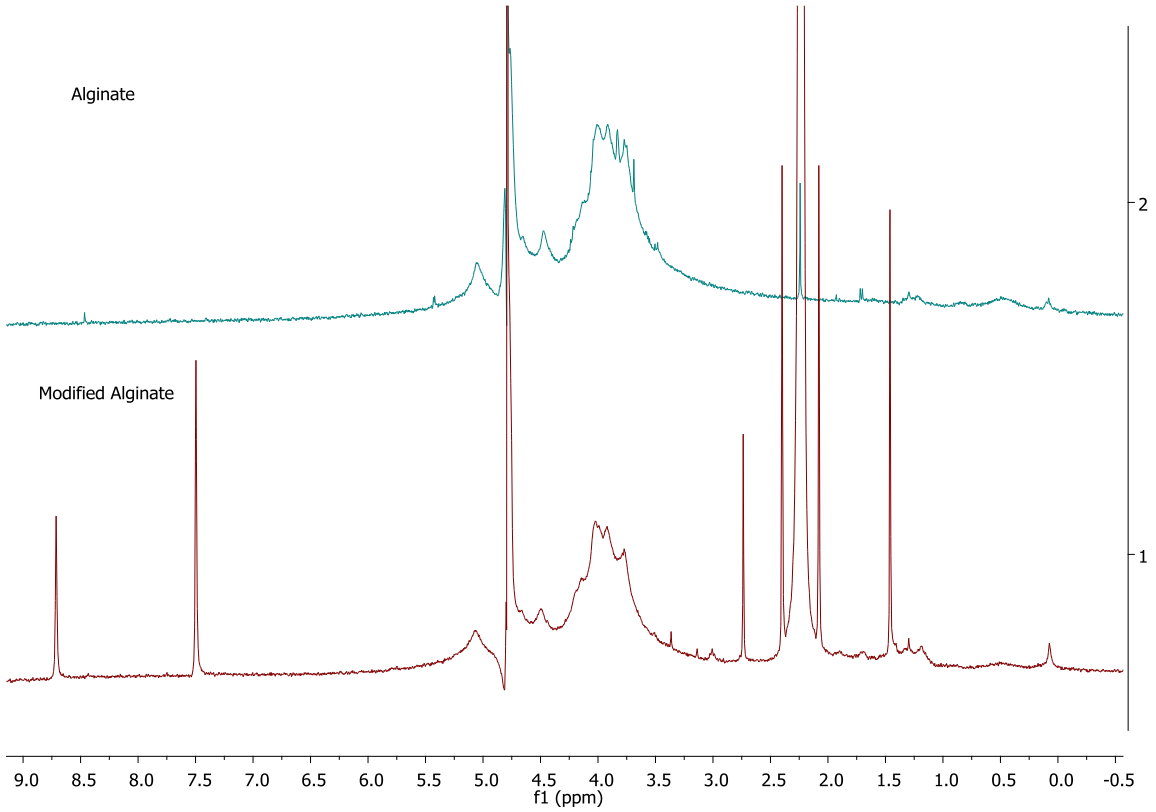

Figure 17 – ^{1}HNMR spectra of sodium alginate and the attempt of modification of sodium alginate with AP-Cl.

The results of the modification of dextran with PA-Cl are shown in appendix C as they are inconclusive in order to evaluate the modification.

The next step was to explain the reason of the appearance those imidazole groups in the structure of the polymer. So we came up with the most reasonable theory that in the reaction process, the AP-Cl molecule instead of breaking the C-N bond yielding the azide groups to the dextran backbone, it was breaking the C-O

bond yielding the imidazole groups instead of the azide. This mechanism is shown in figure 18. In appendix D are presented the FTIR spectra that confirm this result.

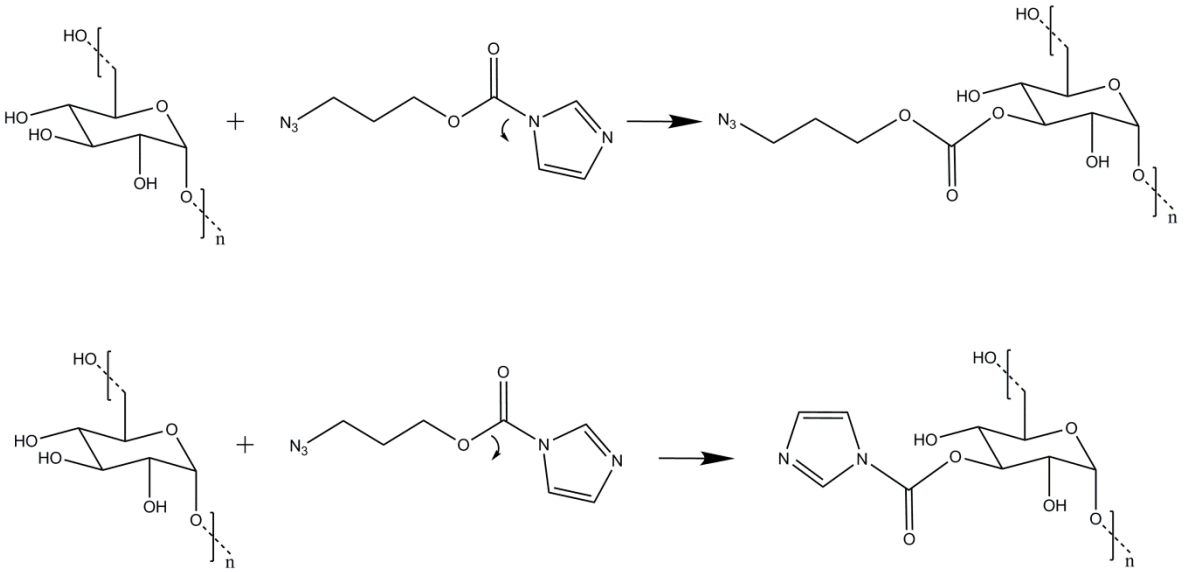

Figure 18 - Schematics of the modification of dextran with AP-Cl. (1) Shows how the modification was intended and expected and (2) shows how it occurred.

With this in mind, it was decided to do some changes to the modification process. The aim was to invert the order of the reaction in order to first modify dextran with CDI to obtain imidazole groups in the dextran backbone, since it was already achieved in the previous procedure, and then input the azide and alkyne groups by taking advantage of the previously grafted imidazole group.

The reaction conditions were pretty similar to the ones applied in the first modifications. However CDI proved to have a large affinity to dextran and the result of this was the formation of a cross-linked gel. To avoid the formation of this compound, were tested several reactions varying the amount of CDI added. Nonetheless the formation of the gel was constant as we can see in table 4.

Table 4 - Data regarding the amounts of dextran and CDI used in the testing of the formation of the dextran-CDI gel.

Reaction	Dextran (M_w~70 kDa) (g)	CDI (g)	Molar ratio	Gel formation
1	0.5	0.8	1.6	Positive
2	1	0.3	0.3	Positive
3	0.5	0.1	0.2	Positive

The products obtained by these procedures were not soluble in any of the solvents available for NMR analysis, so the characterization was made by FTIR spectroscopy. The possible explanation for this crosslinking was the fact that the high reactivity of imidazole groups cause the dextran chains to react on both sides of the CDI molecule. This process is shown in figure 19.

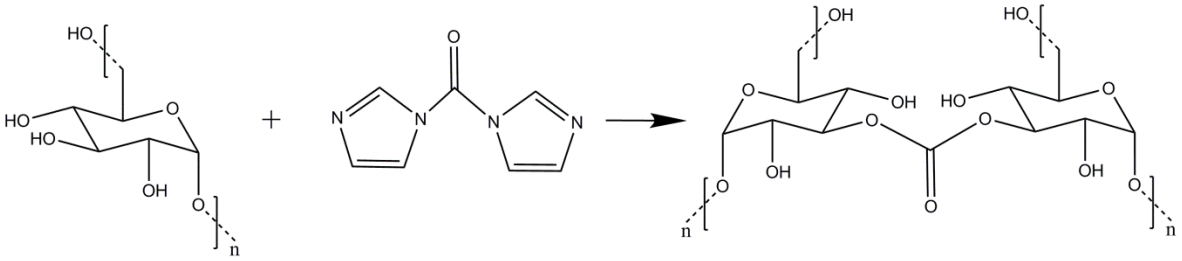

Figure 19 - Schematics of the crosslinking reaction between dextran and CDI.

The FTIR spectra of this product are shown in appendix E, and it shows a band that can be attributed to the carbonate group yielded by the cross-linking reaction. This modification falls a bit off the objective intended when it was realized. However the properties of the product obtained are susceptible to be interesting for other applications out of the scope of this work. With these results in mind, it was crucial to choose another path to input some modifications to the polymers in hand.

3.2. Periodate oxidation of dextran and sodium alginate

The periodate oxidation of dextran and sodium alginate can be contextualized in a new strategy adopted after the results obtained so far. The decision was to synthesize fluorescent compounds out of dextran and sodium alginate that could be good alternatives to the already existing. This strategy was in accordance to the needs of the Ph. D. work that contextualizes this project.

The first step to obtain these fluorescent compounds was to oxidized both polymers with sodium periodate. The procedures of oxidation were carried out according to the literature [16, 34-36, 49]. However some changes had to be made

to the procedures described, like a scale down in the quantities of the reagents. The procedures were carried out as described in sections 2.3.7 and 2.3.8.

The expected outcome of these reactions was, as described earlier, to confer the structures of both polymers with aldehyde groups for further use in nucleophilic addition followed by reductive amination.

The results of these reactions were oxidized dextran and oxidized sodium alginate with oxidation degree of approximately 27% and 19% respectively.

The confirmation of the oxidation process was achieved by FTIR spectroscopy. Initially were acquired ^{1}H NMR spectra of the oxidized samples, however the results of this analysis were inconclusive because the formation of hemiacetals (a more stable structure) between the oxidized units and their vicinities in the chains of the polymers makes it impossible to observe the aldehyde characteristic peaks [16]. The ^{1}H NMR spectra of the oxidized polymers are shown in appendix F.

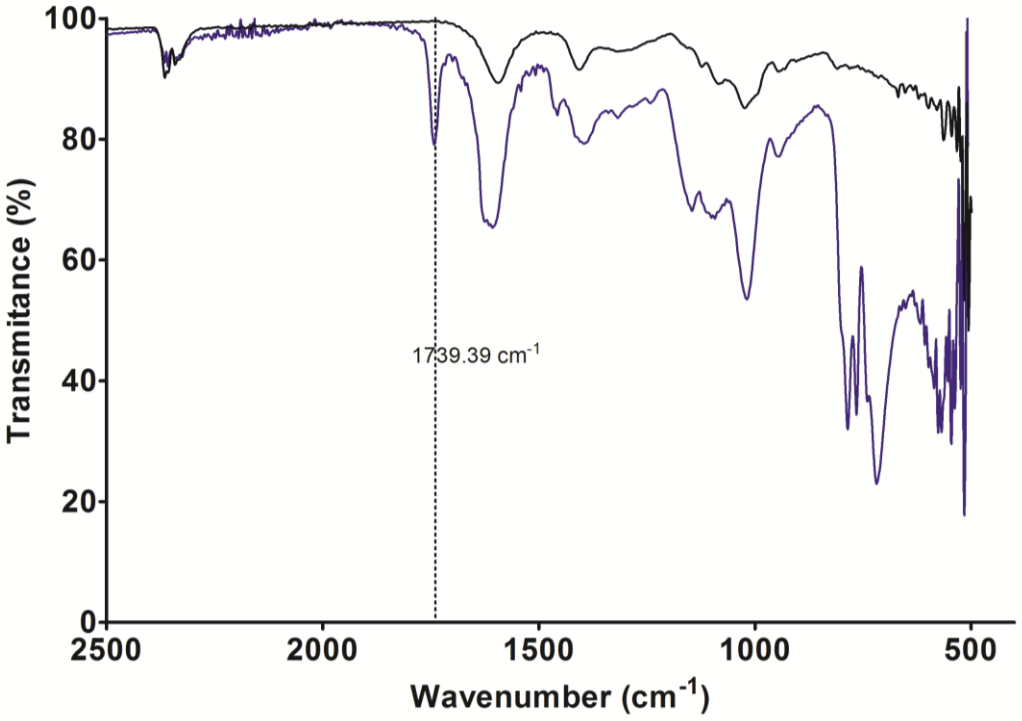

Figure 20 - FTIR spectrum of oxidized (blue) and regular sodium alginate (black).

In figure 20 is shown the result of the oxidation of sodium alginate, as it is visible the aldehyde characteristic band is approximately at 1739.39 cm^{-1}, very close to the reference value of 1730 cm^{-1} referred in the literature [50].

Concerning the oxidation of dextran, figure 21 shows the FTIR spectrum resultant of this procedure. It is visible a low intensity signal corresponding to the aldehyde functionalities at 1732.73 cm^{-1}, thus confirming the theoretical value of 1730 cm^{-1} as expected and described in the literature [38]. The low intensity of the characteristic signal is explained by the low degree of oxidation and also the formation of the hemiacetal structures within the structure of oxidized dextran, making it very hard to observe the aldehyde groups in the spectrum [16, 38].

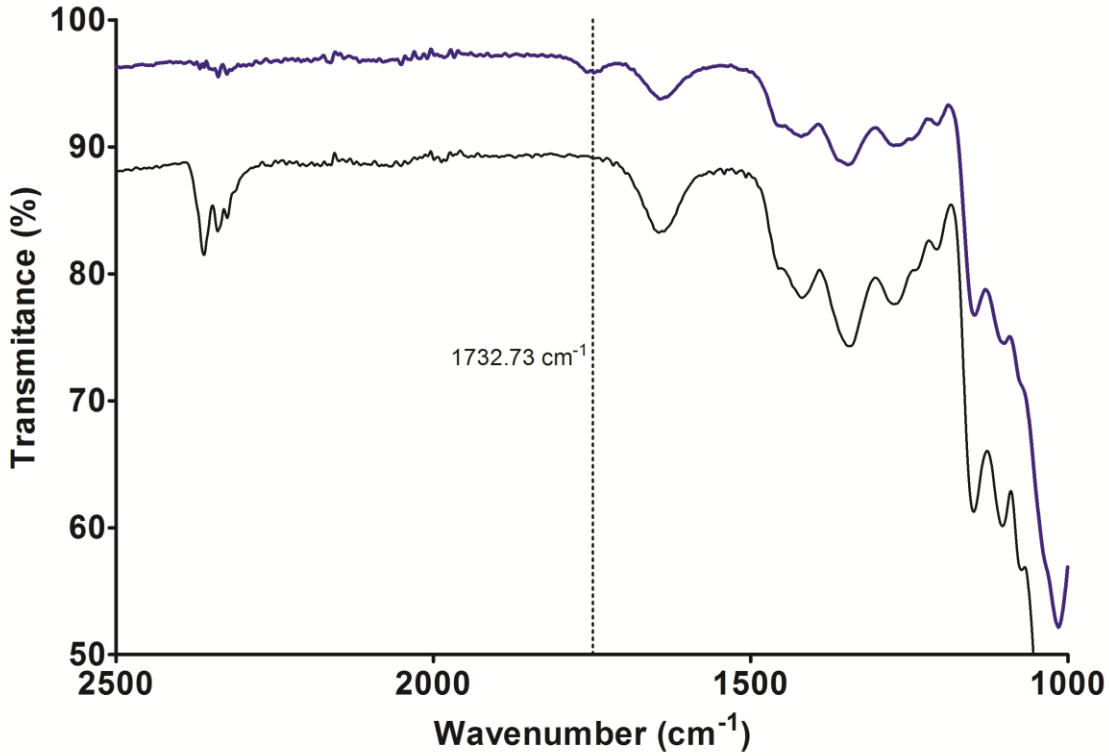

Figure 21 - FTIR spectra of oxidized dextran (blue) and regular dextran (black).

With the confirmation of the success of the oxidation procedures, the first step of this new pathway was complete. The next step was to use the functional groups obtained to attach the fluorescent compound into the polymers structure.

3.3. Nucleophilic addition between 3-amino-7-diethylaminocoumarin and the oxidized polymers

In this phase of the work the objective was to use the affinity between the aldehyde groups of the oxidized polymers and the amine groups of 3-amino-7-diethylaminocoumarin to confer fluorescent properties to the modified polymers.

The reaction mechanism is described in section 1.4.6, and basically involves a nucleophilic addition between the aldehyde and amine groups with subsequent reductive amination in the presence of sodium cyanoborohydride [51].

As mentioned in section 2.3.9 the quantities of 3-amino-7-diethylaminocoumarin (NH_2 - Coumarin) used in the procedures were very low, this is due to the fact that for higher values of this product in solution it was observed the formation of an unknown precipitate (when the two solutions are mixed) which influences negatively the properties of the final product. In table 5 and 6 are shown some data about this fact for both polymers.

Table 5 - Reaction data regarding the formation of a precipitate in oxidized dextran nucleophilic addition.

Reaction	Oxidized Dextran (g)	NH2 - Coumarin (g)	Molar Ratio	Precipitate
1	0.202	0.0576	0.285	Positive
2	0.0404	0.014	0.24	Positive
3	0.5	0.003	$4.2x10^{-3}$	Negative

Table 6 - Reaction data regarding the formation of a precipitate in oxidized alginate nucleophilic addition.

Reaction	Oxidized Alginate (g)	NH2 - Coumarin (g)	Molar Ratio	Precipitate
1	0.0808	0.01858	0.19	Positive
2	0.101	0.0202	0.17	Positive
3	0.1	0.003	0.025	Negative

Because of the low amount of 3-amino-7-diethylaminocoumarin used in the reactions, the results from ^{1}H NMR and FTIR spectroscopy are not conclusive about the success of the modification (see appendix F). Nonetheless when exposed to UV radiation (366nm wavelength) the samples showed fluorescence (figure 22), also the existence of some fluorescent material in the dialysis disposal water suggests that the fluorescent material is linked to the structure and not only adsorbed.

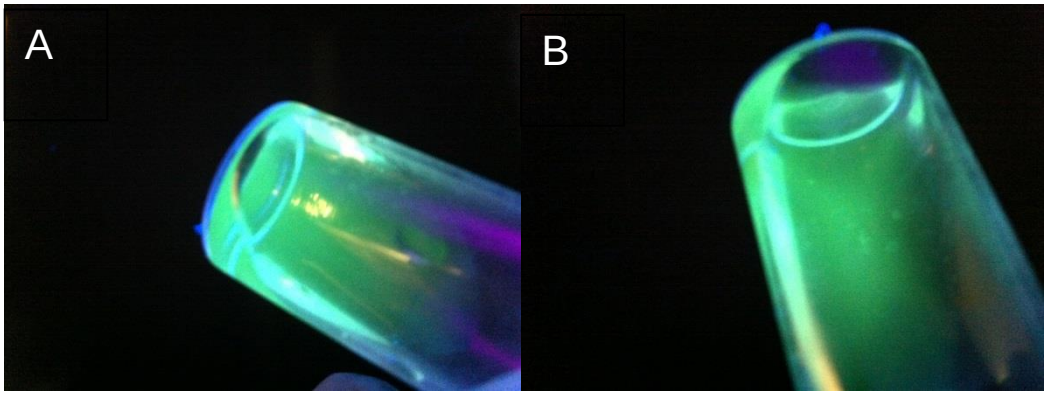

Figure 22 - Aqueous solutions of modified oxidized dextran (A) and oxidized alginate (B) exposed to UV radiation.

The fluorescence seen in the pictures of figure 22 leaded to further investigation about the fluorescence properties of these compounds.

The first step was to obtain an absorbance spectrum by ultraviolet-visible spectrophotometry in order to get to know the peaks of maximum absorbance of radiation by the samples. The results of this analysis (figure 23) were the following:

- For modified oxidized dextran were recorded two absorbance maximums, the first at 237 nm and the second at 393 nm;
- For modified oxidized alginate only one maximum was registered at 280 nm.

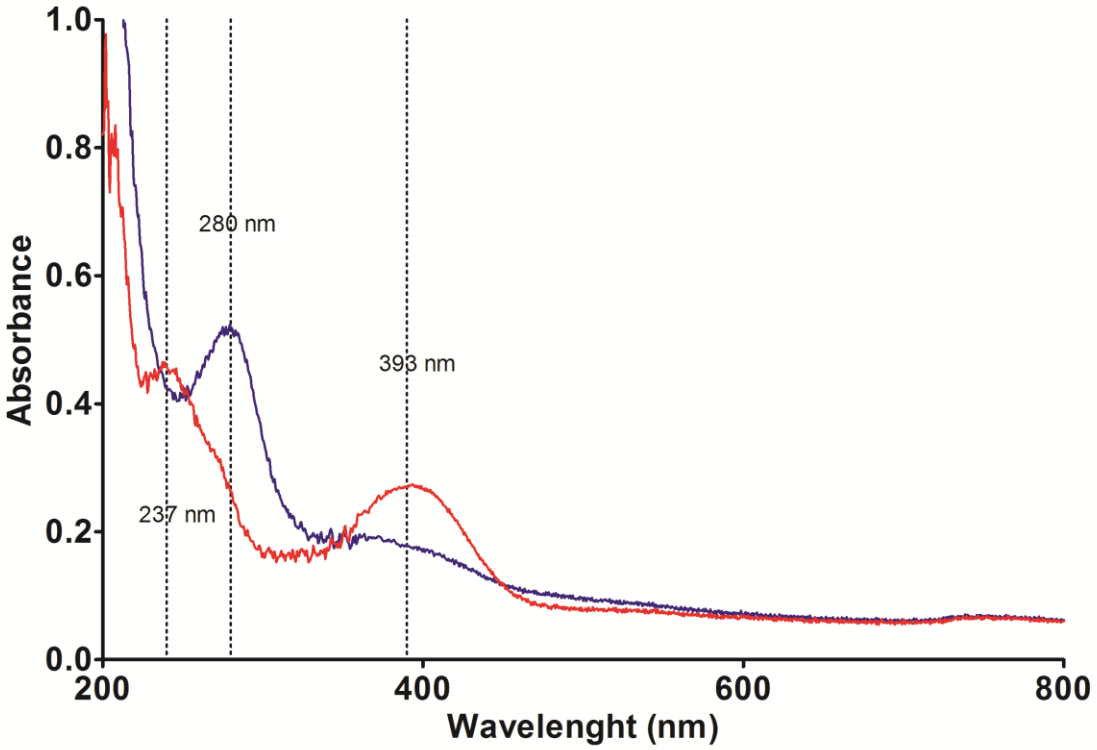

Figure 23 - Absorbance spectrum for the modified polymers, dextran (red) and alginate (blue).

The previous analysis yielded important information about the fluorescent behaviour of the samples. The next step was to use this information to produce an emission and an excitation spectrum to complete the fluorescence study.

The emission spectrum showed the wavelength of the fluorescence emission maximum, is to say the spectrum of emission wavelengths while the excitation light is kept constant in the absorbance maximum wavelengths. The excitation spectrum is obtained with the emission light kept constant in the emission maximum and the excitation light scanning the wavelengths.

The results of this emission and excitation where obtained with a fluorescence spectrophotometer and the results are showed in figures 24 and 25.

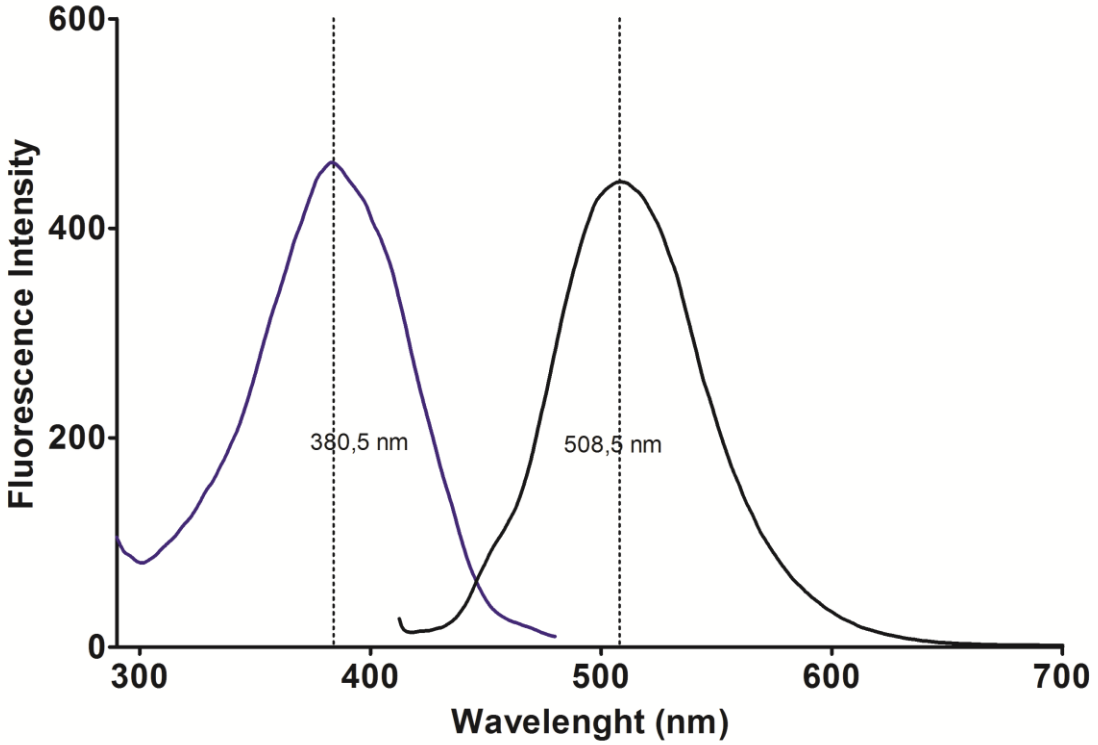

Figure 24 - Fluorescence spectra obtained from oxidized dextran modified with 3-amino-7-diethylaminocoumarin: excitation spectrum (blue) and emission spectrum (black)

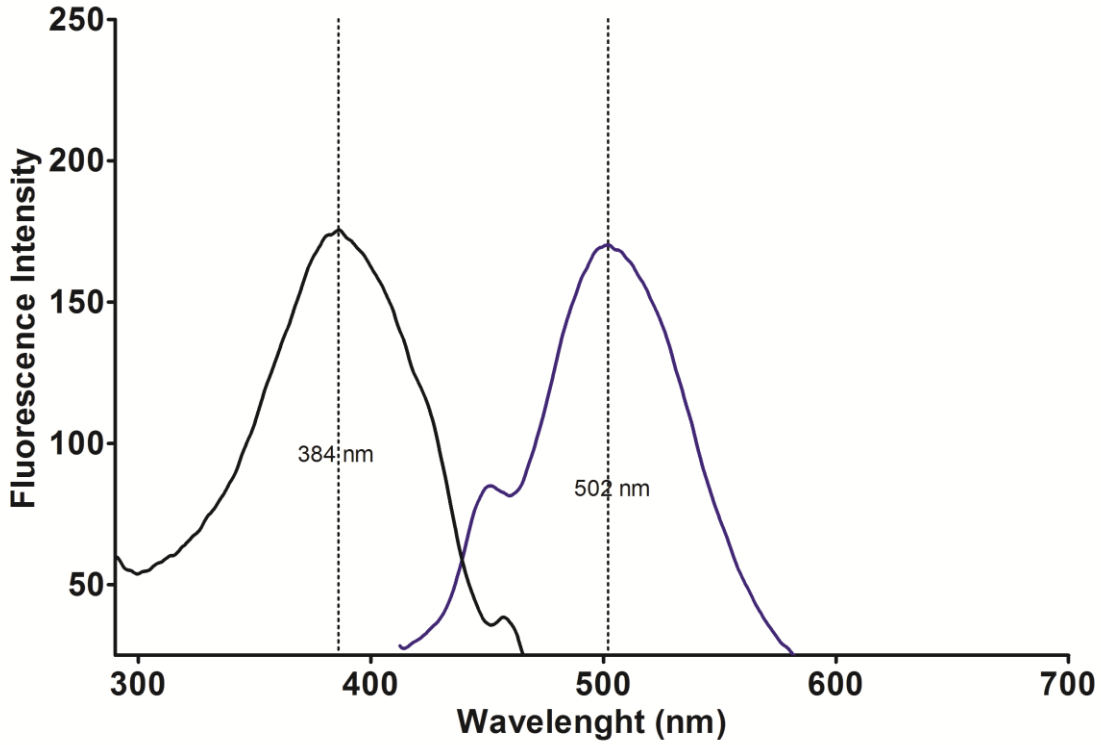

Figure 25 - Fluorescence spectra obtained from oxidized alginate modified with 3-amino-7-diethylaminocoumarin: excitation spectrum (black) and emission spectrum (blue).

The results shown that both products have very similar properties in terms of fluorescence with excitation maximums of approximately 380 nm wavelength and emission maximums of about 500 nm. This data shows the consistency of the procedure of modification for both oxidized polymers. When compared to the fluorescence data obtained from 3-amino-7-diethylaminocoumarin (see appendix G) we can see that the obtained results are pretty consistent with the presence of this compound in the structure of the oxidized polymers.

To further investigate the effect of these modifications on the properties of the original polymers we decided to use one of them, specifically the modified oxidized alginate, to produce alginate beads and see if not only the ion binding properties remained intact but also if the obtained beads would be fluorescent.

The procedure of producing the beads is described in section 2.3.11.

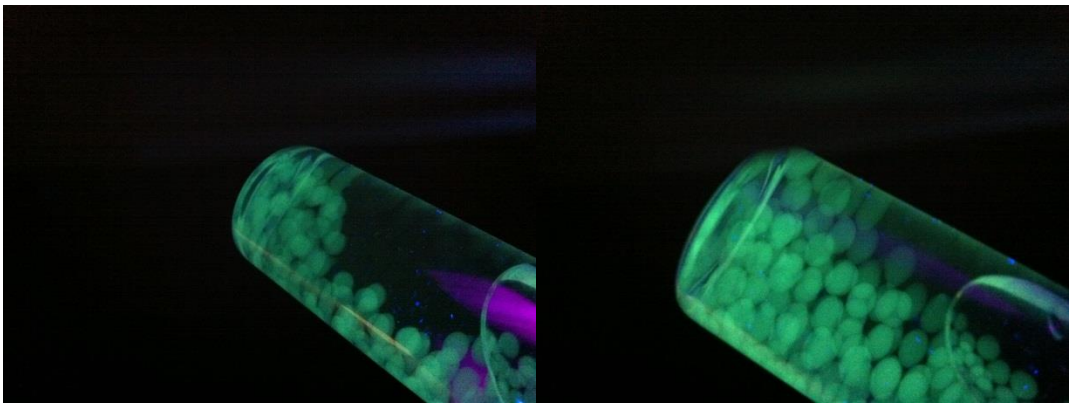

Figure 26 - Fluorescent alginate/modified oxidized alginate beads.

Figure 26 shows pictures of the Alginate/Modified oxidized alginate (80%/20%) beads obtained. It is clearly visible that the beads are fluorescent. Although the fluorescence intensity could be higher if there was available a UV lamp with an excitation wavelength closer to the 380 nm approximate maximum. This excitation was made at 366 nm. It was also attempted to capture the fluorescence with the help of a fluorescence microscope (see appendix H). However the excitation wavelength of the microscope (~494 nm) does not allow the visualization of the beads because it is too distant from the 380 nm of the

product excitation peak. Nonetheless these results proved that besides all the modification steps and new properties acquired, the products obtained possess some of the good properties of the original polymers which grant their usefulness in the same kind of applications.

3.4. Nucleophilic addition between 2-aminoethyl methacrylate and oxidized alginate

The results obtained in this modification are not able to be disclosed for protection of intellectual property reasons.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

Concerning the modification of dextran and sodium alginate with alkyne and azide groups it was difficult to synthesize the products described in the literature. Furthermore the reproducibility of the process was not quite good and alternatives had to be found.

With the results in hand it was decided to realize further procedures with some changes on the order of the reactions to try to achieve the modification by a different path. This pathway leaded to the production of a cross-linked polymer (gel) that can contain interesting properties. The properties of this product were not extensively explored due to time limitation and the focus of the work turned to other direction.

The change in strategy guided the line of work towards the production of fluorescent natural based polymers that could have an interesting application spectrum in uses that require fluorescence to identify structures.

The synthesis of these products involved two phases. The first was the periodate oxidation of dextran and sodium alginate; the second phase was the reaction between the aldehydes of the oxidized polymers and the amines of 3-amino-7-diethylamiocoumarin. The two phases were carried out of this synthesis were successfully accomplished.

The subsequent study of the fluorescence properties of the obtained products showed that they were very well defined and specific in accordance with the fluorescence values of 3-amino-7-diethylaminocoumarin. However it is obvious to conclude that in terms of applications this product has very specific parameters, requiring very specific excitation wavelengths (approximately 380 nm) that sit on the borderline of the most commonly used filter combinations (FITC-Red Texas; DAPI-FITC; FITC-TRITC and DAPI-FITC-TRITC). Nonetheless it has interesting properties for use in applications that involve the formation of particles and encapsulation.

Another use given to the oxidized alginate previously synthesized was the functionalization with methacrylate groups, using a procedure based on the same principles as the one applied for the fluorescent products. The 2-aminoethyl methacrylate modified oxidized alginate was successfully obtained. This modification yields a functionalized product that can be very useful in various biomedical applications.

Other advantages of these products are:

- low cost;
- They are easy to produce and do not require a lot of specific reactions conditions (ambient temperature, normal atmosphere);
- Easy to use (water soluble, easy to manipulate, non-toxic);
- Distinct features (fluorescence, functional groups in their structures).

For future work it would be interesting to develop two aspects resulting of this work. The first aspect is the necessity of a complete study of the properties of the cross-linked polymer obtained by the direct modification of dextran with CDI. The extensive knowledge of this product's properties (i.e. "swelling" capacity, chain mobility among others) may result in the discovery of a product with high interest for biomedical application. The second aspect worthy of future development is the use of the fluorescent products as well as the 2-aminoethyl methacrylate modified oxidized alginate in studies involving the encapsulation of cells or bioactive agents and also investigate their usefulness in drug delivery systems.

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APPENDICES

Appendix A: Reaction tables for the procedures of modification with alkyne and azide groups

Synthesis of PA-Cl

Table 7 - Reaction table for the synthesis of PA-Cl.

	Ratio	mmol	Qtd.	Qtd. Real
Propargyl alcohol	1	50	2,89 ml	2,9 ml
CDI	1,8	90	14,59 g	14,77 g
Dichloromethane	-	-	100 ml	100 ml

Synthesis of 3-azidopropanol

Table 8 - Reaction table for the synthesis of 3-azidopropanol.

	Ratio	mmol	Qtd.	Qtd. Real
3-chloropropanol	1	0,179	14,96 ml	2,9 ml
Sodium azide	2	0.358	23,278 g	23,30 g
THS	8,21	1,47	0,5 g	0,5 g

Synthesis of AP-Cl

Table 9 - Reaction table for the synthesis of AP-Cl.

	Ratio	mmol	Qtd.	Qtd. Real
3-azidopropanol	1	37.5	3.48 ml	2,9 ml
CDI	1,8	56.2	9.12 g	14,77 g
Ethyl acetate	-	-	100 ml	100 ml

Synthesis of dextran-propargylcarbonate (dex-C≡C)

Table 10 - Reaction table for the synthesis of dex-C≡C.

	Ratio	mmol	Qtd.	Qtd. Real
Dextran (M_w~10.2 kDa)	1	6.167	1 g	1.01 g
PA-Cl	0.3	1.86	0.280 g	0.286 g
DMSO	-	-	20 ml	20 ml

Synthesis of dextran-azidopropylcarbonate (dex-N3)

Table 11 - Reaction table for the ynthess of dex-N_3.

	Ratio	mmol	Qtd.	Qtd. Real
Dextran (M_w~10.2 kDa)	1	6.167	1 g	1.005 g
AP-Cl	1	6.16	1.204 g	1.200 g
DMSO	-	-	20 ml	20 ml

Appendix B: ^{1}H NMR spectra of 3-azidopropanol, AP-Cl and PA-Cl

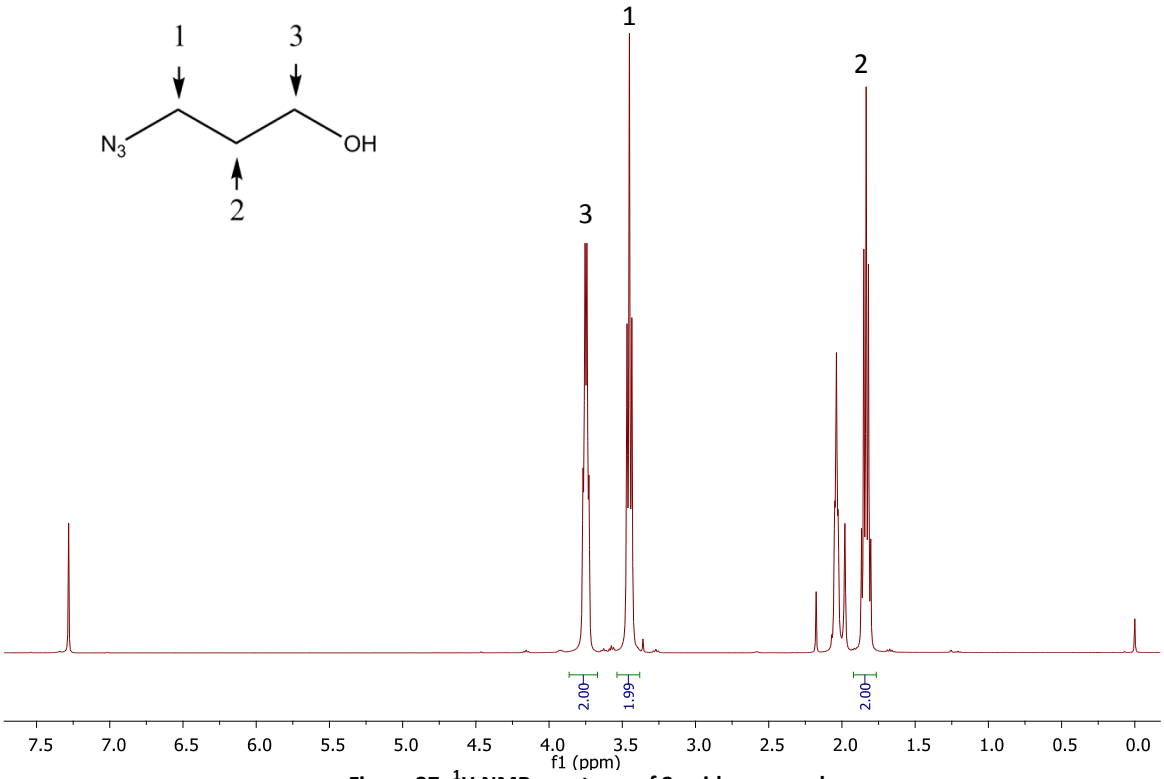

Figure 27- ^{1}H NMR spectrum of 3-azidopropanol.

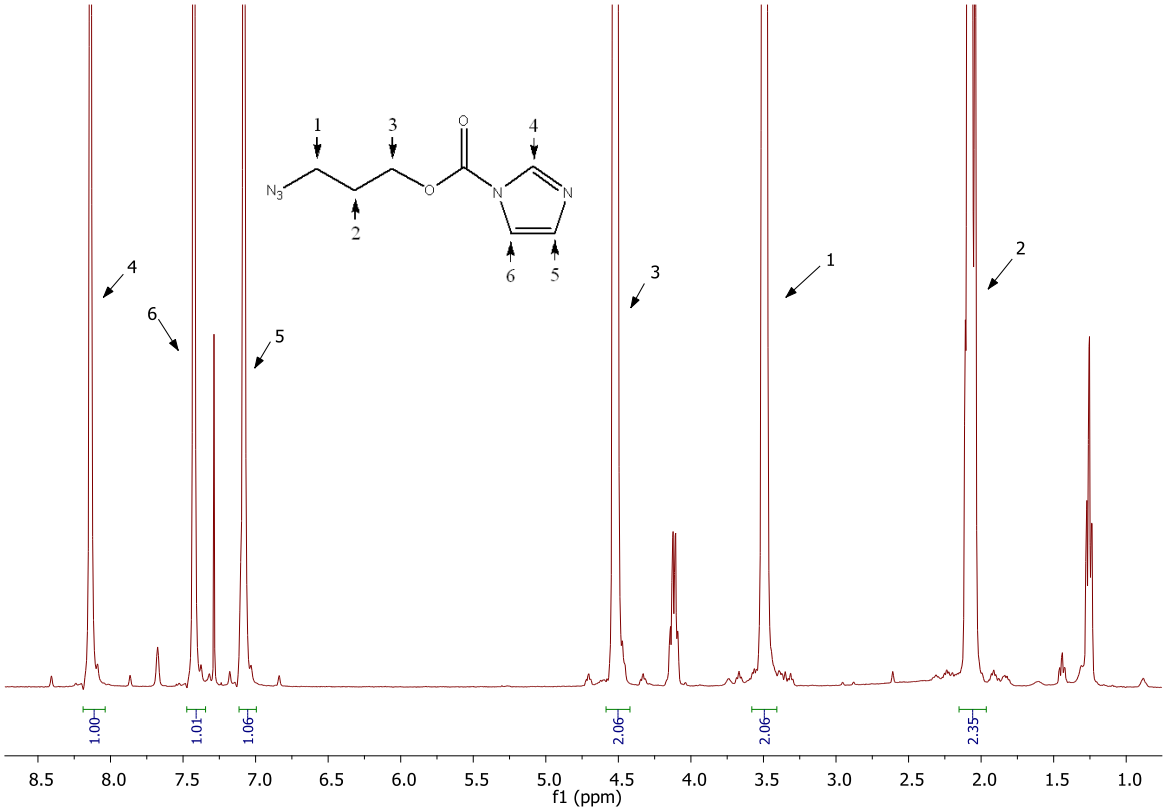

Figure 28 - ^{1}H NMR spectrum of AP-Cl.

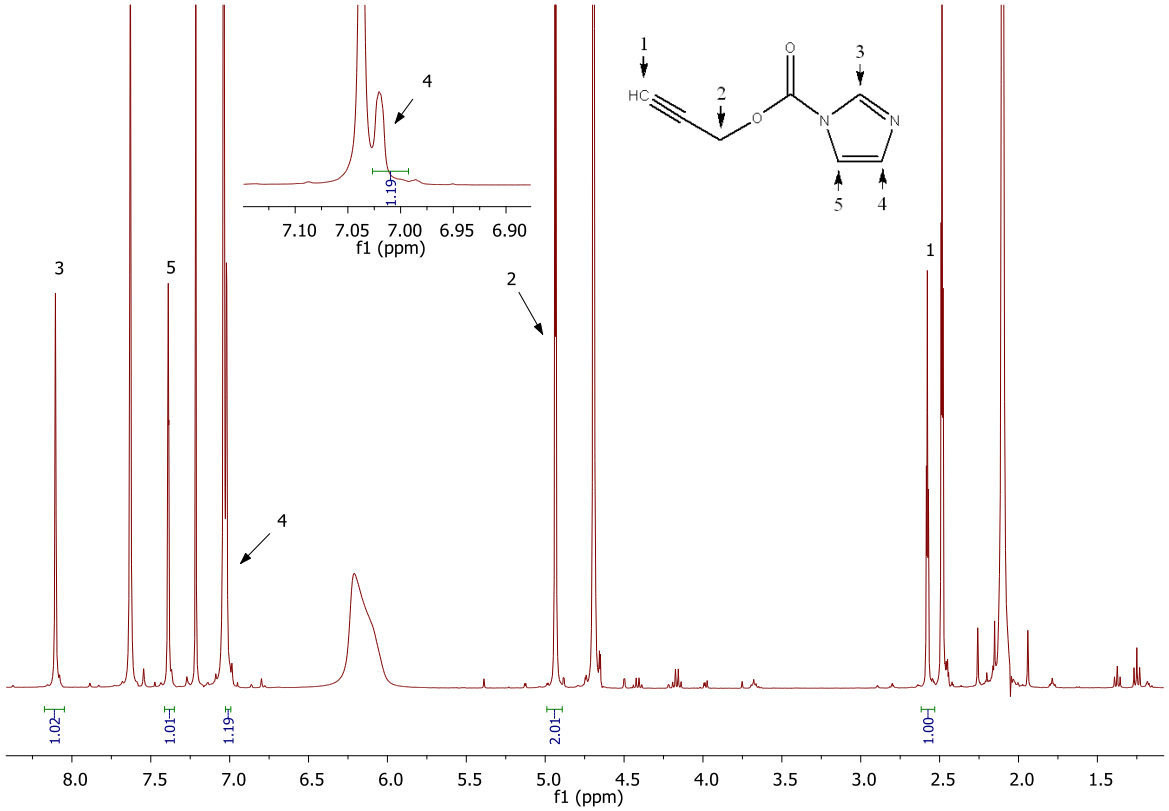

Figure 29 - ^{1}H NMR spectrum of PA-Cl.

Appendix C: ^{1}H NMR spectra of the modification of dextran with PA-CL

In figure 31 is clearly visible a change in the structure of the polymer; however the peaks are not sufficiently defined to confirm the intended modification.

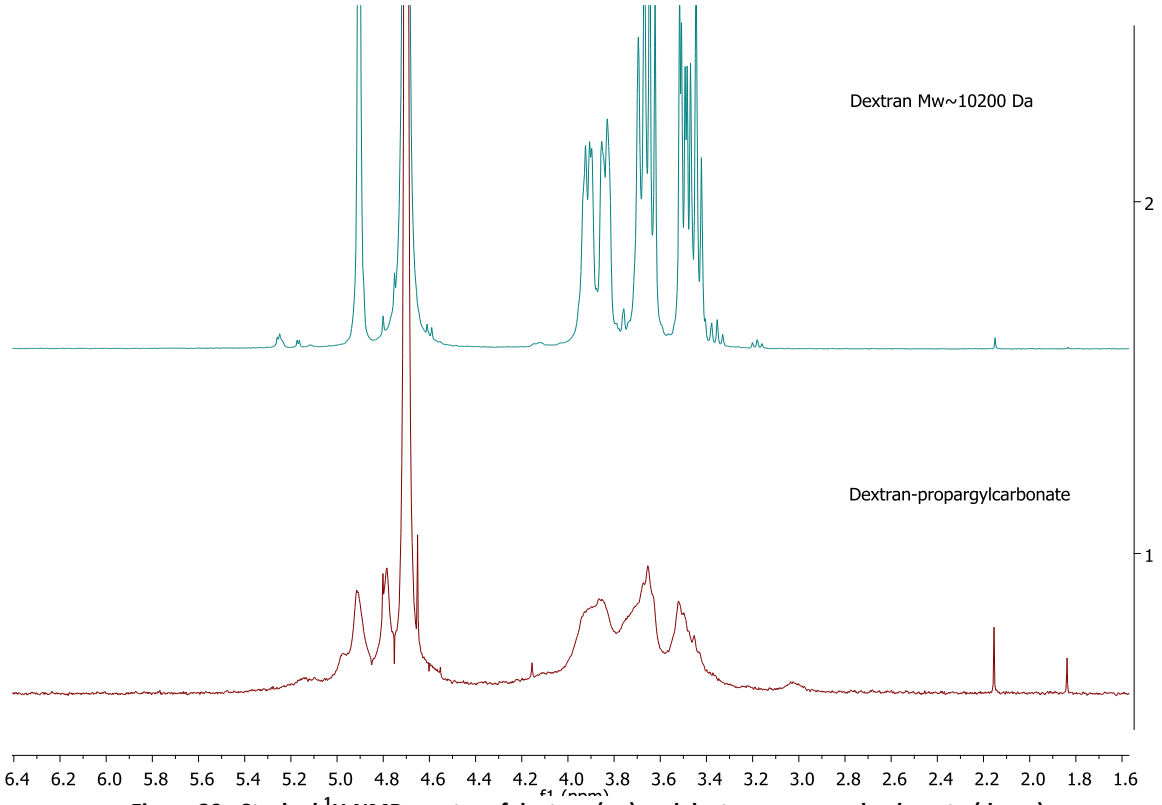

Figure 30 - Stacked ^{1}H NMR spectra of dextran (up) and dextran-propargylcarbonate (down).

Appendix D: FTIR spectra of the modification of sodium alginate with AP-Cl

In figure 32 are highlighted two bands corresponding to the characteristic bands of the carbamate group that resulted from this modification. However only one band is clearly visible (1720.19 cm^{-1}) , the other band his hidden by another band belonging to the polymer.

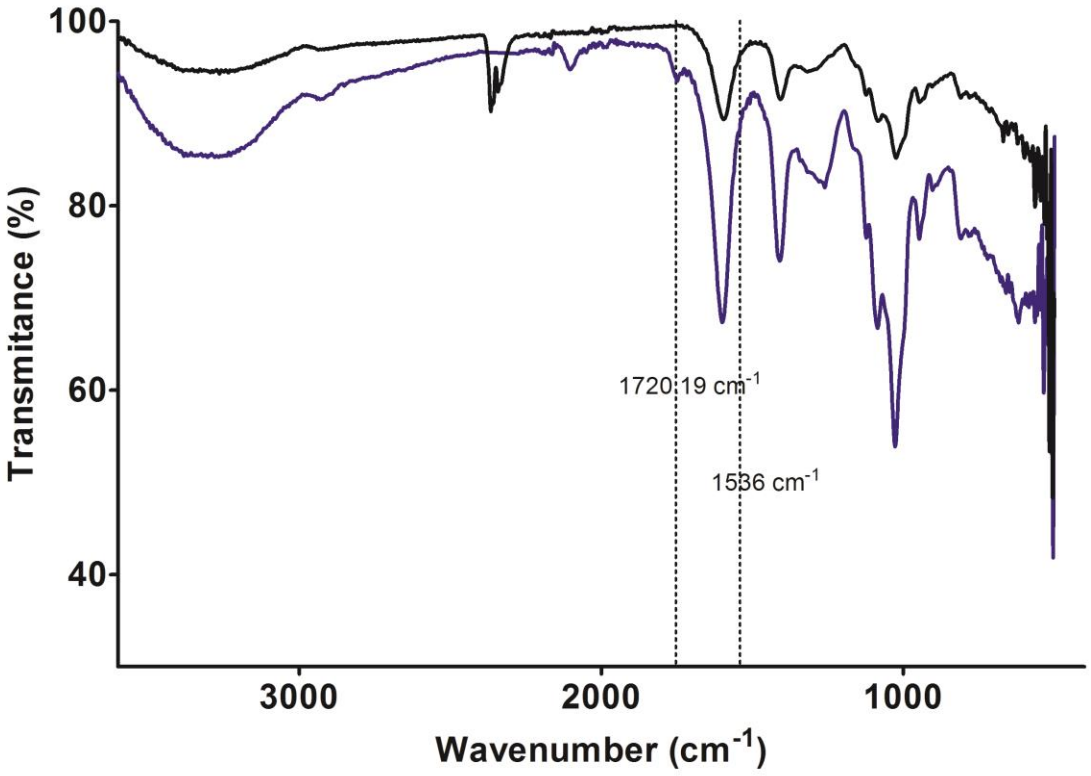

Figure 31 - FTIR spectra of sodium alginate (black) and AP-Cl modified sodium alginate (blue).

Appendix E: FTIR spectra of dextran directly modified with CDI

Approximately between 1678 and 1837 cm^{-1} is visible the appearance of a band (figure 33) which can be attributed to a carbonate group formed in the cross-linking process.

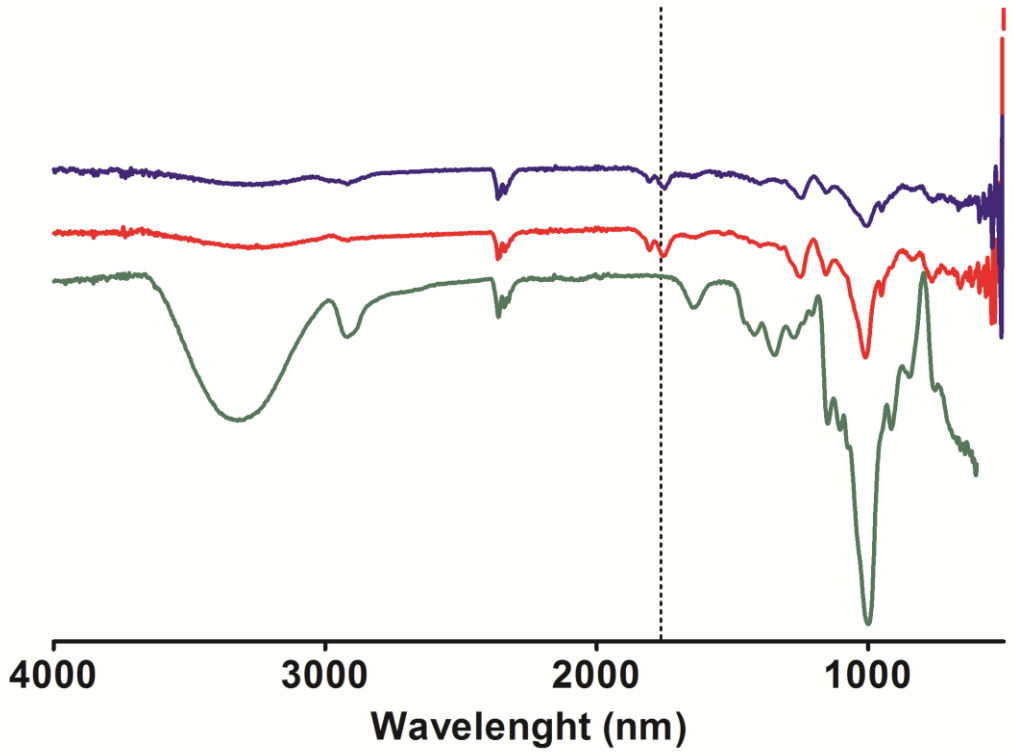

Figure 32 – Stacked FTIR spectra of dextran (Mw~70 kDa) (green) and two samples of dextran modified with CDI in different proportions (blue 0.1g CDI and Red 0.3g CDI).

Appendix F: ^{1}H NMR spectra of the oxidized dextran and sodium alginate

For both polymers, by the analysis of the NMR spectra (figure 33 and 34) it can be affirmed that the structure suffered some alterations. However there is no way to do a precise analysis, because the peaks of the fluorescent products are not visible.

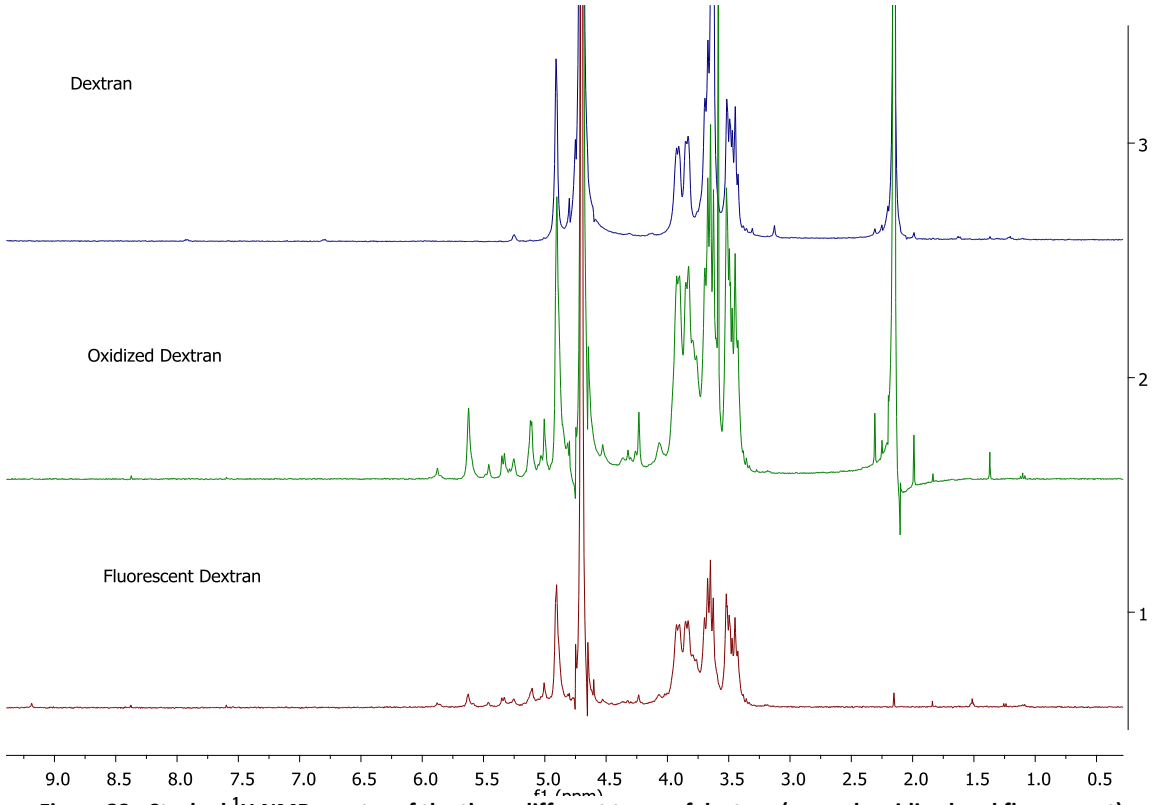

Figure 33 - Stacked ^{1}H NMR spectra of the three different types of dextran (normal; oxidized and fluorescent).

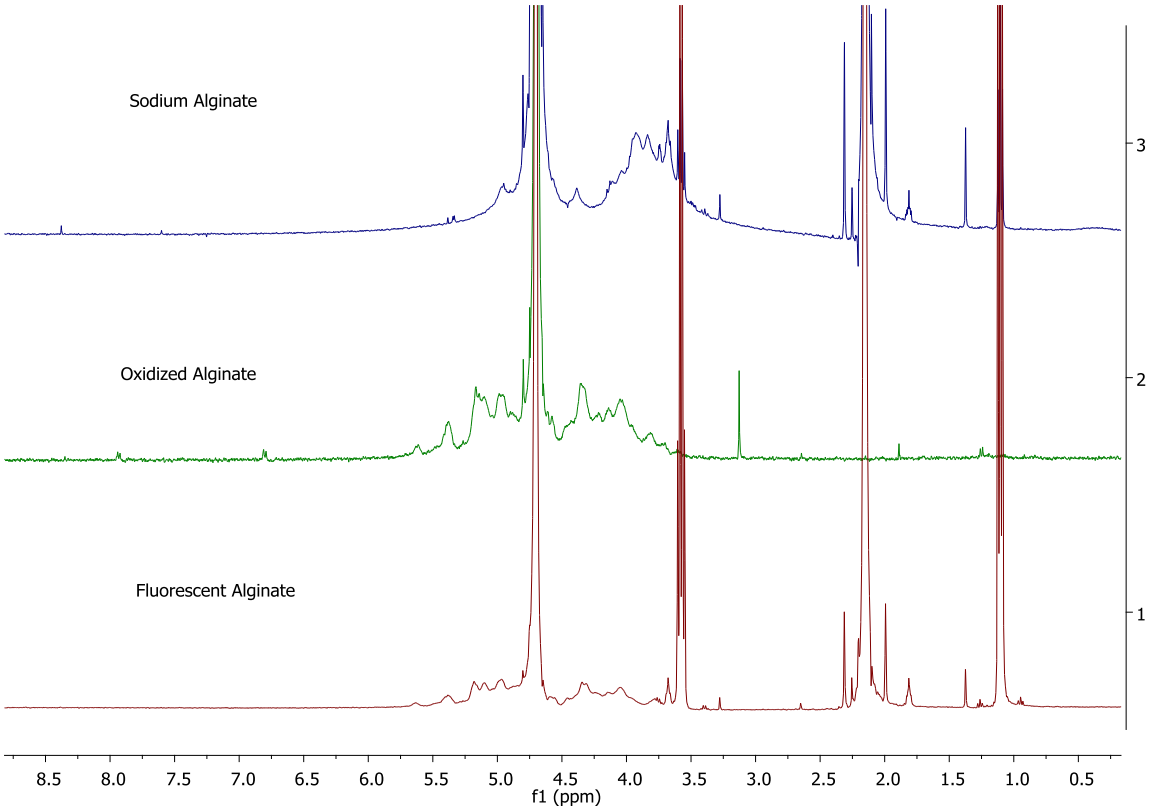

Figure 34 - Stacked ^{1}H NMR spectra of the three different types of alginate (normal; oxidized and fluorescent).

Appendix G: Fluorescence data for 3-amino-7-diethylaminocoumarin

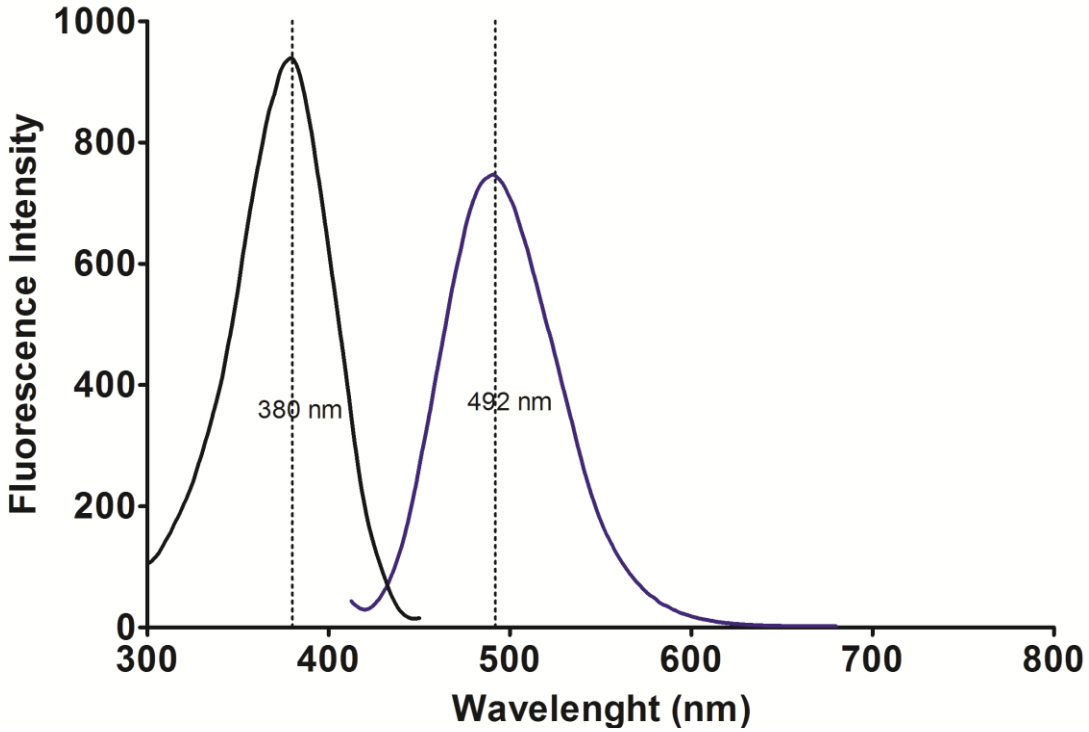

Figure 35 - Excitation (black; maximum at 380 nm) and emission (blue; maximum at (492 nm)) spectra of 3-amino-7-diethylaminocoumarin.

Appendix H: Microscope and magnifier photos of the obtained beads

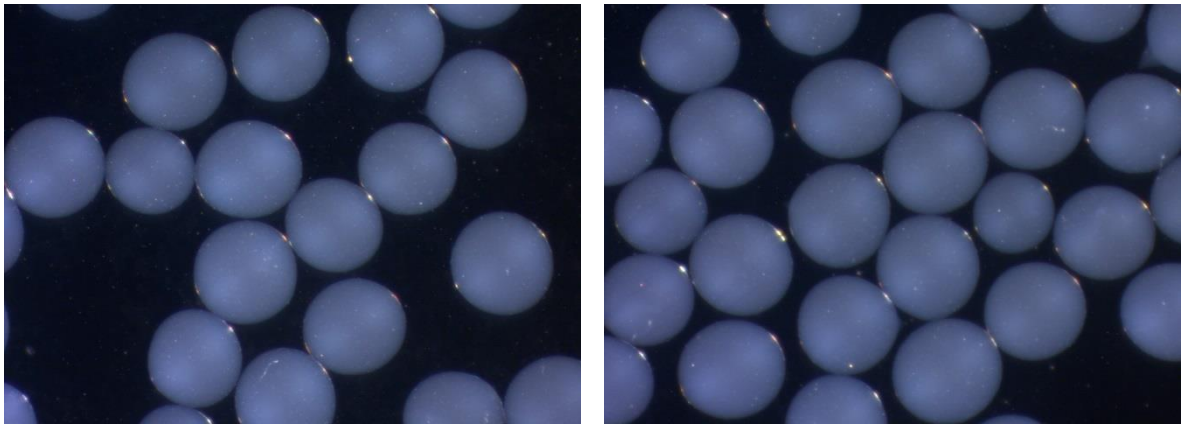

Figure 36 - Photographs of the alginate/fluorescent alginate beads obtained with a magnifier (amplification 3.2x).

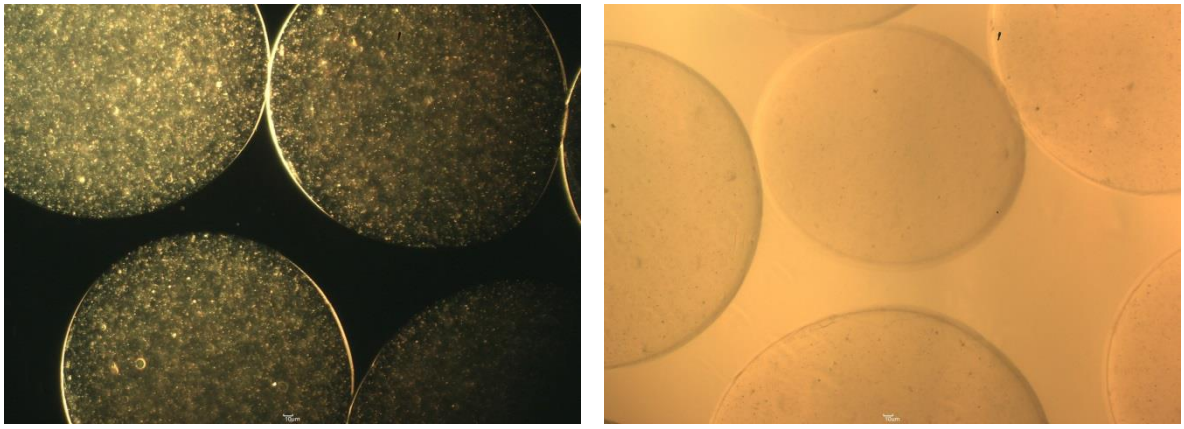

Figure 37 - Photographs of the alginate/fluorescent alginate beads obtained with an optic microscope (amplification 40x). On the left it was used phase contrast microscopy and on the right bright field microscopy.