**Title: "The role of anionic polysaccharides in the preparation of nanomedicines with anticancer applications".**

**Running title: "Anionic polysaccharides in anticancer nanomedicines"**

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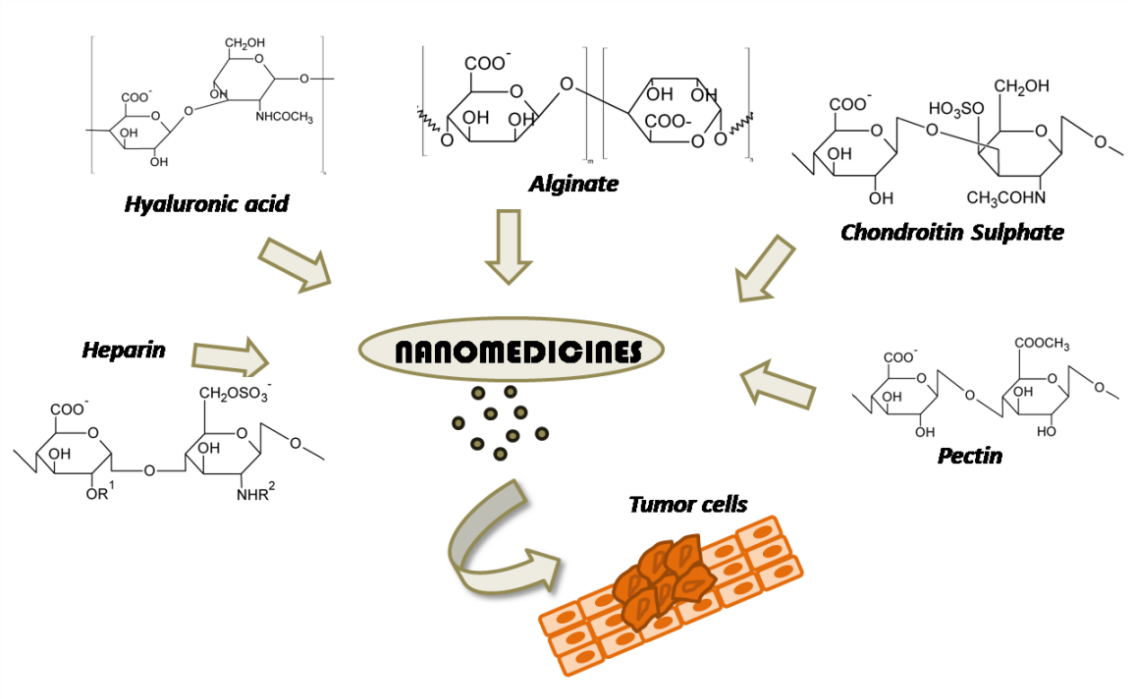
**ABSTRACT**

Cancer has become one of the main causes of death in developed countries, and it is expected to be declared as the disease with the highest worldwide morbidity and mortality indexes in the coming decades. Nanomedicine aims to overcome some problems related of this prevalent disease, particularly the lack of efficient diagnostic and therapeutic tools. The most recent scientific advances conducted to a more personalized medicine have been focused on the production of nanocarriers involved into the transport and the delivery of drugs to specific and targeted cells. A wide variety of nanocarriers composed by different materials have been designed for their use as drug delivery systems. Polysaccharides have emerged as very useful biopolymers among the raw materials used in the preparation of these nanoplatforms. They are highly stable, non-toxic and biodegradable molecules, and present also some chemical properties which are very difficult to reproduce using artificial polymers. Anionic polymers, such as hyaluronic acid, heparine or alginate, present some structural and chemical characteristics which make them ideal polymers to prepare nanosystems with anticancer applications. This review will focus on the description of some anionic polysaccharides and the possibilities that they offer towards the preparation of nanosystems with applications in cancer treatment and diagnostics.

**GRAPHICAL ABSTRACT**




## 1. INTRODUCTION

Carcinogenesis is a multistep disorder caused by genetic and epigenetic alterations where cells mute into malignant forms with an abnormal potential of proliferation and invasion of healthy tissues (1). Cancer has become one of the most important leading causes of death worldwide, partly because of the progressive aging of population or the adoption of some non-healthy lifestyle behaviors (such as smoking, poor diet or physical inactivity) (2). Statistics indicate that a total of 1,658,370 new cancer cases and 589,430 cancer deaths are projected to occur in the United States in 2015 (3). Numbers are not much inspiring in the European Union, since a total of 1,359,100 cancer deaths are predicted in 2015 (766,200 men and 592,900 women) (4). Additionally, cancer is becoming a substantial economic burden on society. The main health-costs are usually associated with prevention and management of the disease, but there are also significant lost earnings associated with costs of unpaid care provided by patients' friends and relatives (5). Consequently, there is an urgent need to achieve more screening measures to prevent and detect cancer, which would lead to a cost-effective public health system.

Cancer treatment is usually based on general therapeutic strategies which combined surgical resection, radiotherapy and chemotherapy. These strategies are normally characterized by non-specific mechanisms where healthy tissues become unnecessarily affected. Therefore, conventional therapies are commonly associated with significant morbidity and mortality rates together with low patients´ quality of life, caused by all serious adverse effects derived from their unspecific action. In this context, nanotechnology emerges as a promising tool to face all these problems and to develop more specific therapeutic agents (6). In fact, nanomedicine, defined as the application of nanotechnology for treatment, diagnosis, monitoring and control of biological systems (7), has become the focus of many researching projects and cherishes the hope of finding new effective tools to fight against cancer.

The main application of nanomedicine in cancer treatment involves the development of different types of drug nanocarriers. Nanoparticles, nanogels, liposomes, micelles or dendrimers are some of the most exploited systems with biomedical applications (8). These devices are designed to reach specifically cancer cells thanks to passive or/and active delivery strategies. Passive delivery is based on some physiological characteristics of solid tumors, such as a defective angiogenic vasculature, the enhanced permeability and retention (EPR) effect or hypoxic conditions. Active targeting implies a better knowledge of molecular mechanisms, like specific recognition of ligands by receptors expressed on tumor cells (9). In contrast to current anticancer treatments, nanocarriers offer some clear advantages, such as high loading capacity of poorly soluble drugs or better biodistribution and bioavailability values (10).

Additionally, the emerging generation of nanocarriers has supposed an authentic revolution in the field of biomaterials, especially in the group of polymers. In the last years, many polymeric materials have emerged to prepare these devices, either synthetic or natural molecules. In the case of synthetic polymers, poly(lactic acid), poly(glycolic acid) or polycaprolactone are some of the most representative molecules; in the case of natural biomaterials, polysaccharides, proteins or polypeptides have been very used to prepare nanocarriers (11).

Polysaccharides have increasingly become important in the field of nanomedicine due to their good properties. They are highly stable, non-toxic and biodegradable molecules, and also present some chemical properties which are very difficult to reproduce using artificial polymers. Additionally, they can be easily obtained from natural sources by using low cost processes (12). However, their versatile chemical structure is maybe their most challenging property for the design of new nanocarriers. Its wide variety of reactive groups, like hydroxyl, carboxyl and amino groups, leads to perform chemical modifications to introduce new physicochemical characteristics (13). Moreover, its reactive structure is also interesting to enhance the interaction with biological tissues and, consequently, increasing the residence time of the drug-loaded nanocarriers.

Taking everything into account, polysaccharides show a promising future as raw biomaterials in the preparation of nanocarriers. There is a wide variety of polysaccharides which have been assayed in the preparation of different drug carriers with anticancer applications (14, 15). The present work is focused on some of the most relevant anionic polysaccharides, and their important role in the preparation of drug delivery systems, including a brief description of their structural features, some of their preparation techniques, and their application in anticancer treatments.

## 2. ALGINATE

### *2.1 Chemical structure and properties of alginate*

Discovered by Edward Stanford in 1883, alginate is the generic name assigned to various natural biomolecules extracted from the intracellular matrix of different brown algae species, such as *Laminaria hyperborea*, *Ascophyllum nodosum, and Macrocystis pyrifera* (16). Bacteria are another source which alginate can be extracted from, including *Azotobacter vinelandii* and some *Pseudomonas* species (17). Despite the existence of bacterial sources, commercial alginates are usually obtained from harvested and dried algae following an acidic extraction.

Alginate is normally found as a salt form of various cations, like $Na^+$, $Ca^{2+}$, or $Mg^{2+}$. These alkaline earth cations are replaced by $H^+$ after an acidic reaction. Then, alginate is converted from the insoluble protonated form to the soluble sodium salt by the addition of sodium carbonate at pH below 10. After their extraction, alginates can be further purified and converted to salt or acid forms (18). Commercial production of alginate started in 1927, and it has been nowadays expanded to about 30,000 metric tons annually. A significant percentage of this tonnage represents the application of alginate in food, pharmaceutical and dental industry (19).

Alginate chemical structure is based on a backbone of (1-4) linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) (Figure 1). However, there exists some variability in the sequence pattern of GG, MG and MM blocks depending on the source and the species chosen to obtain the polysaccharide. Additionally, the enzymatic control during its production and the degree of depolymerization can also affect to its molecular weight. Therefore, there is a wide range of alginates with different composition and

molecular weights (18). Despite this kind of variability, commercial alginates usually have an average molecular weight of approximately 200,000 Da (20).

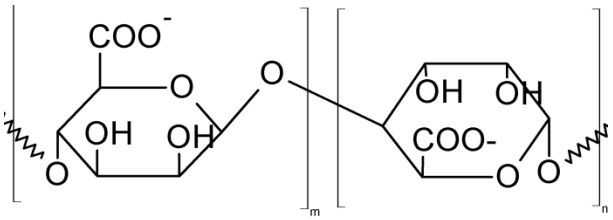



Figure 1. Chemical structure of disaccharide repeating unit of alginate.

Alginate presents some physicochemical properties which make it an attractive molecule for the design of drug carriers. First, it has the capacity of selectively binding divalent (for example, $Ca^{2+}$) and multivalent cations leading to the formation of alginate hydrogels. The stability of alginate hydrogels is directly related to the strong interactions that G-blocks establish with divalent cations, since M and alternating blocks usually form weaker junctions. Consequently, alginates with high G contents yield stronger gels (21). Additionally, alginate presents a high number of free hydroxyl and carboxylic groups along its backbone which are susceptible to be chemically functionalized by different techniques (e.g. oxidation, esterification, amidation, etc.). When these reactive groups are modified, all physicochemical and biological properties of alginate are affected; consequently, alginate becomes a very versatile polymer whose properties can be tailored according to its future applications (22). Finally, the solubility of alginate is directly modulated by three parameters: pH of the solvent, ionic strength of the medium and the presence of gelling ions in the solvent. While alginic acid is not fully dissolved in any solvent system, sodium alginate is completely soluble in water but not entirely dissolved in some organic solvents. Therefore, pH of the solvent should be adjusted above a certain critical value in order to modulate the solubility of the molecule (19, 23).

Regarding biological properties of alginate, its bioadhesion capability has been potentially exploded to develop drug carriers in order to increase the mean residence time of the drug within the body. The bioadhesion of alginate is mediated by its anionic charge, which allows better interactions with biological structures than neutral or cationic molecules (24). Additionally, alginate seems to cause a detectable immunogenic reaction when it is not completely purified from some mitogenic contaminants (like metals or endotoxins) derived from the extraction process. Some authors have taken advantage of the immunogenic capability of alginate to prepare vaccines by conjugating the polysaccharide with toxins (25). To some extent, there is some controversy regarding the immunogenic properties of alginate. Some authors declared that the immunogenicity of alginate was related with a higher content of M units than G blocks along its chain (26, 27); additionally, there are studies that did not find any significant immune reaction after the administration/implantation of highly purified alginates (28).

Taking everything into account, a high number of nanocarriers with biomedical applications based on alginate have been prepared in the last years. The following subsections will go in detail about the methodology used to obtain these alginate-based systems and their applications on anticancer therapies.

### *2.2 Preparation methods of alginate-based systems*

Among scientific literature, it is possible to find various methods to prepare alginate-based systems, such as hydrogels, microparticles or nanoparticles. Although there are some differences between synthesis protocols (like the method selected to obtain an adequate size distribution according to the type of device), most of them are based on two crosslinking procedures: ionic and covalent crosslinking.

Ionic crosslinking is based on the combination of an alginate aqueous solution with multivalent cations, such as divalent cations (i.e., $Ca^{2+}$). These ions bind to G blocks according to egg-box crosslinking model. A three-dimensional gel network is formed as a result of this ionic interaction, where interstices with cations are formed between alginate chains (28). The gelation rate and chemical structure of alginate are critical factors that affect to uniformity and mechanical properties of the devices obtained by this procedure.

Covalent crosslinking methods emerged as an approach to enhance the mechanical stiffness of ionically crosslinked devices. Plastic deformation is observed when mechanical stress is applied to ionically crosslinked gels, whereas an elastic behavior is observed in the case of covalent crosslinked forms (29). Mechanical properties and swelling behavior of alginate based-systems can be tailored by using different types of crosslinking molecules and by controlling crosslinking densities. However, covalent crosslinkers are usually toxic, and their excess needs to be removed completely from the final alginate-based systems.

Considering both gelation processes, there is a wide range of devices which can be prepared using alginate as raw material (30, 31).

#### *2.2.1 Alginate hydrogels*

Hydrogels are three-dimensionally crosslinked networks composed by hydrophilic polymers with the ability to retain water by swelling, mimicking the high water content of the extracellular matrix (32). Alginate is one of the most used biomaterials for their preparation following several chemical and/or physical crosslinking techniques.

Ionic gelation with divalent cations is commonly performed to obtain alginate hydrogels. Since gelation occurs very fast, it is necessary to control the process in order to obtain hydrogels with good mechanical properties. Some alternatives to control the gelation step include the use of phosphate buffers or water soluble calcium salts in the mixture with alginate (33), and also maintaining low working temperature conditions during the gelation process (34, 35). Additionally, chemical structure of alginate might also affect to hydrogel properties. Normally, gels prepared from alginate with high G content will tend to be stiffer and more porous than gels obtained from alginate with low G content (36). Although ionic crosslinking is a gentle method to obtain alginate-based hydrogels, it usually leads to limited long-term

stability in physiological conditions (37). This problem can be partially overcome using covalent crosslinking techniques.

Covalent crosslinking methods usually include the use of poly(ethyleneglycol)-diamines or multi-functional crosslinking molecules, such as poly(acrylamide-co-hydrazide) (PAH) or adipic acid dihydrazide (AAD). It has been demonstrated that these molecules offer good control over degradation rates and mechanical properties of alginate-hydrogels. Additionally, photo-crosslinking reactions using appropriate chemical initiators can be also used to obtain covalent crosslinked alginate gels (28).

Finally, physical factors, like temperature, can be also used to prepare alginate hydrogels. Thermo-sensitive gels can be obtained by the combination of alginate with poly(N-isopropylacrylamide) (PNIPAAm), leading to a semi-interpenetrating polymer network prepared via in situ copolymerization (38).

### *2.2.2 Alginate microparticles*

Generally, the preparation of alginate microparticles follows a protocol that includes the external gelation of the polymer in the presence of suitable crosslinking cations. The material to be encapsulated is previously mixed with the alginate solution, and then the solution is dripped into a bath containing divalent cations, such as a $Ca^{2+}$ (39). The method used to form alginate beads determines the size distribution of the systems.

Large pellets (>1 mm diameter) are usually obtained using a simple syringe or pipette to drip the polymeric solution into the cationic bath and then, after curing the beads in the bath, they can be rinsed with water and air-dried. Coaxial laminar air-flow, electrostatic fields or vibrating nozzles can be introduced to obtain an improved yield, smaller size and narrower size distribution (40). However, the syringe/pipette-based methods usually have problems in large-scale production.

Micropellets (>0.2 mm diameter) can be prepared using atomization, emulsification and coacervation methods. Atomization/spray methods use a syringe pump and a loading syringe, and the alginate solution is delivered through an orifice of about 1mm in diameter. Emulsification methods implies the preparation of a w/o emulsion by mixing the alginate solution with the oil phase. The resulting small alginate droplets are then externally or internally gelled. Finally, coacervation methods use oppositely charged polyelectrolytes to form bilayer equilibrium phases under specific pH and ionic conditions, causing the dense coacervate phase to form microspheres (31).

### *2.2.3 Alginate nanoparticles*

Alginate can be also used to obtain particles with diameters usually ranging from 10 to 1000 nm in size. Nano-aggregates, nanocapsules or nanospheres can be obtained depending on the followed methodology (30).

Nano-aggregates are nanosystems with different morphology where the drug is physically dispersed. Alginate-based nano-aggregates can be obtained following two different methods. The first one is based

on the formation of a pre-gelled solution mixing low concentrations of alginate and calcium chloride. Then, pre-formed nanosized aggregates are dispersed in water as continuous phase and, finally polyelectrolyte complexes are obtained after the addition of a polycationic solution (poly-L-lysine, chitosan or Eudragit E100) (41). The second method developed by Chang and coworkers do not use calcium chloride or policationic solution to form nano-aggregates. Instead of this, they used sonication and oxidation procedures to self-assemble an amphiphilic thiolated alginate (42).

Nanocapsules are devices with an oily/aqueous core, which contains the drug, and are surrounded by a polymeric membrane. Alginate has been successfully used to produce nanocapsules by obtaining pre-formed droplets by an emulsion step, with subsequent gelation and solvent removal (31). The addition to other cationic polymers, like chitosan, to the ionic solution as well as the type of oil phase used for the preparation of the nanocapsules allow the particle size and the drug loading rate to be modulated (30).

Nanospheres are spherical particles with a gelled interior where the drug is physically dispersed. The most common procedure to obtain alginate nanospheres consists of a 2 step procedure (Figure 2) (31).

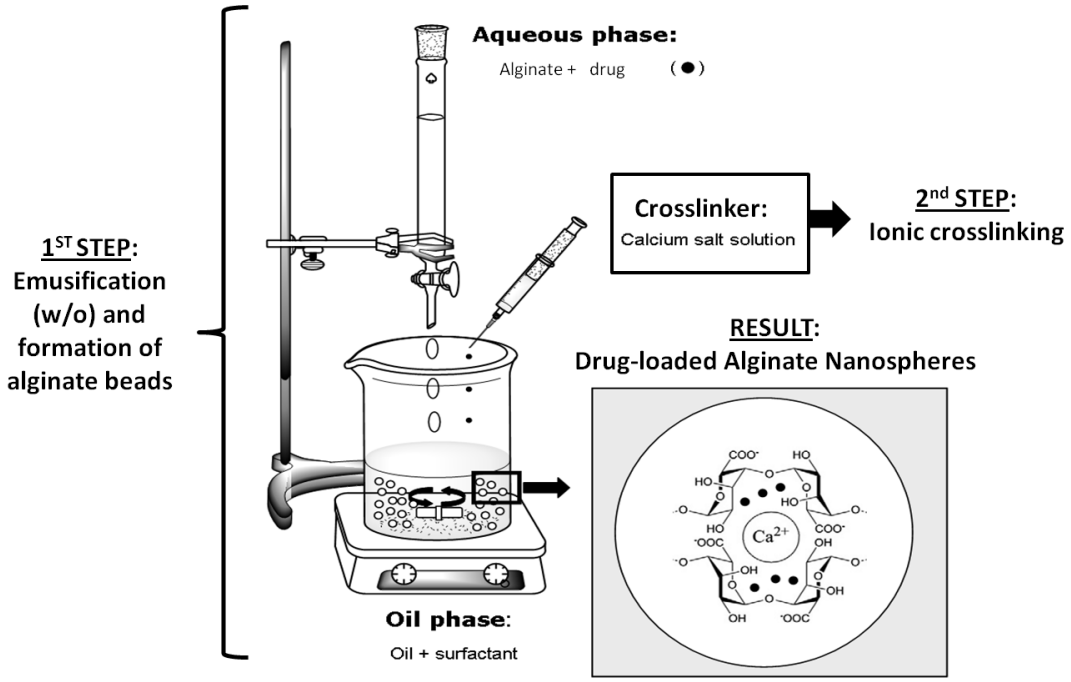



Figure 2. Preparation of alginate-based nanospheres.

First, the encapsulating molecule is dissolved in the alginate solution and then, this aqueous solution is emulsified in an oil phase obtaining a w/o emulsion. Once the alginate droplets have been formed, an ionic crosslinker is added to the emulsion to produce their gelation. The mechanical procedure used to

form the emulsion (e.g. sonication) can highly affect to the size of the particles. Additionally, internal or external gelation can also lead to different homogeneity (43).

Some variations of this methodology include the use of a w/o/w emulsion (44) or a single step procedure (45). Emulsion based technologies present the disadvantage of a difficult adaptation to the industrial scale, in contrast with nozzle procedures.

### *2.3 Alginate-based systems with anticancer applications*

Alginate-based systems offer good characteristics and promising features to deliver anticancer agents locally and specifically into tumor site. Several works have been published in the last years, and have shown how alginate-based devices seem to be interesting approaches to enhance current cancer chemotherapy.

Alginate hydrogels have been widely used as anticancer drug carriers for more than ten years. Bouhadir and coworkers designed an alginate hydrogel using a biodegradable spacer (adipic dihydrazide), and analyzed the delivery of three anticancer drugs: methotrexate, doxorubicin and mitoxantrone. They demonstrated that three different release mechanisms were achieved: diffusion, covalent bond degradation and ionic dissociation-controlled mechanisms. These mechanisms allow the control of drug release kinetics and the localized release of anticancer drugs in a sequential or simultaneous manner (46).

From this starting point, many researchers have recently tried to enhance the delivery of several cytotoxic compounds by designing alginate-based micro/nanohydrogels. Maciel and coworkers prepared alginate nanogels by crosslinking the polysaccharide with cystamine via a miniemulsion method. Then, doxorubicin was included into the nanogels by simply mixing and stablishing electrostatic interactions between the anionic alginate and the cationic doxorubicin. These nanogels showed good cytocompatibility, high drug encapsulation efficiency, an *in vitro* accelerated release of the drug in physiological reductive conditions, and quickly internalization by osteosarcoma cell lines. Consequently, these nanogels could be used for the delivery of doxorubicin and other cationic drugs in applications beyond cancer (47).

Apart from using a covalent crosslinking strategy, pH-sensitive hydrogels can be prepared using alginate as raw biomaterial. Several mixtures of sodium alginate and konjac glucomannan were combined with graphene oxide in order to prepare pH sensitive hydrogels. Wang and collaborators loaded these systems with 5-fluoruracil (5-FU), characterized their physicochemical properties and evaluated the possibility to use them as 5-FU delivery systems. They successfully achieved the control of the release rate of 5-FU from the functionalized KGM/SA/GO hydrogels, and demonstrated these systems could be suitable polymeric carriers for the site-specific drug delivery in the intestine (48).

Additionally, alginate based hydrogels have shown excellent properties for their application in the field of tissue engineering. For example, alginate shows ideal properties to prepare 3D scaffolds for the culture of cancer stem cells (CSCs). CSCs are partly responsible for cancer reoccurrence and metastasis, and the traditional two-dimensional cell culture has revealed some limitations in the maintenance of CSCs (49).

Alginate hydrogels possess some mechanical properties similar to those of native tissues and organs, which make them attractive for tissue engineering applications, and also contributed to solve some unanswered questions about the intersection between mechano-biology and matrix dimensionality (50). In this way, Xu and coworkers showed how alginate beads were a convenient and efficient culture system for the enrichment and characterization of CSC (51). Additionally, alginate hydrogels can be also used as a suitable vehicle in injectable cell delivery systems to facilitate tissue and organ regeneration (52).

Finally, a wide range of nanoparticle systems have been designed using alginate in their composition with anticancer applications. Zhang and coworkers loaded doxorubicin into glycyrrhetinic acid-modified alginate nanoparticles, which exhibited good liver targeting ability due to both passive and active targeting ability of glycyrrhetinic acid. They observed very promising results, such as the reduction of the cardiac toxicity of doxorubicin, and also the enhancement of the antitumor activity of the drug against liver tumors *in situ* (53).

Folic acid has been also used to deliver alginate-based nanoparticles via active targeting. Wanga and collaborators recently prepared self-assembled core/shell nanoparticles from water-soluble alginate substituted by hydrophobic phytosterols. Then, folic acid was conjugated to the systems for targeting folate-receptor-overexpressing cancer cells. After the characterization of their physicochemical properties, doxorubicin was entrapped inside prepared nanoparticles by dialysis method. The release of doxorubicin from drug-loaded nanoparticles was pH-sensitive and more rapidly in an acidic environment, suggesting that anticancer drug release could be triggered within the acidic intracellular environment of cancer cells. These systems showed enhanced cellular uptake, increased targeting capacity, and increased cytotoxicity against KB cells overexpressing folate receptors. Therefore, authors suggested that these self-assembled nanoparticles would be a promising carrier of hydrophobic anticancer drugs for its targeted intracellular delivery (54). Additionally, our group has also the successful experience of folate-decorating nanoparticles based on different mixtures of alginate-cysteine and albumin, which demonstrated the enhanced anticancer efficacy of tamoxifen after *in vitro* (55) and *in vivo* studies (56).

Lastly, doxorubicin and paclitaxel were recently encapsulated into inorganic/organic hybrid alginate/$CaCO_3$ nanoparticles, which were prepared by co-precipitation in an aqueous solution under very mild conditions. These nanoparticles exhibited significantly enhanced cell uptake and nuclear localization as compared with the single drug loaded nanoparticles. Therefore, they show promising applications for the co-delivery of drugs in combination chemotherapy to overcome multidrug resistance (57).

## 3 HYALURONIC ACID

### *3.1 Chemical structure and properties of hyaluronic acid*

Hyaluronic acid (HA) is found in all tissues and body fluids of vertebrates as well as in some bacteria (58). It is very abundant in the extracellular matrix of loose connective tissue, and it is also presented in solution in the synovial fluid of joints and the vitreous body of the eye. Its capability of binding water reversibly makes it one of the major components of articular cartilage. Broadly, hyaluronic acid is linked

to numerous tissue functions, including wound healing, inflammation, angiogenesis, and fibrosis. It is also associated with functions of motility migration, proliferation and differentiation of cells from the mesenchymal linkage (59). Besides its use in many cosmetic products, HA is widely utilized in ophthalmology, rheumatology and dermatology because of its remarkable rheological, viscoelastic and hygroscopic properties (60).

There are two main general procedures to obtain hyaluronic acid: microbial fermentation and extraction from animal sources. HA from microbial fermentation would become the predominant source in medical and cosmetic markets. Hyaluronic acid (HA) fermentations have been performed mostly by *Streptococci* spp., where HA is a capsular biopolymer shedding to the medium. Streptococcal bacteria and other various organisms have HA synthases to synthesize HA as a linear polymer at the inner face of the plasma membrane (61). However, *Streptococcus* is considered a less-than ideal source because of its potential to produce exotoxins, difficult fermentation control and the expensive medium required for their growth (62). In contrast, HA production through fermentation by generally recognized as safe microbial strains (GRAS) has emerged as an attractive alternative. Recombinant GRAS bacteria have been noted as useful sources of bioproducts because of their guaranteed safety status (63, 64).

Regarding HA extraction from animal sources, different tissues have been used for this purpose, but the need of intensive purification steps make HA production a very expensive protocol. In animal tissues, HA is complexed with proteoglycans and proteins, which are potentially allergenic; thus, the production of HA with high purity and high molecular weight is very difficult and costly (61). HA has been extracted from a wide variety of sources, such as rooster combs (65), vitreous humor of fish eyeball (66), marine sources (67) or invertebrate species (68).

Hyaluronic acid, also called hyaluronate or hyaluronan depending on its polyanion states, is the only glycosaminoglycan that does not contain sulfate groups. It is a polysaccharide of disaccharide units, which are composed by D-glucuronic acid bounded by β (1-3) O-glycosidic bond to N-acetyl D-glucosamine; the linkage between disaccharide units is mediated by a β (1-4) O-glycosidic bond between N-acetyl D-glucosamine and D-glucuronic acid (Figure 3). There may be up to 25,000 disaccharide units in a molecule of hyaluronic acid. The molecular mass of hyaluronan can vary from 20 kDa to 10 MDa, depending on the chain length. Additionally, different cellular functions are ascribed to these different molecular masses (69).

Due to its highly negative charge, HA forms a coil-like structure in aqueous solutions, entrapping water within its structure and expanding in volume up to 1,000 times (70). The specific functional groups on the polysaccharide (e.g. carboxyl, hydroxyl, N-acetyl groups) can be modified by using chemical reactions such as esterification, sulphatation or amidation, in order to obtain hyaluronic acid derivatives functionalized with hydrazines, aldehydes, or amines (71).

The molecular weight of HA also affects its biological functions and determines different behaviors of the degradation products from HA. In fact, molecules from degradation of HA with up to 20 disaccharides in length exhibit angiogenic properties, whereas those products obtained from the degradation of high molecular weight HA can actually inhibit angiogenic processes (72).

The biological role of HA is mainly mediated by CD44 and RHAMM (receptor for hyaluronic acid-mediated motility) signaling (73). HA is the principal ligand of CD44 receptor, a glycoprotein widely expressed on the surface of many mammalian cells. There are several CD44 splice variants, but the most expressed one is the shortest CD44 isoform (CD44s) (74). CD44 has a determinant role in several types of cancer. Variants of CD44, specifically CD44v6, promote tumor progression and metastatic potential in lung, breast, and colon cancer (75). In fact, CD44 expression has been lately considered a biomarker in cancer stem cell and cancer initiating cell studies (76, 77). RHAMM (also designated as CD168) is alternatively spliced and present isoforms expressed either on cell inner or cell surface (78). Surface expression of RHAMM is likely required for CD44-mediated cell migration during inflammation, wound healing, and tumorigenesis. However, the cooperative mechanism between RHAMM and CD44 is not yet clearly understood (79).

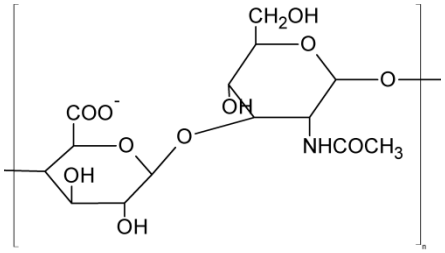



Figure 3. Chemical structure of disaccharide repeating unit of hyaluronic acid.

### *3.2- Preparation methods of hyaluronic acid-based systems*

Several methods have been described in order to obtain HA-based systems, like hydrogels (micro/nano-hydrogels, nanoparticles or HA-drug conjugates.

#### *3.2.1 Hyaluronic acid hydrogels*

Cui and coworkers have prepared micro-hydrogels based on HA by the biotin-avidin system approach (80). Avidin is a tetrameric biotin-binding protein which can be linked simultaneously to four molecules of biotin with high affinity and specificity. In this study, HA was modified with adipic acid dihydrazide and then conjugated with a biotin molecule, resulting in HA–biotin. When HA–biotin solution was mixed with doxorubicin hydrochloride and blended with neutravidin, doxorubicin-loaded HA-micro-hydrogels were formed. Vasi and coworkers have synthesized HA derivatives by the reaction of hydroxyl groups of HA with maleic anhydride (81). These HA derivatives were used for the formulation of new gels by acrylic acid copolymerization in the presence of a redox system in aqueous solution under temperature. They obtained three dimensional structures with elongated and interconnected pores, uniformly distributed, which were successfully used to load pilocarpine, an ophthalmic therapeutic agent. Additionally, HA hydrogel particles with positive and negative surfaces have been also prepared by w/o microemulsion method. Ekici and coworkers used AOT [sodium bis(2-ethylhexyl) sulfosuccinate] in

isooctane to obtain the emulsion, and then crosslinked the particles with divinyl sulfone under vigorous stirring (82). HA particles were conjugated to cysteine residues to obtain thiol groups on the surface of the particles. These thiolated HA particles were further exposed to radical polymerization to generate HA-based ionic hydrogel systems, and have shown great potential in the biomedical field as drug delivery vehicles sensitive to environmental pH changes.

### *3.2.2 Hyaluronic acid nanoparticles*

HA plays an important role in the field of preparation of nanoparticles. Stable HA nanoparticles can be obtained after the covalent crosslinking of HA carboxyl groups with a bifunctional amine in the presence of a water soluble carbodiimide. The reaction conditions highly influenced the formation of HA nanoparticles; for example, salt and HA concentrations in the reaction mixture were determinant in the size of formed spherical shape particles. Although a broad size distribution was obtained, the particle size did not exceed 110 nm (83).

The chemical structure of HA has been commonly modified through carbodiimide mediated reactions in order to obtain self-assembled nanoparticles. Yoon and coworkers prepared amphiphilic PEGylated HA conjugates using HA, aminated 5-β-cholanic acid, and amine-functionalized PEG (84). Aminated black hole quencher-3 was also conjugated to these structures through a carbodiimide reaction. Nanoparticles were finally obtained by dialysis and lyophilization. The hydrophobic photosensitizer, chlorin e6 (Ce6) was loaded into the nanoparticles in order to apply these systems to simultaneous *in vivo* photodynamic tumor imaging and therapy. Also with anticancer applications, Cho and coworkers synthesized self-assembled nanoparticles of polyethylene glycol (PEG)-conjugated HA-ceramide as doxorubicin delivery systems (85). Recently, nanoparticles based on a combination of HA and α-tocopheryl succinate (α-TOS) have been prepared by Liang and coworkers using a carbodiimide mediated reaction, and were then loaded with docetaxel, enhancing the intracellular drug accumulation and MDR-overcoming effect on human resistant breast cancer (86).

Self-assembled HA nanoparticles have been also used in genetic anticancer therapy. Ganesh and coworkers synthesized and evaluated a series of HA based functional macrostructures that can form self-assembled nanosystems encapsulating siRNA payload (87). HA was functionalized with mono-functional fatty amines, bifunctional fatty amines and polyamines via carbodiimide reactions; siRNA was loaded by incubating the respective derivatives with a known concentration of siRNA. The obtained HA based nanosystems showed *in vitro* and *in vivo* gene silencing activity. In the same way, HA has been combined with other polysaccharides, like oleoyl-carboxymethy-chitosan, using a coacervation process to prepare novel potential carriers for gene delivery (88).

### *3.2.3 Hyaluronic acid drug-conjugates*

Finally, HA has been used to obtain anticancer-drug conjugates. Camacho and coworkers have recently reported the use of HA–drug conjugates to co-deliver synergistic doses of camptothecin (CPT) and doxorubicin (DOX) (89). CPT and DOX were conjugated to HA via nucleophilic acyl substitution; the carboxylic acid moieties of HA were conjugated to CPT via ester formation, and to DOX via both ester

formation and aminolysis. CTP and DOX administration by conjugation with HA has the additional advantages of preserved synergistic drug ratios from the site of injection to tumor tissue, and enhanced tumor accumulation.

### *3.3- Hyaluronic acid-based systems for cancer treatment*

As it has been previously mentioned, active targeting of nanosized polymeric systems is emerging as a promising tool to face cancer. HA is a challenging molecule to be used in the preparation of nanoparticles as targeted drug carriers for cancer treatment due to its specific union to CD44 receptor (overexpressed on various tumor cells) (90). There are several works among literature where HA has been used with this purpose and with diagnostic aims. Some of them have been described below.

HA has been used directly to modify the activity of some anticancer drugs. This is the case of camptothecin and doxorubicin, whose combination with HA has allowed the enhancement of the clinical efficacy of these antitumor drugs by promoting drug accumulation in tumors. EPR effect in combination with the specificity of hyaluronic acid by CD44 were responsible for this better antitumor activity (91).

Additionally, HA in combination with some synthetic polymers has been explored to obtain nanoparticles where anticancer drugs are included. In this context, the efficacy of camptothecin and doxorubicin was also enhanced when they were encapsulated into nanoparticles based on a combination of HA with poly(ethylene glycol) (PEG) (92). These systems were internalized into cancer cells via receptor-mediated endocytosis, caused dose-dependent cytotoxicity and were selectively accumulated at tumor site. The combination of HA with PEG, in addition to ceramide, was also used by Cho and coworkers to control doxorubicin delivery (85). Apart from the potential of PEG-HA nanoparticles as therapeutic tools, some studies have demonstrated their additional promising role in early detection of colon cancer (93). In this case, nanoparticles were loaded with Cy 5.5 and showed an excellent antitumor activity after intravenous injection in orthotopic colon cancer models. Furthermore, HA has been combined with poly(DL-lactide-co-glycolide) (PLGA) to obtain doxorubicin-loaded GSH-sensitive nanoparticles by a dialysis procedure (94). CD44 receptor-mediated endocytosis of nanoparticles was confirmed in CD44-positive MDA-MB231 cells, and their efficacy was determined in a xenograft mice model.

HA has been recently used as a targeting ligand of metallic nanoparticles. As a case in point, HA capped gold nanoparticles were loaded with metformin in order to prepare nanomedicines with applications in hepatocellular carcinoma treatment (95). *In vitro* cytotoxicity studies in HepG2 cells (CD44 positive) showed that these systems effectively inhibited the multiplying of cancer cells by principle of active targeting.

Targeting CD44 receptor by HA attachment to nanosystems has shown good expectations in genetic therapy to treat cancer. Some self-assembled nanosystems functionalized with HA have been engineered for the selective delivery of siRNA (87). The siRNA encapsulated in the nanosystems demonstrated specific gene knockdown in both sensitive and resistant A549 lung cancer cells overexpressing CD44 receptors, and also demonstrated tumor selective uptake in *in vivo* studies with metastatic tumors. Apart from siRNAs, small hairpin RNAs (shRNAs) were also included in a nanovector consisting of HA and

poly-L-lysine-graft-imidazole (PLI)-based polyplexes, which was used as CD44-targeted therapy against gastric cancer with good results (96).

Finally, HA has been used in combination with other natural molecules to prepare nanoparticles which were used for cancer treatment. Nanoparticles made of oleoyl-carboxymethy-chitosan (OCMCS) and HA were prepared by coacervation as potential carriers for gene delivery (88). Uptake of these systems into Caco-2 cells was mediated by CD44 giving evidence of their potential for the targeting and further transfer of genes to gastrointestinal system. Additionally, Liang and coworkers recently designed multifunctional nanoparticles based on HA in combination with α-tocopheryl succinate (α-TOS) (HT) conjugates and D-α-tocopheryl polyethylene glycol succinate (TPGS) (86). TPGS and α-TOS act as an inhibitor of multidrug resistance and an activator of mitochondrial apoptotic pathways, respectively. These nanoparticles were loaded with docetaxel and tested *in vivo* on MCF-7/Adr xenografted nude mice, and showed much higher accumulation in tumor tissues and enhanced antitumor efficacy with reduced systemic toxicity.

Taking everything into account, the specific interaction of HA and the overexpressed CD44 receptor is one of the most challenging ways to design targeted systems with anticancer applications. All described cases are just few examples of some of the already developed nanocarriers, but there are more ongoing studies with a promising and inspiring future in this field (97).

## 4 HEPARIN

### *4.1 Chemical structure and properties of heparin.*

Heparin was discovered nearly 100 years ago and has been traditionally employed as a blood anticoagulant since 1935. Natural tissues are the main source to obtain unfractionated heparin, such as porcine intestine or bovine lung. It shows a highly heterogeneous chemical structure with an average molecular weight of 15 kDa (98).

Heparin is involved in various biological processes, like coagulation, inflammation, angiogenesis and apoptosis, which make it an ideal candidate for emerging biomedical applications. Heparin is commonly used for clinical procedures which require anticoagulation conditions. Heparin blocks the coagulation cascade when it binds to antithrombin III. Some of the complications that can occur while using heparin are hemorrhagic incidents or heparin-induced thrombocytopenia. Regarding the role of heparin in inflammation processes, heparin can inhibit opsonization and the activation of complement cascade (99). Additionally, heparin and its fragments show antiangiogenic effects due to their interference with FGF-2 and its receptor. Finally, recent studies have proposed that heparin can induce apoptotic cell death due to its interaction with several transcription factors (98, 100).

The repeating disaccharide unit of heparin is composed of 1→4 linked uronic acid [D-glucuronic or L-iduronic acid] and D-glucosamine residues (Figure 4).

Heparin is one of the most negatively charged natural products due to the high presence of sulfate and carboxylate moieties along its chemical structure. These functional groups facilitate electrostatic connections with many proteins, such as proteases, chemokines and some important growth factors, like fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF). In fact, heparin can stabilize proteins or even increase their affinity for cell receptors. Researchers have taken particular advantage of this characteristic property joining heparin with FGF or VEGF and using it in the design of biomedical materials, as engineered scaffolds for tissue regeneration or controlled release platforms for growth factor delivery (98).

Heparin varieties can be chemically classified according to their molecular weight; thus, two different types of heparin are distinguished: low-molecular weight heparin (LMWH) and unfractionated/high-molecular weight heparin (HMWH). LMWH presents some advantages over HMWH: easier structure, more predictable anticoagulant dose, longer half-life and reduced side effects. Furthermore, some studies have demonstrated that HMWH is less effective in the inhibition of tumor growth, and that LMWH can reduce the activity of heparanase, reducing consequently tumor metastasis (101). Consequently, LMWH is usually selected against HMWH in order to design nanosystems with biomedical applications.

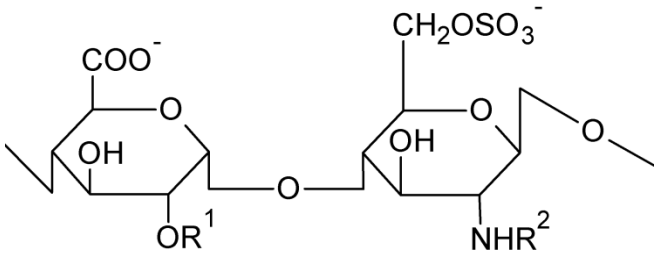



Figure 4. Chemical structure of disaccharide repeating unit of heparin ($R^1$ = H or $SO^{3-}$ and $R^2$ = H, $SO^{3-}$ or $COCH_3$).

### *4.2 Preparation methods of heparin-based systems.*

Heparin has been widely used as raw material to prepare systems with medical applications following different methodologies. These systems could be named as hydrogels or nanoparticles, depending on the characterization of their properties and applications.

#### *4.2.1 Heparin hydrogels*

Heparin hydrogels are generally sensitive to environmental stimuli (pH, temperature, enzyme and light), which results in different hydrogel behaviors (shrinkage, swelling, degradation or sol–gel phase transition). Consequently, heparin hydrogels are versatile systems whose properties can be tailored according their future application. Multiple strategies have been developed to prepare heparin-based hydrogels, but they are normally based on covalent and non-covalent crosslinking strategies.

Non-covalent interactions used to form heparin hydrogels include hydrogen bonding, metal-ligand, host-guest interactions, and stereocomplexation. One of the principal problems of using non-covalent crosslinking strategies is that, sometimes, toxic initiators are needed to form hydrogels; thus, the resulting

structures are less cytocompatible. Heparin–protein interactions are the most used combinations, such as those derived from human platelet factor 4, heparin interacting protein (HIP) and antithrombin III. Growth factors, like basic fibroblast growth factor, VEGF or hepatocyte growth factor, can interact with short sequences of heparin, and could be also potentially employed as multifunctional crosslinkers in the formation of physical networks (98). Additionally, heparin can be previously modified with polymeric molecules to obtain the hydrogel, such as with multi-arm PEG, and then functionalized with different peptides (102).

In contrast, covalent crosslinking methods do not need any toxic initiator; consequently, hydrogels obtained by these procedures are more biocompatible, although they often suffer from poor mechanical properties. However, covalently-formed hydrogels can be easily modified to achieve the required mechanical properties according to the specific biomedical application. The most common reactions to obtain covalent crosslinked heparin hydrogels are Michael-type addition, photo-induced radical polymerization, enzyme-catalyzed reactions and amide coupling reactions (98).

#### *4.2.2 Heparin nanoparticles*

There is an increasing interest on the design of heparin based nanoparticles due to the challenging biological possibilities that heparin shows at nanoscale dimension.

On the one hand, heparin can be attached to the surface of metallic nanoparticles, which are normally designed to be used as diagnostic tools in magnetic resonance imaging techniques. Heparin plays different roles when it is used as superficial ligand; it improves the stability of the formulation, avoiding self-aggregation, and also provides more targeted action, increasing cellular uptake (103, 104).

On the other hand, there is a wide variety of heparin-based nanoparticles prepared with the aim of being used as therapeutic tools. There are several methods described to prepare these systems, but generally the most common approach is made based on the interactions that carboxyl ($COO^-$) and sulfate ($OSO_3^-$) groups of heparin can establish with different molecules. For example, heparin can be combined with deoxycholic acid to form nanoparticles using a carbodiimide-mediated reaction (105). The possibility of forming amide linkages between carboxilate groups of heparin and aminated-compounds has been explored in several works, even using directly aminated-drugs (i.e. paclitaxel) to obtain spherical nanoparticles with high aqueous solubility (106, 107). Finally, the highly hydrophilic behavior of heparin can be also used to form self-assembled core–shell nanoparticles in aqueous solution when heparin is conjugated with hydrophobic molecules (108).

### *4.3 Heparin-based systems with anticancer applications*

Traditionally heparin has been clinically used as an anticoagulant agent. However, some of its biological properties, such as its role in the inhibition of adhesion of cancer cells, inactivation of heparanase, activation of NK cells or interference with the activity of growth factors (bFGF and VEGF), make it an ideal candidate to be used in the design of systems with anticancer applications (15). Consequently, in the

last years many researching projects have been focused on the development of heparin-based nanosystems as drug carriers (105-107, 109).

Cancer treatment with paclitaxel (PTX) presents serious disadvantages, such as problems of solubility or serious side effects; therefore, many researchers purposed its delivery by heparin-based nanosystems (109). When PTX is administered included in heparin-nanosystems, there is an increase of cytotoxicity with respect to the administration of the isolated drug (106, 108, 109). Additionally, PTX-nanoparticle conjugates enhance the solubility values of PTX (108, 109) and also display longer systemic circulation times (107).

Doxorubicin is another example of drug included in heparin-based systems in order to enhance its effectiveness and reduce its side effects. Park and coworkers modified heparin with deoxycholic acid to form self-assembled nanoparticles where doxorubicin was included in. These doxorubicin loaded-nanoparticles induced a higher decrease in tumor size in contrast to free doxorubicin, with more potent anti-angiogenic, cytotoxic and apoptotic effects (105).

In conclusion, the attractive aspect of using heparin as biomaterial in the design of new anticancer tools is undoubtedly confirmed due to its ability to control angiogenesis, tumor growth and metastasis. However, researchers are still trying to overcome the disadvantage of its anticoagulant effect, developing derivatives of low molecular weight heparin with low anticoagulant effect but great antiangiogenic activity (110).

## 5 CHONDROITIN SULPHATE

### *5.1. Chemical structure and properties of chondroitin sulfate.*

Chondroitin sulfate (CS) is a linear, poly-disperse natural polysaccharide composed of two alternating units of β-1,3-linked glucuronic acid (GlcA) and (β-1,4) N-acetyl-galactosamine (GalNAc) (Figure 5). It is often modified with sulphate groups, replacing one or more of the hydroxil groups on C4 and C6 of GalNAc, and C2 and C3 of GlcA (111). Therefore, there is a wide variety of modified CS molecules which can be obtained with different relative molecular mass, chemical properties and biological/pharmacological activities, which depends also on the type of tissue and/or organ source (111, 112).

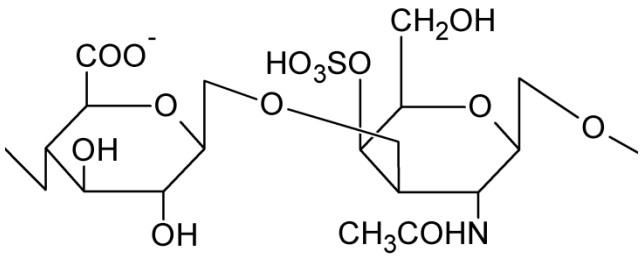



Figure 5. Chemical structure of disaccharide repeating unit of chondroitin sulphate

CS is mainly intercalated into the cell membrane, tightly associated with the surface of cell membrane or the extracellular matrix, producing proteoglycan components such as aggregan, versican, neurocan or phosphacan (111, 113). As a consequence of its location, CS seems to be relevant in signaling pathways, cell orientation and cell-cell recognition via extracellular matrix interaction (114), which could be essential in the modulation of tumor cell behavior, tumor progression, and metastasis (115).

This glycosaminoglycan is an ubiquitous structural component of cells, tissues and organs, widely distributed in mammals and invertebrates, though commercially available CS is mostly extracted from trachea, nasal septa, chicken keel, shark cartilage and fish (111, 112) with different sulphation degree according to tissue location or animal age (116, 117). However, the epidemic diseases associated with terrestrial animals, including mad-cow disease, foot-and-mouth disease, and hog cholera, as well as the increasing price of raw material extracted from the endangered shark, reduces the number of natural sources available for the extraction of this polysaccharide (112).

For these reasons, current research studies are focused on searching new alternative sources to obtain chondroitin sulfate. Maccari and collaborators (118) have recently investigated five different common fishes (monkfish, cod, spiny dogfish, salmon and tuna) as alternative sources for commercial CS extraction. In addition, other authors have already confirmed the chemical characteristics and antithrombotic effect of CS purified from other natural sources, such as sturgeon skull and sturgeon backbone (119).

Additionally to the antithrombotic effects mentioned above, CS–based drugs have also demonstrated relevant physiological activity due to its biological anti-inflammatory, antioxidant, allergic, chondroprotective, antirheumatic and central nervous system development effects (112, 120, 121). Recent *in vitro* osteoarthritis studies, based on the best described CS function within cartilage, suggested that CS may induce the synthesis of the damaged cartilage proteoglycans, inhibit/reduce gene expression of proteolytic enzymes and could reduce subcondral bone resorption (111, 112). Moreover, CS can act as receptor of various pathogens and nonsulfated chondroitin can interfere in the morphogenesis and cell division of *C.elegans*, as revealed RNA interference experiments (122).

As a result of the high number of functions displayed by CS, current research studies carried out by the chemical industry are focused on the development of new isolation, purification and modification techniques (111).

### *5.2. Preparation methods of chondroitin sulfate-based systems.*

In the past few decades, several systems have been designed in combination with CS, due to its good biocompatibility and high hydrophilicity. This negatively charged glycosaminoglycan usually forms surface layers via electrostatic interactions with other natural or synthetic polymers. Moreover, CS could be modified with additional moieties, such as polylactide (123), acetyl groups (124), methacrylate (125-127) or fucose sugar (128), to form self-assembled carriers and enhance their drug loading capacity (129).

#### *5.2.1. Chondroitin sulfate hydrogels*

The synthesis of CS-based hydrogels can be achieved using different methods and polymer combinations. For instance, Zhao and coworkers (126) prepared methacrylated CS-based hydrogels by the gamma-irradiation technique. This method is easy to control, is environmentally friendly and is performed at room temperature. Methacrylated modified CS was also used by Fajardo and coworkers (127) to prepare a novel type of hydrogel which combined chemically and physically crosslinking methods. Authors obtained first a conventional chemically crosslinked hydrogel based on methacrylated chitosan, and then, a physically crosslinked network was formed between the chitosan-gel and CS, due to the electrostatic interactions.

Oprea and colleagues studies (130, 131) described the synthesis of cellulose/CS-based hydrogels for their use as drug delivery systems by a chemically crosslinking technique, using epichlorohydrin as crosslinking agent. In both research studies, the amount of CS content in composition seemed to be essential to swelling capability of the matrix. Besides, Yu and coworkers (132) obtained novel pH-sensitive biodegradable micelles based on histamine-CS combination by direct dissolution technique in aqueous medium, without organic solvent. The combination with CS polymer also gave them the ability to target tumor cells and facilitate cellular uptake via binding hyaluronic acid receptors, which are overexpressed on cancer cells.

### *5.2.2 Chondroitin sulfate microparticles*

It is well known that CS is able to form micro-particulate systems, although this type of device is currently less investigated. Different synthesis techniques were developed to obtain CS-based systems. For instance, Ganza-González and coworkers (133) prepared chitosan and CS-A microspheres by spray drying. These authors selected spray-drying technique because of its rapid high-yield and easy industrial scale application properties, obtaining small microspheres from hydrogel-forming polymers. Additionally, Huang and coworkers (134) also prepared Ch/CS complex microcapsules to encapsulate 5-fluorouracil. These microcapsules were prepared by emulsion-chemical crosslinking method, using glutaraldehyde compound as crosslinker agent.

### *5.2.3 Chondroitin sulfate nanoparticles*

Metal-based nanoparticles are very common devices in cancer therapy and imaging, due to their use in highly sensitive diagnostic assays, thermal ablation and radiotherapy enhancement, as well as their use in drug and gene delivery applications (135). Therefore, current studies are focus on improving the biocompatibility and stability properties of metal-based particles by using natural polymers such as chondroitin sulfate.

In this context, Li and coworkers (136) obtained biocompatible gold nanoparticles (AuNPs), using sodium borohydride as the reducing agent and employing CS as the stabilizing agent. *In vitro* characterization studies demonstrated that polyanionic CS molecules acted as excellent stabilizing agents during the synthesis process, and improve biocompatibility of those nanoparticles. This stability role could be attributed to the electrostatic repulsion and steric hindrance established between the AuNPs with the presence of CS molecules around the surface of gold particles, which prevent their agglomeration.

Chen and coworkers (137) prepared silver nanoparticles (AgNPs) with silver nitrate as precursor and CS as both reducing agent and stabilizing agent. These nanoparticles were obtained in a stirring aqueous medium at the room temperature without any assisted by microwave, autoclave, laser irradiation, γ-ray irradiation or UV irradiation. Then, the obtained CS-capped AgNPs were coated with N-[(2-hydroxy-3-trimethyl-ammonium) propyl] chitosan chloride (HTCC) via an ionic gelation method in order to modify the surface charge of AgNPs from negative to positive (137).

Other method for obtaining CS-metal based nanoparticle formulations is *in situ* sol–gel technique, recently used by Kandiah and coworkers (138) to obtain $TiO_2$/CS-4 nanocomposites. Briefly, CS was added at different concentrations into the titanium isopropoxide solution and the mixture was vigorously stirred until to obtain the gel-like constituent. Then, the obtained constituents were dried in hot-air oven to evaporate. An increase in swelling degree and in degradation percentage was observed at high CS concentrations (138).

In addition to the previous described techniques, Toth and coworkers (139) recently obtained CS-coated core–shell magnetite nanoparticles by post-coating method. Firstly, they synthesize magnetite nanoparticles by co-precipitation method and then, the surface of the purified, bare magnetite was modified by CS-A (CSA). Results demonstrated that increasing amounts of CS-A shift the isoelectric point gradually to a more acidic pH value and narrow the pH-range of nanoparticle aggregation (139).

In addition to its utility to obtain metal-based nanoparticles, CS is routinely used in combination with other natural polymers, such as chitosan. Several authors have prepared CS-ChS-based nanoparticles by ionic gelation method in order to encapsulate and deliver therapeutic agents. Thus, Jardim and coworkers (140) have designed novel chitosan/CS nanoparticles loaded with curcumin. Interactions between $NH_3^+$ groups of chitosan and $SO_3^-$ groups from CS determined nanoparticle properties.

### *5.3 Chondroitin sulfate -based systems with anticancer applications*

CS-based systems can interact with cancer-overexpressed CD44 receptor (141, 142), leading to receptor-mediated endocytosis. For this reason, Tomiyama and coworkers (143) recently introduce CS into plasmid DNA-polymer-peptide conjugates complex (pDNA/PPC) in order to enhance tumor-targeted gene delivery. Results showed that introduction of negatively charged CS into polymers with a low charge density may lead to low stability and gene regulation of complexes. In the same way, Lee and coworkers (144) also produced bile acid-conjugated CS A-based nanoparticles for CD44-overexpressed tumor targeting. In this case, doxorubicin-loaded nanoparticles were obtained when hydrophobic deoxycholic acid derivative was conjugated to the hydrophilic CS-A backbone via amide bond formation. The interaction between CS-A and CD44 receptor was shown in cellular uptake studies developed with CD44 receptor-positive MDA-MB-231. *In vivo* experiments with the MDA-MB-231 tumor-xenografted mouse model confirmed that CS-based nanoparticles could be further developed into an efficient tumor-targetable theranostic nanosystem for CD44 receptor-positive cancers.

Besides, CS has also been used to prepare potential MRI contrast agents (145). CS-coated core–shell magnetite nanoparticles could be promising candidates for theranostic application (139). In this case, CS

acted as superparamagnetic iron oxide nanoparticles cover to prevent aggregation and dissolution of these magnetite nanoparticles under physiological conditions. Results showed changes in the colloidal state of samples (from aggregated to stable) when the amount of CS concentration increased.

Nanoparticle stabilization properties of CS were also used in other research studies. For instance, Huang and coworkers (146) designed amphiphilic nanocarriers based on Pluronic® F127 (PF127), which can inhibit drug efflux transporters in cancer therapy. Authors covered these nanoparticles with methacrylated CS in order to stabilize these doxorubicin-loaded nanocarriers, which reacted with acrylated hydroxyl groups on both termini of PF127 polymer. Moreover, the introducing CS-surface cover had carboxylic acid groups which could be used to react with a folic acid-polyethylene glycol for active targeting to folate receptor-overexpressed tumors. Overall, the synthetized particles showed a nano-scale size, an effective doxorubicin-loading and a better cellular uptake of chondroitin sulfate-coated particles, which could be explained by the presence of unspecified sugar receptors in the cellular surface.

## 6. PECTIN

### *6.1. Chemical structure and properties of pectin.*

Pectin is a major complex polysaccharide found in cell walls of all land plants, located in the soft parts of the plant and in the middle lamella and cell corners. New developments have demonstrated several relevant functions in plant growth, morphology, development and plant defense (147).

Pectin is a term which refers to a family of complex oligosaccharides and polysaccharides with common features, such as the containing galacturonic acid (GalA) linked at the O-1 and the O-4 position, but extremely different in chemical structures (148). Thus, various pectic polysaccharides have been isolated from primary cell walls: homogalacturonan, rhamnogalacturonan-I, and substituted galacturonans (Figure 6), although currently it is not well established how these pectic polysaccharides are linked to each other, or to other polymers in the wall (147, 149).

Multiple research studies evidence the wide variety of pectin applications. This polysaccharide can act as a stabilizer, thickener, gelling agent, emulsifier and drug vehicle in the food and pharmaceutical industries. It has also demonstrated multiple positive effects on human health including lowering cholesterol and serum glucose levels, reducing cancer and stimulating the immune response (147, 150). For these reasons, multiple current studies are focused on pectin production, improving the existing biosynthesis methods, chemical modification techniques and purification process.

As described above, pectin has a complex chemical structure that provides multiple structural epitopes, where more than 67 pectin transferases enzymes can interact during the pectin synthesis process. Thus, the identification of genes encoding those proteins is necessary to understand pectin structure/function relationships (147, 149). Several recent advances in pectin analysis include the use of monoclonal antibodies, microarray technologies, enzymatic fingerprinting, electron spray ionization mass spectrometry, capillary electrophoresis and chemometrics technology (148). Although commercial pectin

is mainly extracted from citrus, apple, or other higher plants, producers are currently beginning to develop a new generation of sophisticated designer pectins with specific functionalities (148, 150).

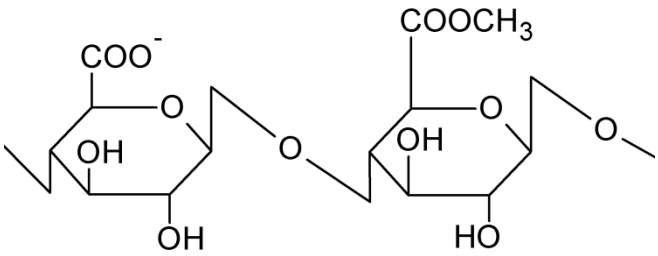



Figure 6. Chemical structure of disaccharide repeating unit of pectin

### *6.2. Preparation methods of pectin -based systems.*

#### *6.2.1. Pectin hydrogels*

The most abundant pectic polysaccharide component, homogalacturonan, can be methoxylated in galacturonic acid (GalA) residue, determining pectin functionality. Pectin with high degree of GalA methoxylation can form gels in acidic media, whereas pectin with low degree of GalA methoxylation can produce a continuous network due to the interactions among free carboxylic acid groups and divalent ions (such as $Ca^{2+}$, according to "egg-box" model) (151). Despite these electrostatic interactions, van der Waals interactions and hydrogen bonds can stabilize the chain when egg-boxes are formed between neighboring chains (152). Fraeye and coworkers demonstrated that both intrinsic (degree and pattern of methoxylation, degree of polymerisation) and extrinsic ($Ca^{2+}$ and pectin concentration) parameters determine the texture of pectin-calcium gels (153).

According to pectin properties described above, research groups are currently investigating new strategies for the synthesis of hydrogels from the natural polymer of pectin. Moreira and coworkers (154) proposed internal gelation method to obtain injectable pectin hydrogels with calcium carbonate. One of the advantages of using that technique is avoiding the addition of D-glucono-δ-lactone, which might induce difficulties in the control of the final pH and of the gelling kinetics of the hydrogels. Moreover, *in vitro* characterization studies revealed easy gelling kinetics and rheological properties modification.

Besides, Takei and coworkers (155) developed *in situ* gellable hydrogels composed of periodate oxidized citrus pectin for localized anticancer drug delivery. Thus, antitumor drug, doxorubicin, was coupled to oxidized citrus pectin by imine bonds and adipic dihydrazide acted as cross-linking agent. In contrast to the reaction conditions described above, oxidized citrus pectin and adipic dihydrazide were previously dissolved in $Ca^{2+}$ and $Mg^{2+}$-free phosphate buffered saline (pH 7.4), respectively. They observed that an increase in the molar ratio of sodium periodate to monosaccharide units of citrus pectin resulted in an increase in the oxidation degree (the number of aldehyde groups), which might influence pectin-based hydrogel characteristics such as mechanical strength and the amount of the incorporated doxorubicin.

### *6.2.2. Pectin microparticles*

According to scientific literature pectin-based microparticles are mainly used as nutraceutical compound carriers for preservation of functionality and targeted delivery of bioactive food components. Thus, a wide variety of methods has been developed in the past few decades (156) such as spray drying (157), ionotropic gelation method (158) or electrostatic extrusion (159). However, current research studies rarely investigate pectin-based microparticles for biomedical applications.

### *6.2.3. Pectin nanoparticles*

Currently, most of the pectin-based nanoparticles are used as hydrophobic drugs carriers. Thus, Hu and coworkers (160) prepared coating cationic zein nanoparticles with anionic pectin molecules using an electrostatic deposition method. Burapapadh and coworkers were able to encapsulate poorly water-soluble drugs (itraconazole) in pectin-based nanoparticles, prepared from nanoemulsion templates (161). As it is well known, the presence of methoxyl groups might increase the hydrophobic properties of pectin molecules, turning them in suitable interfacial agents in oil-in-water emulsions. In this work nanoemulsions containing itraconazole were prepared by high-pressure homogenizer using pectin as a polymeric emulsifier, at different concentrations, and chloroform as an oil phase.

In other research studies, authors have synthesized nanoparticles from a solution of modified pectin. For instance, Tummalapalli and colleagues (162) obtained oxidized pectin by mixing the polyssaccaride with periodic acid, at hydrochloric acid and sodium bicarbonate solution. Once oxidized pectin was prepared, silver nitrate ($AgNO_3$) was added to oxidized pectin solution and the reduction was carried on under constant stirring and temperature (60ºC). At the end of the reaction, oxidized pectin-nanosilver was precipitated from the solution using an excess of isopropanol and separated out by vacuum filtration.

## *6.3. Pectin -based systems with anticancer applications*

Despite the most common pectin applications in food industry as gelling and stabilized agent (158, 159), current research studies have demonstrated its application in cancer therapy as dietary pectin, due to its tumor growth inhibition (163), anti-mutagenic potential (164) and the regulation of transformation-related micro-RNA/oncogenes (165).

In addition to those dietary benefits, recent research studies are focused on investigating antitumor applications of this non-toxic polymer as drug delivery systems. Its easily modified functional groups (i.e., -COOH, -OH) allow to obtain a wide range of modified pectin fragments with low molecular mass, which demonstrated antitumor activity (166). For instance, it was observed that pH-modified citrus pectin can inhibit tumor growth, angiogenesis and galectin-3, a key target in metastasis (167). In addition, other examples of modified pectin fragments have also revealed that antitumor effect, such as heat-treated ginseng pectin derivative and pectin treated with 20 kGy of γ-irradiation, which demonstrated a key role in the inhibition of HT-29 colon cancer cells proliferation and other tumor cells (163, 168).

Pectin-based drug delivery systems can be developed for enteral and parenteral administration using different types of devices: hydrogels (155, 169), microparticles (170, 171) and nanoparticles (160, 161, 172, 173). Dev and coworkers (174) designed pectin-based 5-fluorouracil matrix tablets, dually coated with Eudragit S 100, for colon-cancer targeting after enteral administration. *In vivo* evaluation of these devices demonstrated a suitable bioavailability, without any premature drug release in the small intestine or in the stomach medium. Besides, pectin also can be chemically cross-linked with anti-cancer drugs to create a pro-drug, as observed in Tang and coworkers study (175). Here, authors obtained an injectable novel pectin–adriamycin conjugate which was synthesized by attaching adriamycin to low-methoxylated pectin via an amide linkage. This novel system avoids some of the limitations in clinical administration of the drug, such as cardiotoxicity, multidrug resistance and a short half-life.

## 7. CONCLUSIONS

A new generation of anticancer therapies, based on the use of drug delivery nanosystems, is likely to have immediate clinical advantages compared with the conventional use of anticancer drugs, which produce several side effects due to non-selective uptake by various tissues. As reviewed above, anionic polysaccharides have extensively contributed to the development of several types of nanocarriers with the purpose of being used in cancer treatment and diagnosis. Much of the systems have been investigated in terms of their physicochemical properties, drug-loading capability and *in vitro* and *in vivo* effectiveness, showing in general very promising results. Although there is much work to do yet, it could be clearly state that anionic polysaccharides are ideal biomaterials to obtain new tools to fight against cancer, and probably contribute to obtain more anticancer systems in the coming years.

## 8. ACKNOWLEDGEMENTS

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