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From Chitin to Chitosan

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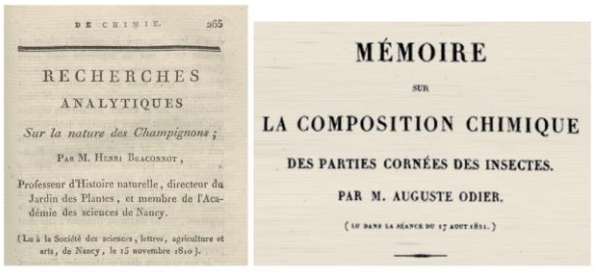
INTRODUCTION

Henri Braconnot, who was the director of the Botanical Gardens at the Academy of Sciences in Nancy, France, discovered chitin in 1811 after the report of a "material particularly resistant to usual chemicals" by A. Hachett, an English scientist in 1799. The substance, named "fungine," was extracted from mushrooms that would not dissolve in sulphuric acid and that contained a substantial fraction of nitrogen. Incidentally, that discovery stemmed for investigations on the composition of edible mushrooms and their nutritional value. (Braconnot, 1813) In 1823. Antoine Odier published an article on the cuticle of insects, in which he noted that similar substance was present in the structure of insects as was in the structures of fungi. (Odier, 1823) He gave the name of the alkaline-insoluble fraction as chitin, from the Greek word, meaning "tunic" or "envelope."


DE CHIMIE. 265

RECHERCHES ANALYTIQUES

Sur la nature des Champignons ;

PAR M. HENRI BRACONNOT,

Professeur d'Histoire naturelle, directeur du Jardin des Plantes, et membre de l'Académie des sciences de Nancy.

(Lu à la Société des sciences, lettres, agriculture et arts, de Nancy, le 15 novembre 1810).

MÉMOIRE

SUR

LA COMPOSITION CHIMIQUE

DES PARTIES CORNÉES DES INSECTES.

PAR M. AUGUSTE ODIER.

(LU DANS LA SÉANCE DU 17 AOUT 1821.)

Figure 1. Facsimile of the essays published by the French Academie des Sciences, by H. Braconnot and A. Odier, respectively

The concept was further known in 1843 when Jean Louis Lassaigne while working of the exoskeleton of silkworm butterfly Bombix morii, demonstrated the presence of nitrogen in chitin. (Lassaigne, 1843a,b,c) The same year, Anselm Payen, who had reported the identification of cellulose in 1838 initiated interrogation as regard to the difference between cellulose and chitin. (Payen, 1843) In 1876, Leddorhose identified glucosamine and acetic acid as structural units of chitin. (Ledderhose. 1876) Glucosamine, as the repeated unit of Chitin, was confirmed by Gilson in 1894. However, it took 50 years before Earl R. Purchase, and Charles E. Braun elucidated the final chemical nature of chitin (Purchase & Braun, 1946).

The history of chitosan dates back to 1859 when French physiologist Charles Rouget described the deacetylation of chitin through its boiling in the presence of concentrated potassium hydroxide. (Rouget, 1859) Immediately, he recognized that the newly obtained product was soluble in acidic solutions, contrasting with the water-insoluble nature of native chitin, thus opening new possibilities for its use. However, it was not until 35 years later that the modified chitin received the name “chitosan,” as given by the German physiologist and chemist Felix Hoppe-Seyler. (Hoppe-Seyler, 1894).

By the early 20th century, a great deal of research had been accomplished on the subject of chitin and chitosan. In 1930, G. Rammelberg’s researches headed to the verification on the uniqueness of chitosan from the sources of chitin, specifically crab shells and fungi, and the production of fibers from these different systems. Later on, came the elucidation of glucosamine, as the monosaccharide constituent of chitin. In the 1950’s x-ray analysis, along with infrared spectroscopy, and enzymatic analysis advanced the study of chitin and chitosan in fungi. The first monograph on chitin and chitosan appeared in 1951.

In the 20th century, scientists began to investigate the polymer’s potential uses and discovered a lot of beneficial properties. Chitin is non-toxic and biodegradable, and therefore more environmentally friendly than most synthetic polymers. It is also an anti-microbial, providing the fungi and animals it coats with anti-disease defenses.

In the early 1960s, the studies on chitosan dealt for its ability to bind with the red blood cells. The substance was considered a hemostatic agent. For three decades now, chitosan has been used at water purification plants for detoxifying water. It spreads over the surface of the water, where it absorbs grease, oils, and other potential toxins. For a while, chitosan was sold over the counter as a “fat blocker” or “fat trapper”. The claim was that chitosan taken as as supplement might reduce the amount of fat absorbed in the gastriointestinal tract. Such a claim has not be substantiated by any reliable scientific evidence

An essay entitled "Historical landmarks in the discovery of chitin" describes the 220 years of the development of chitin. (Crini, 2019) The essay covers five periods: (i) discovery from 1799 to 1894, (ii) a period of confusion and controversy from 1894 to 1930, (iii) exploration in 1930–1950, (iv) a period of doubt from 1950 to 1970, (v) the period of application from 1970 to the present day.

The present chapter collates the developments and conclusions of many of the extensive studies that have been conducted on chitin and chitosan. We aim to provide a comprehensive presentation of the scientific and technological advances that populate the vast panorama of chitin and chitosan.

THE OCCURRENCE OF CHITIN

Chitin (β-1,4-linked 2-acetamido-2-deoxy-D-glucose) is found throughout the biosphere. Its estimated production is 10^{10} to 10^{12} tons per year. Chitin is the second most abundant polymer after cellulose. Sea animals, insects, and micro-organisms are the sources of chitin. In more than 90% of all animal species and insects, chitin-based composites are the major constituents of the exoskeletons of arthropods. This is the case of crustaceans (crabs, lobster, and shrimps) and insects as well for the radulae of mollusks, cephalopods beaks, and the scales of fish and lissamphibian.

The exoskeletons of crustacean provide firm support and protection to the soft tissues of these animals. They are not only composed by chitin, as other proteins occur, including an elastic rubberlike substance called resilin, which is an intrinsically disordered protein. The identity and nature of these proteins determine whether the exoskeleton will be rigid, like a beetle’s shell, or soft and flexible like the joints of a crab leg. Non-protein compounds are found such as calcium carbonate, that determines whether the exoskeleton is rigid, or soft and flexible. They form structurally and mechanically graded biological nanocomposites

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Figure 2. Sources of chitin and chitosan. • Sea animals: Crustaceans, Coelenterata, Annelida, Mollusca, Lobster, Shrimp Prawn, Krill, Crab. Fish-scales; • Insects: Scorpion, Brachiopod, Cockroach, Spider, Beetle, Ant; • Microorganisms: Green algae, Yeast, Fungi (cell wall), Mycelia penicillium, Brown algae, Chytridiaceae, Ascomycetes, Blastocladiaceae, Spores

In fungi, chitin is a major constituent of the supramolecular network formed by structural polysaccharides and proteins outside the plasma membrane. Chitin is an important structural building block of the fungal cell wall. It has a role in the plant innate immunity in the microbe-associated molecular patterns (MAMPs). Several plant receptors for chitin have been characterized as well as different strategies adopted by fungi to evade chitin recognition. Despite its strong activity as an elicitor of plant defense chitin represents only a small percentage of the cell wall of most fungi compared to other complex sugars. β-glucan, to which chitin and chitosan may be bound.

The partial or complete de-N-acetylation of chitin, under alkaline conditions, yields chitosan, which contains varying amounts of GlcNAc and D-Glucosamine (GlcN) units. Chitosan, is a polycation and is found less frequently in the biosphere and is associated with some fungal cell walls and green algae. Chitosan is a random copolymer with a fraction of β-(1→4)-N-acetyl-D-glucosamine and a fraction (1-DA) of β-(1→4)-D-glucosamine

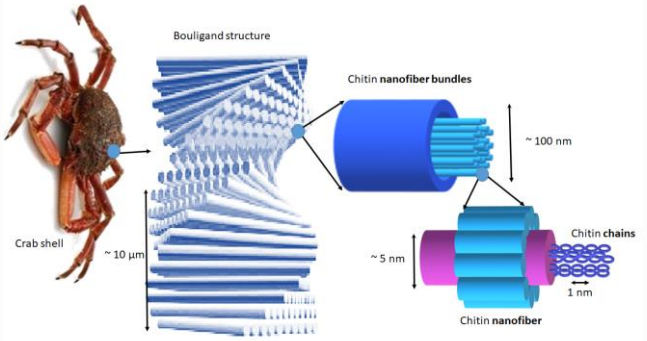

Figure 3. Schematic representation of the exoskeleton structure of crab shell, over several orders of magnitude. The exoskeleton material of arthropods consists of mineralized fibrous chitin-based tissue, which follows a strict hierarchical organization over several order of magnitutes, ranging from the millimeter to the nanometer range. Nanofibrills having dimensions of about 2-5 nm diameter and about 300 nm lengths are formed by the side-by-side arrangement of 18-25 chitin chains chains, wrapped by proteins. The next step of organization results from the clustering of these nano-fibers to form long chitin-protein microfibrils of about 50-300 nm diameter. They are further organized and form a planar woven and periodically branched network in the form of chitin-protein layers. Proteins and biominerals (essentially in the form of microcrystalline CaCO3) fill the spacing between the fibers).The helicoidal stacking sequence of the fibrous chitin-protein layers constitutes the following meso-scale level of overall hierarchy. This is referred to as a twisted plywood or Bouligand pattern. This structure is formed by the helicoidal stacking sequence of the fibrous chitin-protein layers (translation and rotation). This determines a stacking density of planes which are gradually rotated about their arrangement with respect to the normal axis, About 20 layers of chitin-protein layer constitute one picth of the helical arrangement.

A critical evaluation of potential sources of chitin and chitosan (Allan et al., 1978) concluded that shrimp, prawn and crab waste were the principle source of chitin and chitosan and would remain so for the immediate future. However, it was envisaged that both Antarctic krill and cultured fungi would complement these major sources of raw materials.

CHITIN METABOLISM

The coordination of chitin synthesis and its degradation requires strict control of the participating enzymes for subsequent growth and development in insects and fungi. As for insects, growth and morphology depend on the capability to remodel, in time and space, chitin-containing structures. Insects, repeatedly produce chitin synthases and chitinolytic enzymes in different tissues. (Merzendorfer & Zimoch, 2003) A similar interplay between synthesis and degradation occurs in cell division and sporulation of yeast and other fungi.

3.1. Chitin biosynthesis

Chitin biosynthesis follows three distinct steps. In the first step, the enzymes' catalytic domain facing the cytoplasmic site forms the polymer. In the second step, the nascent polysaccharide chain translocates throughout the membrane from where it is released into the extracellular space. The third step completes the process, and single polysaccharide spontaneously assembles to form crystalline microfibrils.

All the different chitin forms are the result of biosynthesis by glycosyltransferases : chitin synthases (UDP-N-acetyl-D-glucosamine : chitin 4-β-N-acetylglucosaminyltransferase). These Glycosyltransferases (GTs) are plasma membrane-associated. They are highly conserved enzymes present in every chitin-synthesizing organism. The CAZy database classifies chitin synthases as belonging to the GT-2 family. (Henrissat, 1993) On top of chitin synthases, this family contains other inverting GTs synthases, such as cellulose synthases, and hyaluronan synthases. These enzymes utilize UDP-N-acetylglucosamine (UDP-GlcNAc) as a substrate and require divalent cations as co-factors. Addition of GlcNAc to the nonreducing end of the polymer elongates chitin polysaccharide. In this process, the nucleotide sugar donor UDP-GlcNAc is transferred to the α-linked GlcNAc sugar in an inverting mechanism onto the non-reducing end of the growing acceptor chain.

There exists a large literature available on the synthesis of chitin. All characterized fungi have chitin synthases and most have multiple genes encoding chitin synthases. *S. cerevisiae* has three genes that encode chitin synthases. (Bansal et al., 2012) The chitin synthases are large transmembrane proteins with theoretical molecular masses ranging from 160·kDa to 180·kDa. Three distinct domains termed A, B, and C constitute the enzyme. Domain A is located at the N-terminus and has limited sequence conservation among different species. In fungal class, I–III and VI CHSs, the A domains do not contain any transmembrane helices, whereas class IV + V and VII enzymes contain 2–3 transmembrane helices. The B domain comprises about 400 amino acids and hosts the catalytic center of the protein. It is highly conserved and it contains two unique motifs that are present in all types of chitin synthases including those essential for the catalytic mechanism. (Breton et al., 2001) Then follows the C-domain that contains 3-7 transmembrane helices. These transmembrane domains form a channel through which the elongating chitin chain is extruded into the cell wall space, reducing end first. Despite being the essential enzymes, chitin synthases have resisted protein expression, solubilization, and crystallization for structural studies or high throughput ligand screening. As a result, the detailed understanding of the mode of action of the chitin synthase remains to be established. The similarities that exist between the structure and function of membrane-integrated processive glycosyltransferases might help establishing firmer the understanding of chitin biosynthesis. (Bi et al., 2015)

In the cell wall space, chitin polysaccharide chains assemble into microfibrils, having dimensions of about 0.3 nm, throughout intermolecular interactions involving hydrogen bonding and van der Waals interactions. Within these microfibrils, there exist significant differences between the relative arrangements of the chitin chains, leading to two distinct polymorphic types. These crystalline types are referred to as α, β chitin. In the α chitin, the polymer chains are arranged in an antiparallel fashion, whereas a parallel orientation of the chains forms β chitin crystals. These two forms of chitin vary in packing and polarities of adjacent chains in the succeeding sheets. The length of the chitin microfibrils can be as long as 0.5 µm. Up to 10 or more of these microfibrils further organize in the form of bundles. Such type of architecture provides both a thermodynamic stability and prevents accessibility to chemicals.

Many investigations concern the fungal kingdom, where multiple genes encoding chitin synthases have been identified. Such a wide diversity suggests that different chitin synthases might be used for chitin production to varying stages of the fungal life cycles such as in septum formation, hyphal growth, and development, or particular cell types and specific species. (Horiuchi, 2009) Such an occurrence is far from being a surprise when one considers the different life histories, developmental processes, and ecological niches that characterize the more than, 1.5 million different species that constitute the fungal kingdom. Interestingly, some reports mention that chitin is not required for all fungal cell walls and that chitin can be a cell-type specific cell wall. (Lui et al., Scientific reports, 2017) Some of the results established for fungi systems seem to be valid for the insect chitin synthases as well. In contrast to fungi, molecular analysis of Chitin Synthase genes has so far revealed a limited number of gene copies of nematode and insect chitin synthase genes (CHS).

3.2. Chitin degradation

In chitin-producing organisms, chitinolytic enzymes are essential for maintaining normal life cycle functions such as morphogenesis of arthropods or cell division and sporulation of yeast and other fungi. Since chitin is hard to break due to its physicochemical properties, its degradation usually requires the action of more than one enzyme type. The degrading enzymes include the following chitinases poly[1,4-(N-acetyl-β-D-glucosaminide)] glycanohydrolase, β-N-acetylglucosaminidases (β-N-acetyl-β-D-hexosaminide N-acetyl hexosaminohydrolase. As for lytic polysaccharide monooxygenases, N-acetylglucosaminidases different carbohydrate-binding modules enable tight binding to insoluble substrates. All of them catalyze the hydrolysis of β-(1-4)-glycosidic bonds of chitin polymers and oligomers. Endo-splitting chitinases produce chito-oligomers that are subsequently converted to monomers by exo-splitting β-N-acetylglucosaminidases. Chitinases belong to the glycosyl hydrolase 18 families (Lombard et al., 2013), which is comprised of various proteins found in a wide range of organisms, including plants, bacteria, fungi, insects, protozoa, and mammals

3.2.1. Insects. Insect growth and development is strictly dependent on the capability to remodel chitinous structures. Therefore, insects consistently synthesize and degrade chitin in a highly controlled manner. During each molt cycle, a new cuticle is deposited simultaneously with the degradation of the inner part of the chitinous procuticle of the overlying old exoskeleton by molting fluid enzymes including epidermal chitinases. Degradation of cuticles by chitinolytic enzymes certainly needs the assistance of molting fluid proteases to degrade proteinaceous components. The mechanism of catalysis seems to be similar to that postulated for the cellulase complex and other multi-enzyme systems hydrolyzng polysaccharide assemblies.

Insect chitinases are endo-splitting enzymes that retain the anomeric β-(1-4) configuration of the cleavage products. They belong to family 18 of the glycoside hydrolase superfamily (GH18). However, some of them have lost their catalytic activity but retained the chitin binding activity and possess growth factor activity. In all sequenced insect genomes, multiple genes encode chitinases. They are differentially expressed during development and in various insect tissues. Some of them have non-redundant functions and are essential for growth and development. A particular property is their multi-domain architecture, which comprises varying numbers of catalytic and chitin-binding domains that are connected by glycosylated serine/threonine linker regions. Based on sequence similarities and organization, they have been classified into eight different groups.

1.2.2. Fungi. Fungal cell walls confer mechanic stability during cell division and polar growth. Chitinases play a housekeeping function in plasticizing the cell wall or can act more specifically during cell separation, nutritional chitin acquisition, or competitive interaction with other fungi. In the case of phytopathogenic fungi, the cell wall is the first constituent that establishes intimate contact with the host plant. Depending on the species and lifestyle of fungi, there is a considerable variation in the number of encoded chitinases and their function.

EXTRACTION OF CHITIN AND PREPARATION OF CHITOSAN

4.1. Extraction of Chitin

The main commercial sources of chitin are crab and shrimp shells. The isolation of chitin begins with the selection of shells, which has an important bearing on the subsequent quality and property of the final isolated material. For example, for lobsters and crabs, shells of the same size and species are chosen. In the case of shrimps, where the cell wall is thinner, the chitin isolation is easier than from other types of shells. The selected shells are then cleaned, dried and ground into small shell pieces. In industrial processing, chitin is extracted by acid treatment to dissolve the calcium carbonate followed by an alkaline solution to dissolve proteins. Also, a decolorization step is often added to remove pigments and obtain colorless pure chitin. All those treatments must be adapted to chitin source, owing to differences in the ultrastructure of the original material, to produce first high-quality chitin, and then chitosan (after partial deacetylation). Chitin is infusible and sparingly soluble during the transformation into different conformations. The question of its solubility is a major problem in the development of both processing and use of chitin as well as its characterization.

4.1.1. Chemical extraction

4.1.1.1. Chemical demineralization. The process consists in the removal of minerals, primarily calcium carbonate, using hydrochloric acid as a preferred reagent. Demineralization is easily achieved because it involves the decomposition of calcium carbonate into the water-soluble calcium salts with the release of carbon dioxide. Most of the other minerals present in the shellfish cuticle react similarly and give soluble salts in the presence of acid. Then, salts can be separated by filtration of the chitin solid-phase followed by washing using deionized water.

Chitin Source	Mineral Before Process (%)	Number Baths		Bleaching H_2O_2 HCl 9.1;V/V	Residual Mineral (%)	Amount Chitin (%)		
		HCL 0.55 M	NaOH 0.3M 80°C				Chitin	DA (%)
Barnacle	-	2	4	Yes	-	07	α	100
Red Crab	31.13	5	3	Yes	0.025	10	α	98
Marbled Crab	-	3	3	Yes	-	10	α	99
Spider Crab	25.96	3	3	Yes	0.014	16	α	96
Lobster	33.99	3	3	Yes	0.062	17	α	-
Spinny lobster	-	3	7	Yes	0.168	32	α	100
Slipper lobster	-	2	3	Yes	-	25	α	-
Crayfish Fresh water	-	2	3	Yes	-	36	α	-
Prawn	21.5	3	3	Yes	0.019	22	α	100
Shrimp	12.95	2	2	Yes	0.02	14	α	100
Squille mante	18.99	3	3	yes	0.010	24	α	100
Cuttlefish	-	3	3	No	-	20	β	-
Squid	1.70	3	2	No	0.017	42	β	100

Table 1. Extractions of chitin form different sources. Recomposed from ref Tolaimate et al., 2008)

4.1.1.2. Chemical deproteinization. The deproteinization step requires the disruption of the chitin protein-complex and the solubilization of proteins. Such a step is performed heterogeneously using chemicals that also depolymerize the biopolymer; the results depend from the experimental conditions used. The complete removal of protein is especially essential for biomedical applications, as a percentage of the human population is allergic to shellfish, the primary culprit being the protein component.

The processes of chemical deproteinization and demineralization affect the molecular weights and degree of acetylation of chitin. The use of solid-state NMR provides a way to assess the degree of acetylation. The higher the degree of acetylation, the less degradation has occurred to the native polymer sample. The use of successive baths of lower HCl concentrations (0.55M) and NaOH (0.3 M) concentrations preserves the native chitin as shown from the results obtained for 12 different species of crustaceous and cephalopods for which the degree of acetylation was varying between 96% and 100%. (Tolaimaite et al., 2008)

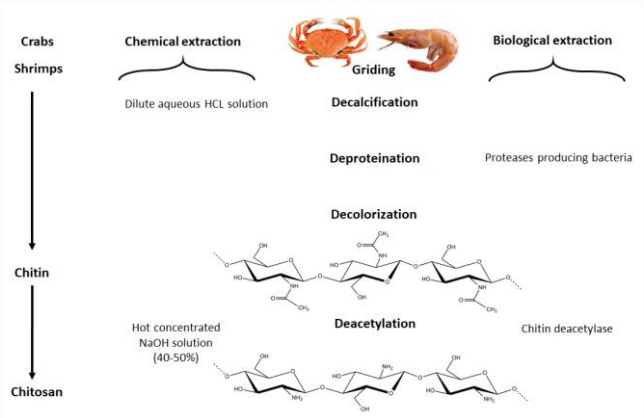

Figure 4.The chitin and chitosan chemical and biological processes

4.1.2. Biological extraction

The use of enzymes in the deproteinization step appears as early as in 1934, which lead to the foundations and the developments of the fermentation process. Comparative studies between chemical and biological processes for extracting chitin from shrimp's shells point toward a preference for the uses of enzymes and microorganisms for chitin extraction. While offering several advantages such as simplicity, producibility, low solvent, and energy consumption, the process yields the chitin of high molecular weight. The fermentation is achieved by a process called auto-fermentation, by endogenous microorganisms or by adding selected strains of microorganisms. Fermentation methods follow two different pathways; namely lactic acid fermentation and non-lactic acid fermentation. The lactic acid fermentation requires to inoculate the well ground shellfish waste with a lactic acid culture and a carbohydrate source while being mixed thoroughly. The production of lactic acid decreases the pH, thereby dissolving the $CaCO_3$, and concomitant proteolysis by the enzymes from the shellfish viscera. (Younes & Rinaudo, 2015)

A somehow related approach to this, in which the deproteinization and demineralization steps are separated, requires the action of both bacteria (*Bacillus* strains) and fungi. Many processes and substrates have been investigated. One of this used *Aspergillus niger*, taking advantage of the action of the enzymes released by the fungi for their deproteinization and partial demineralization of crustacean shells. Many factors influence the fermentation process and the subsequent efficiency of deproteinization and demineralization. The biotechnological process must be completed by further mild chemical treatment to remove the extra protein and minerals and yield highly purified chitin.

4.2. Chitosan preparation

The term usually refers to a family of polymers obtained after chitin partial deacetylation to varying acetylation degrees (DA). The acetylation degree, which reflects the balance between the two types of residues differentiates chitin from chitosan. All (1-4)-linked copolymers of β-D-GlcNAc and β-D-GlcN units should be designated as chitin or chitosan based on their insolubility or solubility, respectively. During deacetylation, acetyl groups are removed randomly or non-random but also depolymerization reaction occurs, indicated by changes in MW of chitosan. Several factors influence the structures and properties of chitosans. These are: the degree of polymerization (DP), the heterogeneity of the molecular weight, the fraction of acetylation, and the pattern of acetylation.

Although chitosan occurs naturally in some fungi and green algae, it is primarily produced industrially from chitin by chemical treatment using alkali. These methods are used extensively for commercial purpose of chitosan preparation because of their low cost and suitability to mass production. From a chemical point of view alkalis can be used to deacetylate chitin. Commonly, in the heterogeneous method, chitin is treated with a hot concentrated solution of NaOH during a few hours and deacetylated to up to ~85%–99%, and chitosan is produced. According to the homogeneous method, alkali chitin is prepared after the dispersion of chitin in concentrated NaOH (30 g NaOH/45 g H2O/ 3 g Chitin) at 25 °C for three hours or more, followed by dissolution in crushed ice around 0 °C. This method results in soluble chitosan with an average degree of acetylation of 48%–55%. Many parameters in the deacetylation reaction can limit the characteristics of the final chitosan. The results shown in Table 2, illustrate the advantage of successive bathes compared to a single treatment of longer time; the importance of addition of $NaBH_4$ preventing oxidation is clearly demonstrated.

Starting Material	Deacetylation Process				DA	Intrinsic	Molecular
	NaOH (w/w %)	T (°C)	Time (h)	Addition $NaBH_4$	(%)	Viscosity ([η] mL/g)	Weight (Mv g/mol)
	40	80	9h	-	**17**	1127	298 000
Chitin β	40	80	3h x 3	-	**1**	1675	500 000
Calmar	40	80	3h x 3	+	**0**	2027	644 000
DA:100%	40	80	6h	-	**20**	1368	384 000
	40	80	3h x 2	-	**3**	1906	590 000
	40	80	3h x 2	+	**5**	2927	1 040 000
	50	120	12h	-	**3**	269	45 000
Chitin α	50	120	6h x 2	-	**3.9**	430	84 000
Shrimp	50	120	6h x 2	+	**1.8**	679	153 000
DA:100%	50	120	4h x 3	+	**1**	658	147 000
	50	120	3h x 4	+	**0**	667	140 000
	50	120	3h x 3	+	**1**	801	190 000

Table 2 Deacetylation of chitin throughout different experimental protocols along with the resulting structure and some properties of the resulting chitosans (revomposed from : Tolaimate et al., 2008*)*

Chitin is converted to chitosan by enzymatic preparations. Such methods, exploiting chitin deacetylases offer the possibility of a controlled non-degradable process that yields well-defined chitosans, both in size, degree of acetylation, and pattern of acetylation. Chitin deacetylase (EC 3.5.1.41) catalyzes the hydrolysis of N-acetamido bonds in chitin to produce chitosan. Since chitin is hard to break due to its physicochemical properties, its degradation usually requires the action of more than one enzyme type. In several instances, pretreatment of chitin substrates before enzyme addition is required to improve the accessibility of the acetyl groups to the enzyme and therefore to enhance the deacetylation yield.

Endo-splitting chitinases produce chitooligomers that are subsequently converted to monomers by exo-splitting β-N-acetylglucosaminidases. The latter enzyme cleaves off N-acetylglucosamine units from non-reducing ends and prefers smaller substrates than chitinases. (Koga et al., 1982, 1983, 1997 ; Fukamizo and Kramer, 1985a,b ; Kramer and Koga, 1986 ; Kramer et al., 1993 ; Zen et al., 1996 ; Filho et al., 2002) As a consequence, of these properties, the overall rate of chitin hydrolysis is limited by the action of the chito-oligomer-producing chitinase, which drastically increases.

NOMENCLATURE

The descriptions and depictions of chitin and chitosan require several levels that are linked to the carbohydrate nature and the polymeric nature of these polysaccharides. As regard to their chemical compositions, it is a common practice to use of IUPAC nomenclature, in the condensed form. (McNaught, 1977) A perspective drawing of the ring offers a simplified model to represent the monosaccharides. The ring is oriented almost perpendicular to the plane of the paper, but viewed from slightly above so that the edge closer to the viewer is drawn below the more distant edge, with the intracyclic oxygen behind and the anomeric carbon at the right-hand end. To define the perspective, the ring bonds closer to the viewer are often thickened. The naming of the ring atoms of the monosaccharides provides a convenient way to describe those substituents attached to them (OH, NAc, NH_2, NH_3^+). As chiral macromolecules, polysaccharide chains have a polarity, the direction of which that is usually described on going from the non-reducing to the reducing residue.

In addition to the sequence representation of structures, other graphical representations have been developed favoring cartoon representations that facilitate the visualization of the monosaccharides present. This graphical representation, called SNFG (Symbol Notation for Glycans) (Varki et al., 2015) has been proposed as the result of a concerted international agreement with the hope it will cope better with the rapidly growing information on the structure and functions of glycans in chemical and biological systems. In view of the ubiquitous occurrence of polysaccharides and oligosaccharides, along with their properties (Birch et al., 2019) and functions (Clerc et al., 2018, 2019), their introduction in databases is growing. In anticipation of the need to interconnecting and interoperating such databases, the representations and computational processing require the development of standards. Bioinformaticians have developed a collection of tools to parse and translate glycan and polysaccharides structures across different encoding systems. In the recent years, different databases have introduced GlycoCT (Herget et al., 2008) as a standard encoding system. Figure 5 exemplifies the use of these distinct modes of representations of chitin, chitosans and their oligomers.

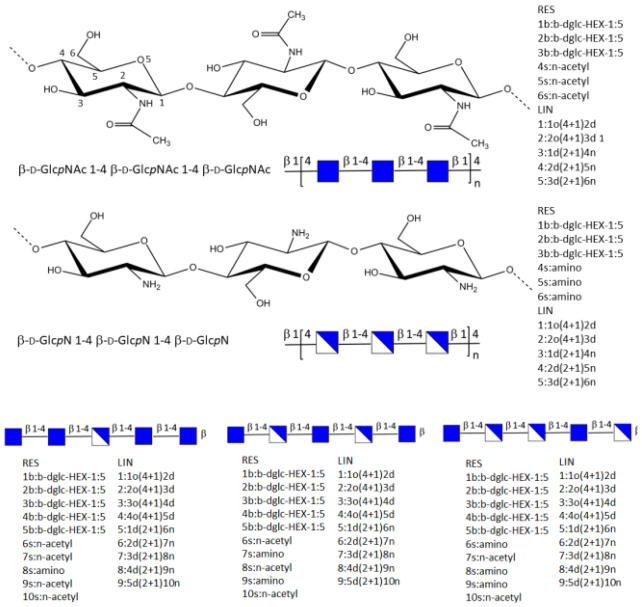

Figure 5. Different modes of structural depictions and representations of chitin and chitosan.

Because of their polymeric natures, the descriptions of the structures of chitin and chitosan require several descriptors: the molecular weight distribution; the weight-average molecular weight; the number-average molecular weight; the degree of polymerization (DP) and the dispersity of the molecular weight.

CHITIN AND CHITOSAN IN THE SOLID STATE

6.1. Crystallography of Chitins

The availability of chitin in the form of a solid polycrystalline material made it a substrate of choice for X-ray diffraction studies, first on powders and later on oriented fibrillar forms. Clark and Smith were the first to make crystal studies of chitin and chitosan using X-ray diffraction. (Clark & Smith, 1937) The powder X-ray diffraction patterns recorded from shrimp shells and squid pens exhibit differences that reveal the existence of two polymorphs, namely α-chitin and β-chitin. (Rudall & Kenchington, 1973; Blackwell,

1973, Atkins, 1985, Atkins et al., 1979; Saito et al., 1995; Chanzy, 1988) Further structural information was revealed throughout fiber diffraction methods that led the way to the proposal of the occurrence of two structural arrangements that differ in the packing and polarities of adjacent polysaccharide chains. (Blackwell, 1973, Gardner & Blackwell, 1975; Minke & Blackweel, 1978) The structure of α-chitin is formed by an arrangement of antiparallel chains, whereas a parallel arrangement of chains forms β-chitin crystals. Further characterizations of the crystalline structure of α-chitin and β-chitin were conducted using electron diffraction experiments on crystalline sample. (Persson et al., 1990; Helbert & Sugiyama, 1998) They confirmed the unit cell parameters and space group assignments.

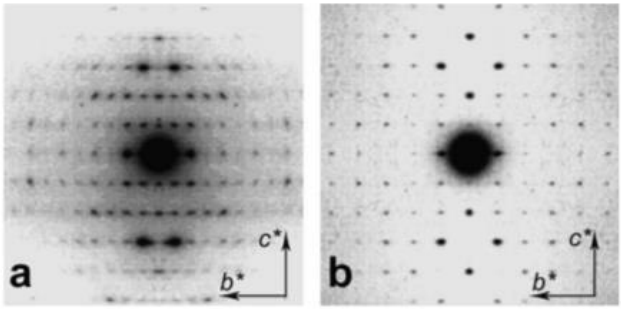

Figure 6. Electron diffractograms of highly crystalline chitin a) is taken on a fragment of a Sagitta grasping *spine and b) on a microfibril extracted from a tube synthesized by a vestimentiferan worm* Tevnia jerichonana. *These two patterns, corresponding to b*c* projections, indicate clearly that along the b* direction, the cell parameter of α-chitin is close to twice that of β-chitin, whereas the c* parameter is the same in both patterns. In addition the a*c* projections (not shown) of α- and β-chitin are nearly identical in both allomorphs*

It is only recently that highly accurate determinations of the crystalline structures have been obtained using synchrotron X-ray and neutron diffraction method. (Sikorski et al., 2009; Nishiyama et al., 2011; Sawada et al., 2012)) While confirming the gross structural features reported previously, these investigations provide the detailed description of the conformations about the glycosidic torsional angles and the resulting two fold helical conformation of the chains. The orientation of the N-acetyl groups, as well as some detailed about the occurrence of disordered orientations of some primary hydroxyl groups, are distinctively characterized. Within the network of intra-chains and inter-chain hydrogen bonds, there is an important involvement of strong C-O…NH hydrogen bonds. In both structures, there is a packing of chitin chains in sheets where intra-sheet hydrogen bonds strongly hold them.

In α-chitin, further hydrogen bonds and van der Waals forces strongly link the sheets together, with the distinct feature displayed by the occurrence of two distinct conformations of the primary hydroxyl groups. β-chitin lacks such a feature, which explains why it is more soluble and more reactive towards solvents and has a greater affinity towards them. The susceptibility to intra-crystalline swelling and the penetration of several polar guest molecules (ranging from water to alcohol and amines) takes place without major distortion of the sheet organization. Once a guest molecule has penetrated the crystalline lattice of β-chitin, another one of a different chemical family can displace it. This feature produces a wide distribution of crystalline β-chitin complexes. However, the reversibility of this swelling and even the crystalline state do not survive in strong acid media typically 6-8 M HCl or concentrated nitric acid.

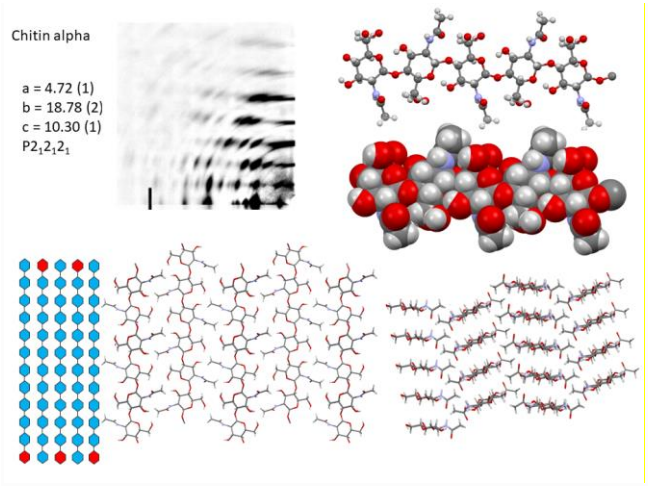

Figure 7. Structural features of α-Chitin in the crystalline state, having unit cell having unit cell parameters: a= 4.72, b = 18.78, c= 10.30 (Ang) in the $P2_12_12_1$ space group as established by synchrotron X-ray fiber diffractometry. Structural drawings of the crystallographic conformation of a segment of α-chitin. The bottom row shows the longitudinal anti-parallel chain packing perpendicular to the chitin chains, with the red hexagon indicating the reducing GlcNAc residue and the transverse packing of the chains (hydrogen atoms are not shown).

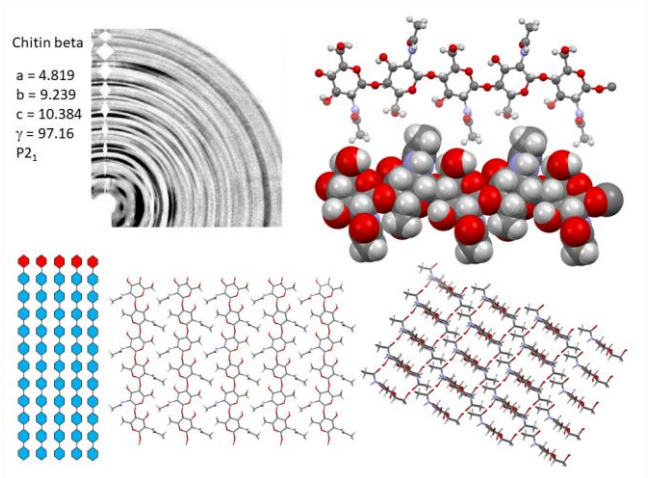

Figure 8. Structural features of β-Chitin in the crystalline state, having unit cell having unit cell parameters: a= 4.819, b = 9.239, c= 10.384 (Ang) γ = 97.17° in the $P2_1$ space group as established by synchrotron X-ray fiber diffractometry. Structural drawings of the crystallographic conformation of a segment of β-chitin. The bottom row shows the longitudinal parallel chain packing perpendicular to the chitin chains, with the red hexagon indicating the reducing GlcNAc residue and the transverse packing of the chains (hydrogen atoms are not shown).

Nevertheless, removal of the acid restores the crystallinity in the form of α-chitin. This process destroys all the β-chitin crystals and new crystal of α-chitin form during recrystallization. The irreversibility of the β-α conversion indicates that α-chitin is thermodynamically more stable than β–chitin. The fact that α-chitin is always obtained in recrystallization from solution confirms this stability.

6.2. Relevance to the Biosynthesis of Chitins

There do exist strong similarities between the polymorphic features observed for chitin and cellulose. In both cases, occurs the irreversibility of the parallel chains to the more stable antiparallel chains arrangements. However, only in the case of chitin, the two polymorphs occur in native tissues. The crystalline structure of β-chitin (as that of native cellulose) results from a continuous biosynthesis coupled with a chain extension, out of the synthesizing organelle (located in the plasma membrane) and simultaneous crystallization. The case of α-chitin would require a distinct process in the form of a two-step mechanism. The first step would happen in a close organelle (also called chitosome), where it would occur in a somewhat fluid manner. This particular state would allow the chains to reorient and to organize in the most thermodynamically stable antiparallel arrangement. In a second step, the chitosome burst opens, and the microfibrils unravel. At present, such a two-step process works well *in vitro*. The question remains as to whether such a mechanism operates *in vivo* and relates to the widespread occurrence of α-chitin microfibrils in cuticles and not in cocoons where β-chitin occurs. (Merzendorfer & Zimoc, 2003) These differences might help to understand the function of chitin in different organisms as significant differences arise between cuticles and peritrophic matrices. As described in the previous section, the architecture of the cuticle results from a hierarchy of structural arrangements starting from α-chitin micro-fibrils embedded into a protein matrix. By contrast, in peritrophic matrices, the β-chitin micro-fibrils are generally arranged as a network of randomly organized structures embedded in an amorphous matrix and do not display high ordered arrangements.

6.3. Crystallography of Chitosan and its Polymorphs

Following the first attempt to characterize the crystalline arrangement of chitosan by X-ray diffraction, the results of many investigations highlighted the influence of experimental conditions: the degree of acetylation, molecular weight, on the crystallinity. The number of reported chitosan crystal structures matches the versatility of chitosan properties themselves. Numerous chitosan complexes resulting from the crystallization of chitosan with acids or transition or post-transition metal ions have been identified, but the detailed characterization of their molecular structures is lacking. (Cartier et al., 1992; Ogawa & Inukai, 1987; Ogawa, Oka, & Yui, 1993) In the absence of any adduct, chitosan crystallizes at least into two forms: (i) a hydrated orthorhombic $P2_12_12_1$ allomorph, proposed with a unit cell containing four molecular chains and eight water molecules. (Okuyama et al., 1997) (ii) a highly crystalline anhydrous allomorph, the structure of which has been progressively refined in a series of reports, following successive improvements in preparing and analyzing the crystals. (Ogawa et al., 2019)

In the most recent X-ray structure determination at atomic resolution, anhydrous chitosan crystallizes as a two-chain unit cell within the $P2_12_12_1$ space group. (Ogawa et al., 2019) Strickly speaking this structure corresponds to poly-glucosamine (more than "real" chitosan, which by definition, always contains N acetyl Glucosamine residues). The most detailed characterization of the structural feature of anhydrous crystal structure has been obtained using a combination of neutron crystallography and quantum chemical calculation. In the orthorhombic unit cell, the anhydrous chitosan chains adopt a two-fold helical conformation and pack in an antiparallel fashion. The conformational features of the primary hydroxyl groups, their participation in the network of hydrogen bonding network along with the amino groups are unambiguously established. There is a similarity with the hydrogen-bonding network of cellulose II in helping to stabilize the cohesion between antiparallel chains. Small differences occur due to the presence of the amino groups that act as poor hydrogen-bonding donors compared to the hydroxyl groups that act as both donors and acceptors.

6.4. Solid State Analysis of Chitin and Chitosan

The crystallographic features of α– and β– chitin and chitosan described above are established on "ideal" samples of well-assessed purity. When it comes to "real" samples, differences in the acetate contents and their distribution, molecular weight, polydispersity and level of crystallinity, generate changes in the overall structure and impact on the properties. Other spectroscopic methods have to be used for analyzing the structure and determining the physicochemical properties of chitin, chitosan, and their derivatives. A series of spectroscopic techniques using X-ray excitation offers an excellent complementarity to Infrared, NMR, Raman, and UV-Vis analytical techniques. (Kumirska et al., 2010)

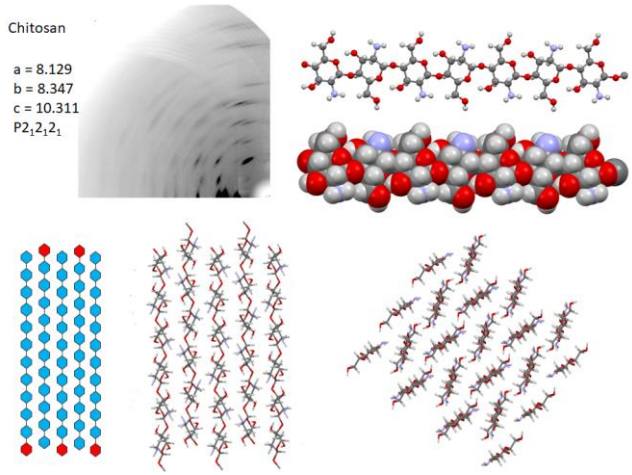

Figure 9. Structural features of anhydrous chitosan in the crystalline state, having unit cell having unit cell parameters: a= 8.129, b= 8.347, c= 10.311 (Ang) in the $P2_12_12_1$ space group as established by synchrotron X-ray fiber diffractometry. Structural drawings of the crystallographic conformation of a segment of chitosan. The bottom row shows the longitudinal anti-parallel chain packing perpendicular to the chitinosan chains, with the red hexagon indicating the reducing GlcN residue and the transverse packing of the chains –hydrogen atoms are not shown.

The first level of characterization is performed using X-ray powder diffractometry to identify the nature of the polymorph. The same method provides the determination of the crystallinity index of the samples as well as the degree of N-acetylation of chitin and chitosan. Such X-ray measurements are often used to characterize new derivatives in their processed form or as part as components of materials.

Infrared spectroscopy is and has been widely used to identify the occurrence and nature of chitin and chitosan from different sources. (Kumirska et al., 2010) Because of the high crystallinity of the samples, they display a series of very sharp absorption bands, which define specific signatures for α-chitin and β-chitin. The description and interpretation of the infrared spectra of the two forms of chitin have been published by many scientists. (Darmon & Rudall, 1950, Pearson et al., 1960; Brunner et al., 2009) The C=O stretching region of

the amide moiety, between 1600 and 1500 cm^{-1}, is quite interesting as it yields different fingerprints for α–chitin and β–chitin. For α-chitin, the amide I band is split at 1656 and 1621 cm^{-1}, whereas it is unique, at 1626 cm^{-1} for β-chitin. In contrast, the amide II band is unique in both chitin allomorphs: at 1556 cm^{-1} for α-chitin and 1560 cm^{-1} for β-chitin. The availability of the characterized 3D network of hydrogen bonds in α-chitin, explains the main features of its polarized FTIR spectra and sheds new light on the origin of the splitting of the amide I band observed on α-chitin IR spectra. (Sikorski et al., 2009)

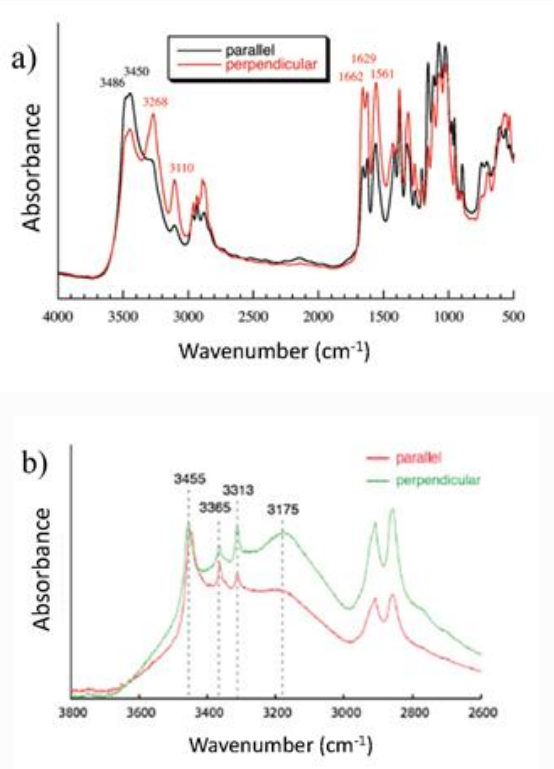

Figure 10. a) Polarized FTIR spectra of α-chitin (b) ATR-FTIR spectra of anhydrous chitosan with two different orientations of the fibrous specimen with respect to the incident beam

The characterization of the solid-state features of chitins and chitosans with a high level of acetylation is most suited to be studied by solid-state ^{13}C and ^{15}N NMR spectroscopy that does not require the dissolution of the polysaccharide. The most crystalline samples yield the best-resolved spectra. (Heux, et al., 2000) When recorded at 7.05 T, each spectrum consists of six single-line signals and two doublets at C-2 and C=O, but these doublets are in fact singlets that are split by the effect of the ^{14}N quadrupole coupling. In accounting for this phenomenon, there are therefore only eight signals for the eight carbon atoms of α- and β-chitins. Thus, in both allomorphs, the N-acetyl-D-glucosamine moiety is the independent magnetic residue, in full agreement with the crystal structure of α- and β-chitin where this residue is also the independent crystallographic unit. ^{13}C and ^{15}N CP-MAS NMR spectroscopies are also particularly suited to calculating the whole range of acetyl content from 0% to 100% with a small distortion below 5% due to the spectrum baseline and signal broadening. ^{15}N CP-MAS NMR spectroscopy was particularly powerful for calculating the fraction of acetylation FA (or DA).

FRACTION & PATTERNS OF ACETYLATION

Chitosan is the collective name for a group of fully and partially deacetylated chitins. Their fraction of acetylation FA influences the properties of chitosans, (also described as degree of acetylation (DA) The pattern of acetylation (PA) and their degree of polymerization. These parameters influence the physicochemical properties strongly from which the solubility in acidic conditions and interchain aggregation. They influence the biological activities of chitosan and chitosan oligosaccharides. Hence, accurate structural characterization is a key factor in understanding the structure-function relationships of chitosans.

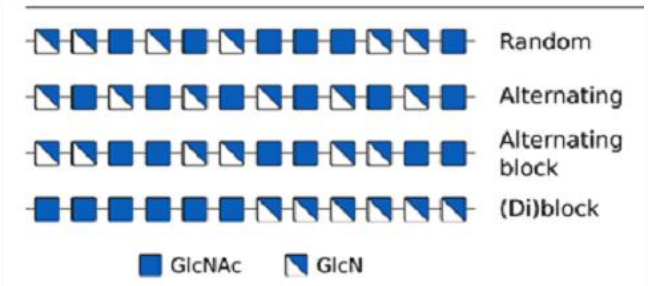

Figure 11. Illustration of theoretical patterns of acetylation (PA) of a chitosan polymers, all having the same degree of polymerization and FA = 0.5

The characterization of a chitosan sample requires the determination of its average DA. Various techniques, in addition to potentiometric titration (Rusu-Balaita, 2003), have been proposed, such as InfraRed (Brugnerotto et al., 2001; Miya et al., 1980; Baxter et al., 1992; Domszy et al.,1985), elemental analysis, an enzymatic reaction (Peletier et al., 1990) UltaViolet (Muzzarelli & Rochetti, 1985),^{1}H liquid-state NMR (Rinaudo et al., 1992) and solid-state ^{13}C NMR. (Saito et al., 1987; Raymond et al., 1993; Heux et al., 2000) Those different methods were discussed. (Kumirska et al., 2010)

The fraction of $–NH_2$ in the polymer (which determines the DA) can be obtained by dissolution of neutral chitosan in the presence of a small excess of HCl, followed by neutralization of the protonated $–NH_2$ groups by NaOH, in excess, using pH or conductivity measurements and then followed by titration with HCl to confirm the NH^{3+} content. These techniques and the analysis of the data obtained have been described. (Rusu-Balaita et al., 2003) ^{1}H NMR is the most convenient technique for measuring the acetyl content of fully soluble chitosan samples.

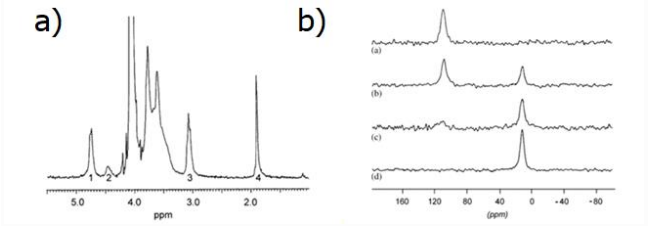

Figure 12. (a) ^{1}H spectrum obtained for chitosan dissolved in D_2O containing DCl (pD ca. 4). The signal at 1.95 ppm allows determination of the acetyl content by reference to the H-1 signal at 4.79 ppm for the D-glucosamine residue and at 4.50 ppm for the H-1 of the N-acetyl-D-glucosamine unit at 85 °C. ^{13}C (b) ^{15}N solid-state NMR were also tried and discussed; these techniques were used over the whole range of acetyl content from 0% to 100%.(Heux et al.2000)

As an example, Table 3 lists the values of DA obtained on four samples : A is an α-chitin, B is a homogeneous re-acetylated chitosan and C, D are commercial samples. (Heux et al., 2000) ^{15}N NMR gives only two signals related to the amino group and the N-acetylated group. This technique can be used in the solid state, whatever the DA. ^{13}C was also compared with ^{1}H NMR, and ^{15}N NMR. A good agreement was found over the entire range of DA, irrespective of the state of the sample.

Samples	A	B	C	D
DA from ^{1}H NMR (solution)	insoluble	0.58	0.21	Acetyl traces
DA from ^{13}C NMR (solid state)	0.99	0.61	0.20	~ 0
DA from ^{15}N NMR (solid state)	1	0.63	0.20	0

*Table 3. Degrees of acetylation of chitin and chitosan obtained by liquid state (^{1}H) and solid state ^{13}C and ^{15}N NMR on the same (*Heux et al., 2000*)*

The distribution of acetyl groups along the chain (PA) (random or blockwise) may influence the solubility of the polymer and also the inter-chain interactions due to H-bonds and the hydrophobic character of the acetyl group. This distribution was evaluated from ^{13}C NMR measurements; (Varum et al.,1991; Varum et al., 1991) diad and triad frequencies were determined for homogeneous and heterogeneous chitosan with different values of DA. More recently, ^{13}C NMR is analyzed following Varum *et al*. but introducing also alternated frequency of the two different types of units. (Kumirska et al., 2009; Kumirska et al., 2010; Kumirska et al., 2009) Additionally, mass spectrometry was developed and discussed. (Kumirska et al., 2010)

Several Infrared techniques are used to assess the acetylation faction. They all require the knowledge of a few reference sample of known FA. The PA can be assessed by the ratio of the intensity of the characteristic band of N-acetylation (which is a measure of the N-acetyl or amine content) to that of the intensity of a band that does not change with different FA value. There exist variations around these methods making use of different absorption band

The use of infrared spectroscopy for characterization of the composition of chitin and chitosan covering the entire range of degree of acetylation (DA) and a wide variety of raw materials is examined. The ratio of absorbance bands selected was calibrated using ^{1}H liquid and ^{13}C CP-MAS solid-state NMR as absolute techniques. IR spectra of the structural units of these polymers validated the choice of baselines and characteristic bands. The bands at 1650 and 1320 cm^{-1} were chosen to measure the DA. As internal reference, the intensities at 3450 and 1420 cm^{-1} were evaluated. The absorption ratio A1320/A1420 shows superior agreement between the absolute and estimated DA-values (Brugnerotto et al., 2001)

Fingerprinting techniques have expanded the possibilities for polysaccharide analysis, typically involving partial depolymerization and yielding mixtures of oligomers, which are subsequently analyzed. When combined with mass spectrometry (MS), these fingerprinting techniques have the advantage of extremely high sensitivity while at the same time reducing the sample amount from the milligram to the micro- or nano-gram range. (Weikert et al., 2017) Fingerprinting techniques require partial depolymerization of the sample, which is achieved using chemical, physical, or enzymatic treatments. Enzymatic depolymerization might be preferred because enzymes often have higher cleavage specificities than the other methods. In the case of chitosan, an enzymatic sequencing approach uses a combination of an exo-β-N-acetylhexosaminidase and an exo-β-glucosaminidase, which removes in an alternative fashion, GlcNAc and GlcN from the non-reducing end of the analytes. A broader range of chitosan hydrolyzing enzymes, particularly chitinases and chitosanases, can be involved. Chitinases cleave the glycosidic linkage between two adjacent GlcNAc units (A/A), and some chitinases may cleave the GlcNAc-GlcN (A/D) or the GlcN-GlcNAc (D/A) linkage. Similarly, chitosanases cleave the glycosidic linkage between two adjacent GlcN units (D/D), and some can cleave A/D or D/A. The more specific enzymes, which selectively cleave only A/A or only D/D, are preferred for fingerprinting. The amounts of the different chito-oligosaccharides derived from the enzymatic hydrolysis of chitosans are determined using Ultra-high performance liquid chromatography – electrospray ionization – mass spectrometry (UHPLC-ES-IMS). Data interpretation in the form of Partial Least Squares Regression yields determination of FA.

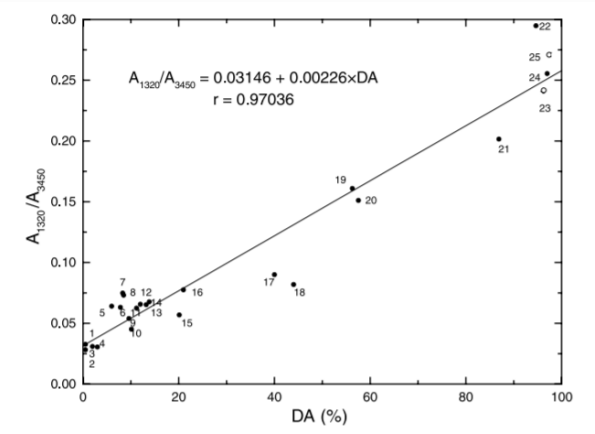

Figure 13. Calibration curve giving the degree of acetylation (DA) %, calculated from the rations of absorbance A1320/A1420. (Brugnerotto et al., 2001)

The PA analysis of chitosan polysaccharides relies on the analysis of dyad frequencies of the C-5 carbon resonances area in the ^{13}C NMR spectra. These studies were performed on chitosan samples derived from chemical preparation methods, including homo- and heterogenous de-N-acetylation and homogeneous N-acetylation. These investigations did not find evidence for a clear non-random PA in any of the samples. As the consequence of a continuous interplay between the results of the analysis of the enzymatic hydrolysis and the nature of these enzymes, new degrading enzymes generating non-random patterns of acetylation can be imagined and produced in a recombinant form. By this token, it is easy to produce enzymatically partially N-acetylated chitosans. By controlling and keeping FA and DP of the polymers constant, differences in functionalities of the polysaccharides could be assessed to the individual differences of their PA.

CHITIN AND CHITOSAN: SOLUBILITY

8.1. Solubility of Chitin

Chitin occurs naturally partially deacetylated (with a low content of glucosamine units), depending on the source. (Mathur & Narang, 1990) Nevertheless, both α and β forms are insoluble in all the usual solvents, despite natural variations in crystallinity. The insolubility is a major problem that confronts the development of processing and uses of chitin. An important mechanism is the solid-state transformation of β-chitin into α-chitin which occurs by treatment in strong aqueous HCl (over 7M) and washing with water (Saito et al., 1997). In addition, β-chitin is more reactive than the α form, an important property concerning the enzymatic and chemical transformations of chitin. (Kurita et al., 1993)

Because of the solubility problem, only limited information is available on the physical properties of chitin in solution. The first well-developed study (Austin, 1984, 1975) introduced the solubility parameters for chitin in various solvents. This gave rise to the formation of a complex between chitin and LiCl (which is coordinated with the acetyl carbonyl group). The complex is soluble in dimethylacetamide and in N-methyl-2-pyrrolidone. The same solvents and, especially, LiCl/DMAc mixtures, are also solvents for cellulose. Formic, dichloroacetic and trichloroacetic acids for dissolution of chitin chains are also used. Experimental values of parameters K and a relating intrinsic viscosity [η] and molecular weight M for chitin in several solvents according to the well-known Mark–Houwink equation $[\eta] = KM^a$. are given in Table 4.

Solvent	*K* (mL/g)	*a*	T(°C)	dη/dc	Ref
2.77M NaOH	0.1	0.68	20	0.145	Einbu et al., 2004
DMAc / LiCl 5%	7.6 x 10-3	0.95	30	0.091	Poirier & Charlet, 202
DMAc / LiCl 5%	2.4 10-3	0.69	25	0.1	Terbojevich et al., 1988

Table 4. Mark-Houwink parameters for chitin in various solvents (Rinaudo, 2006)

For a long time the most widely used solvent for chitin was a DMAc/LiCl mixture, though $CaCl_2$ $2H_2O$-saturated methanol was also employed, as well as hexa-fluoro-isopropyl alcohol and hexa-fluoro-acetone sesquihydrate. (Tamura et al., 2003; Carpozza et al., 1976) Concentrated phosphoric acid at room temperature dissolves chitin. (Vincendon et al., 1994) In this solvent, decreases of the viscosity and of the molar mass were observed with time with no change in the degree of acetylation. The use of a fresh saturated solution of lithium thiocyanate was instrumental to record a well-resolved NMR spectra at 90 °C. (Gagnaire et al., 1982; Vincendon, 1985)

A few papers deal with the preparation of alkali chitin by the dissolution of chitin at low temperature in NaOH solution. The chitin is first dispersed in concentrated NaOH and allowed to stand at 25 °C for 3 h or more ; the alkali chitin obtained is dissolved in crushed ice around 0°C. This procedure allowed the authors to cast transparent chitin film with good mechanical properties. (Einbu et al., 2004; Sannan et al., 1975; 1976) The resulting chitin is amorphous and, under some conditions, can be dissolved in water, whereas chitosan with a lower degree of acetylation (DA) and ordinary chitin are insoluble. The authors interpreted this phenomenon as related both to the decrease of molecular weight under alkaline conditions and to some deacetylation. They confirmed that to get water solubility, the DA has to be around 50%. Presumably, the acetyl groups must be regularly dispersed along the chain to prevent packing of chains resulting from the disruption of the secondary structure in the strong alkaline medium. (Sannan et al., 1976; Kubota & Eguchi, 1997) A study, utilizing techniques such as rheology, turbidimetry, and fluorescence, demonstrated that alkali chitin solubilized in cold (0 °C) aqueous NaOH (16% w/w), according with the protocol of Sannan et al. (Sannan et al., 1975; 1976), forms an LCST solution with a critical temperature around 30 °C. (Arguelles-Monal et al., 2003)

A chitin gel, obtained from the solution by washing to extract NaOH, was found to be temperature and pH-sensitive. (Sannan et al., 1975,1976, Goycoolea et al., 2006) These authors reported the occurrence of a volume phase transition at 21 °C as the result of the influence of temperature on polymer–polymer and polymer–water interactions such as hydrogen bonding and hydrophobic interactions. This transition occurs only within a narrow range of pH (7.3–7.6) and modifies the mechanical shear modulus as a function of oscillating variation in temperature.

Chitin can be dissolved and regenerated from various imidazolium based ionic liquids such as 1-butyl-3-methylimidazolium acetate and 1-butyl- 3-methylimidzolium chloride (Wu et al., 2008) 1-allyl-3-methylimidazolium bromide (Prasad et al 2009), and 1-ethyl-3-methylimidazolium propionate (Mundsinger et al., 2015) have been employed. Other Ionic Liquid systems have been investigated. For example, tris (2-hydroxyethyl)methylammonium acetate with added ethylenediamine, could dissolve chitin without heating. (Shimo, et al., 2006) Nevertheless, in addition to the nature of the Ionic Liquids, the solubility of chitin seems to be moderate and depends on the molecular weight and on the Degree of Acetylation. (Wang et al., 2010) Xie et al., 2006 reported that 1-butyl-3-methylimidazolium chloride ([C4mim]Cl) can dissolve pure chitin and chitosan with solubilities of ca. 10 wt% in 5 h at 110° C. Yamazaki et al.(Yamazaki et al., 2009) obtained similar solubilities with 1-allyl-3-methylimidazolium bromide [Amim]Br at 100 °C for 24 h. (Wu et al., 2008) 'Native' chitin could be dissolved using the acetate salt [C4mim]OAc with 3–7 wt% solubility at 110 °C. It is claimed that dissolution of dried schrimp shell in Ionic Liquids allows to get pure chitin while byproducts (such as calcium carbonate) remain undissolved and could be centrifuged out. Following coagulation in a non-solvent (water or methanol) proteins and fatty acids remain in water-Ionic Liquid mixture after coagulation and are removed during regeneration. (Rahman et al., 2009)

The rheology of chitin in solution is that of a semi-rigid polysaccharide for which the conformational analysis has been developed in comparison with chitosan. Chitin has been completely dissolved in NaOH/urea aqueous solution at low temperature (5°C) to obtain a transparent solution to determine the persistence length Lp in absence of aggregates. In this solvent, chitin behave as a worm like chain with Lp= 30 nm. (Fang et al., 2015) The solution must be diluted to avoid the formation of aggregates which increases with polymer concentration and temperature. A series of functional chitin-based materials such as hydrogels, aerogels, films, fibers, and microspheres with homogeneous structure and excellent properties have been obtained. (Fang et al., 2015) Several biocompatible chitin-based aerogels, fibers, and hydrogels have been directly constructed. (Zhang, 2015) A facile method for the construction of nanofibrous microspheres from chitin in NaOH/urea aqueous solution through thermally induced self-assembly was reported for the first time. (Zhang 2015)

8.2. Solubility of chitosan

When the degree of deacetylation of chitin reaches about 50% (depending on the origin of the polysaccharide), it becomes soluble in aqueous acidic media and is called chitosan. The solubility is a complicated parameter to control: it is related to the DA or FA, the ionic concentration, the pH, the nature of the acid used for protonation, and the distribution of acetyl groups along the chain, as well as the conditions of isolation and drying of the polysaccharide. The intra-chain H bonds involving the hydroxyl groups are also important. The microstructure of the polysaccharide plays a significant role when a fully deacetylated chitin is reacetylated in solution; the critical value of chitosan DA required to achieve insolubility in acidic media is then greater than 60%. Also, solubility at neutral pH has been reported for chitosan with DA around 50%. (Aiba, 1991) A water-soluble form of chitosan at neutral pH was obtained in the presence of glycerol 2-phosphate. (Chenite et al., 2000; Chenite et al., 2001; Molinaro et al., 2002; Cho et al., 2005) Stable solutions were obtained at pH 7–7.1 and room temperature, but a gel formed on heating to about 40 °C. The sol-gel transition was partially reversible, and the gelation temperature depended slightly upon experimental conditions. (Rinaudo, 2006)

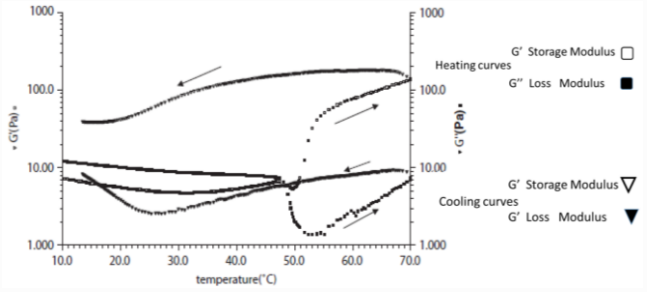

Figure 14. Dynamic rheology giving the moduli G' and G" at 1 Hz frequency as a function of temperature for chitosan-phosphate-glycerol solution showing evidence of a thermogelation at pH = 7.19. Polymer concentration 15g/L.

A highly deacetylated polysaccharide has been used to explore the different methods of characterization. (Domard & Rinaudo, 1983).

The solution properties of a chitosan depend not only on its average DA but also on the distribution of the acetyl groups along the main chain, in addition of the molecular weight. (Kubota & Eguchi, 1997;Aiba, 1991;Rinaudo & Domard, 1989) The deacetylation, is usually performed in the solid state; it gives an irregular structure due to the semi-crystalline nature of the initial polymer. Examination of the role of the protonation of chitosan in the presence of acetic acid (Rinaudo et al., 1999) and hydrochloric acid on solubility (Rinaudo et al., 1999) showed that the degree of ionization depends on the pH and the pK of the acid. The solubilization of chitosan with a low DA occurs for an average degree of ionization α of chitosan around 0.5; in HCl, α = 0:5 corresponds to a pH of 4.5–5. The solubility is also a function of the ionic concentration. An excess of HCl (1M HCl) creates a salting-out effect allowing the preparation of the chlorhydrate form of chitosan. When the chlorhydrate and the acetate forms of chitosan are isolated, they are directly soluble in water giving an acidic solution. (Rinaudo et al., 1999) Study of these solutions allows the determination of the intrinsic pK (pK_0=6 ± 0.1) by extrapolation of pK for a degree of protonation α=0. This intrinsic value of the pK agrees with the previous measurements. (Domard, 2000) Thus, chitosan is soluble at pH below 6. The solubility of chitosan is usually tested in acetic acid by dissolving it in 1% or 0.1M acetic acid. The amount of acid needed depends on the quantity of chitosan to be dissolved. (Rinaudo et al., 1999) The required concentration of proton must be at least equal to that of $-NH_2$ units involved.

Solvent	*K* (mL/g)	*a*	T(°C)	dη/dc	Ref
0.1 M AcOH/0.2M NaCl	1.81 x 10-3	0.93	25		Roberts et al., 1982
0.1 M AcOH/0.02M NaCl	3.04 x 10-3	1.26	25		Roberts et al., 1982
0.2 M AcOH/0.1M AcONa/4 M urea	8.93 x 10-2	0.71	25		Lee, 1974
0.3 M AcOH/0.2M AcONa (DA= 0.02)	8.2 x 10-2	0.76	25	0.163	Rinaudo et al., 1993
0.3 M AcOH/0.2M AcONa (0<DA<0.03	7.9 x 10-2	0.796	25	0.190	Brugnerotto et al., 2001
0.02M acetate buffer/0.1M NaCl	8.43 x 10-2	0.92	25	0.203	Berth & Dautzenberg, 2002

Table 5. Mark-Houwink parameters for chitosan in various solvents (Rinaudo, 2006)

The aqueous solution containing LiOH/KOH/urea/H_2O in the weight ratio (4.5 wt % LiOH/7 wt % KOH/8 wt % urea) via the freezing-thawing process was used as an alkaline solvent of chitosan (Duan et al., 2015). To prepare the solutions, chitosan powders (with DA=0.11) were dispersed into the alkaline aqueous solvent with stirring for 5 min and then were stored under refrigeration (−30 °C) until completely frozen. The frozen solid was fully thawed and stirred extensively at room temperature. After removing air bubbles by centrifugation at 7000 rpm for 10 min at 5 °C, a transparent chitosan solution with the concentration of 4 wt %. A temperature increase yields a different morphology.

CHITIN AND CHITOSAN: MOLECULAR WEIGHT, PERSISTENCE LENGTH, RHEOLOGY

9.1. Molecular Weight.

A complete analysis of the molecular weight distribution by SEC using triple detection (Brugnerotto et al., 2001) (viscosity, concentration, molecular weight) was reported. The samples under investigation were heterogeneous chitosans: some were from commercial sources after solid-state treatment; others were some homogeneous chitosans of different molecular weights obtained by re-acetylation of a highly deacetylated chitosan. (Roberts & Domszy, 1982) The DA of these acid-soluble chitosans varied from 0.02 to 0.61. The data confirm that the stiffness of the chain is nearly independent of the DA and demonstrate that the other descriptors (including the persistence length) depend only slightly on the DA. The relationship obtained between the intrinsic viscosity [η] and the radius of gyration Rg and the molecular weight is:

$[\eta](mL/g) = 0.0843M^{0.92}$ and $Rg(nm) = 0.075\ M^{0.55}$

Within a window covering the total range of DA, average values for the Mark–Houwink were established; their validity holds for heterogeneous as well as for homogeneous samples.

DA (%)	*K* (mL/g)	*a*
0.3	0.079	079
12	0.074	0.80
22-24	0.070	0.81
40	0.063	0..83
56-61	0.057	0.825

Tableau 6. Mark-Houwvink parameters for chitosan having different average DA in 0.3 M AcOH/0.2 M AcONa (Rinaudo, 2006)

The relatively high values for the parameter "*a*" agree with the semi-rigid character of this family of polysaccharides. Computer simulation performed on chitin, and chitosan confirmed this conclusion, (Mazeau et al., 2000; Mazeau & Rinaudo, 2004) in agreement with the experimental results obtained by SEC. It is important to mention the usual method of preparing chitosans with various molecular weights using nitrous acid in dilute HCl aqueous solution. (Allan & Peyron, 1995) The influence of the ionic strength on the Mark–Houwink parameters K and *a* (Rinaudo et al., 1993; Anthosen et al., 1993; Varum & Smidsrod, 2005) was also investigated. Two series of solvents were used: 0.3M acetic acid/variable Na acetate content and 0.02M acetate buffer (pH = 4.5) buffer with various concentrations of NaCl. This experiment allowed the determination of the relationship between the intrinsic viscosity and the salt concentration. From these experimentally determined values, the extrapolation to infinite ionic strength is used to approach the θ-conditions.

9.2. Persistence Length

The semi-rigid nature of the polysaccharide chains dictates the dimensions of chitosan chains, their related hydrodynamic volume and ultimately, their viscometric contribution. Since chitosan in an acid medium is a polyelectrolyte, the ionic concentration influences these properties. This point can be addressed using static and dynamic light scattering experiments in the dilute and semi-dilute regimes. (Bulher & Rinaudo, 2000; Buhler et al., 2000) The actual persistence length Lt at a given ion concentration results from an intrinsic contribution Lp and an electrostatic contribution Le calculated following Odijk's treatment. (Odjik, 1979) The worm-like model for a semi-flexible chain has been developed by several groups and successfully applied to polysaccharides. (Rinaudo et al., 1993; Brugnerotto et al., 2001; Reed, 1984)

A computer simulation performed on chitin and chitosan having different degree of acetylation (Mazeau et al., 2000; Mazeau & Rinaudo, 2004) confirmed the semi-rigid nature of chitin and chitosan. The calculated persistence length (asymptotic value obtained at a high degree of polymerization) depends moderately on the DA of the polysaccharide. From this analysis, chitosan without acetyl groups has an intrinsic persistence length Lp = 9 nm at 25°C when the electrostatic repulsions are screened. Lp increases as DA increase up to Lp =12.5 nm for DA= 0.60, then remains constant up to pure chitin. The local stiffness is related to the conformation of the molecule, and especially to the intra-chain H bond network formed. The decrease of the stiffness of chitosan as temperature increases, as shown by ^{1}H NMR (Brugnerotto et al., 2001), agrees with the prediction from molecular modeling. A critical temperature around 40°C is found where Lp starts to decrease more rapidly; this behavior relates to the destabilization of H bonds as the temperature increases. The difference in Lp values between experiment and prediction is not dramatic for chitosan, and it is difficult to determine for chitin because of its low solubility. The decrease of the stiffness of chitosan chain when the DA decreases has been confirmed and analyzed in terms of the destabilization of the local conformation by intra-chain H bonds. (Fang et al., Biomacromolecules, 2015)

The stiffness of the chain contributes to the rheological behavior of the polysaccharide. Even in dilute solution, the stiffness influences the formation of interchain H-bonds throughout the creation of multimeric assemblies that perturb all characterization of these polysaccharides. The aggregation has been discussed and analyzed; it seems that H-bonds, as well as hydrophobic attractions, have a role, irrespective of the DA. (Philippova et al.. 2001)

9.3. Rheology

The rheological behavior of polymeric solutions is generally characterized using flow and dynamic measurements.

Flow experiments. In this type of experiment, the viscosity is determined as a function of the shear rate at a constant temperature. Usually, the viscosity increases when the shear rate decreases going to a Newtonian plateau at low concentration. The viscosity obtained at zero shear rate on the diluted and semi-diluted solution of the polymer has been studied. A general relationship could be proposed for perfectly soluble polymers in good solvent relating the specific viscosity at zero shear rate to the polymer concentration and molecular weight expressed by the overlap parameter C[η]:

$$(\eta sp)_0 = C[\eta]\{1 + k_1(C[\eta]) + k_2(C[\eta])^2 + k_3(C[\eta])^3\}$$

with $k_1 = 0.4$; $k_2 = k_1^2/2!$; $k_3 = k_1^3/3!$.

These parameters are used to establish a master curve for the polymer as a function of the Mw and the polymer concentration. Solutions obtained with chitosan in acetate buffer and hyaluronan in 0.1M NaCl at different polymer concentrations were studied to establish the experimental dependence of the specific viscosity at zero shear rate on the overlap parameter (Figure 15):

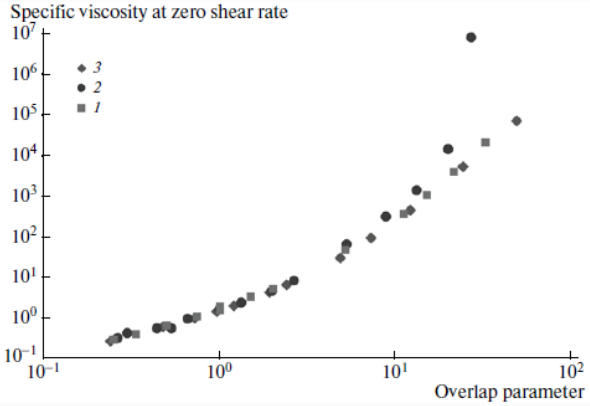

Figure 15. Influence of the overlap parameter on the specific viscosity at zero shear rate and at 25°C: **1** *HA in presence of 0.1 M NaCl;* **2** *Chitosan in 0.3 M acetic acid/0.1 M sodium acetate;* **3** *predicted value from relation established by (*Mazeau & Rinaudo, 2012*)*

As seen on this figure, hyaluronan a perfectly soluble polysaccharide fits with the theoretical prediction. On the opposite, when the overlap parameter is larger than 10 (i.e. in semi-diluted solution) the viscosity increases more sharply (look at the points •) indicating some aggregation when polymer concentration increases. (Mazeau & Rinaudo, 2012) This aggregation was discussed previously. (Philippova et al., 2012)

From flow experiments, the determination of the intrinsic viscosity [η] obtained at zero concentration and zero shear rate, allows to access to the viscometric-average molar mass Mv using the Mark Houwink relationship determined in the same solvent at the same temperature. (Brugnerotto et al., 2001; Rinaudo, 2006).

Dynamic experiments. In the linear viscoelastic regime, a steel cone-plate geometry rheometer is used. The storage (G′) and the loss modulus (G″) as a function of the angular frequency (ω) are determined at a constant temperature. On polymeric solution, one

obtains usually, G' < G'' (as a function of ω) at low frequencies. These curves cross at a given ω_0 which decreases when polymer concentration increases. Chitosan solutions in good solvent have the normal behavior of the polymer solution. This behavior is described on xanthan dissolved in 0.1M NaCl aqueous solution. (Milas et al., 1990)

CHITOSAN: COMPLEX FORMATION

10.1. Complex formation with metals

Chitosan exhibits good complexing ability throughout the involvement of the $–NH_2$ groups along the chain, in specific interactions with metals. Many articles have been devoted to the formation of complexes for the recovery of heavy metals from various waste waters. (Muzarelli, 1973) A mechanism for complex formation with copper at pH>5, was proposed (Domard, 1987) (in agreement with X-ray data on chitosan–copper stretched films. (Ogawa et al., 1984). The mechanism of complex formation with copper in dilute solution was re-examined; two different complexes were proposed to occur, depending on the pH and copper content. (Rhazi et al., 2002)

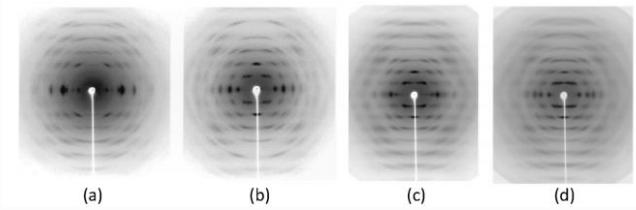

Figure 16. X-ray diffraction pattern of chitosan complexed to (a) HNO_3 salt; (b) $ZnCl_2$ salt; $CdCl_2$ salt; and (d) $CdSO_4$ salt. (Okuyama et al., 2000)

This chelation depends on the physical state of chitosan (powder, gel, fiber, film). Higher degrees of deacetylation of chitin generates better chelation. Thus, chelation is related to the $–NH_2$ content as well as to their distribution. (Kurita, et al., 2002) It is also related to the DP of oligo-chitosans; the complex starts to form, at a degree of polymerization of 46. (Rhazi et al., 2002) The two following forms are proposed:

$[Cu\ (\text{-}NH_2)]^{2+}$, $2OH^-$, H_2O and $[Cu\ (\text{-}NH_2)2]^{2+}$, $2\ OH^-$

The first complex is formed at pH between 5 and 5.8, whereas the second forms above pH 5.8; the maximum amount of copper fixed is $[Cu]/[\text{-}NH_2] = 0.5$ mol/mol. The nature of the cation is critical in the mechanism of interaction. The affinity of chitosan for cations absorbed on film shows selectivity following the order:

$Cu^{2+}>>Hg2^{+}>Zn^{2+}>Cd^{2+}>Ni^{2+}>Co^{2+}+ \sim Ca^{2+}$; $Eur^{3+}>Nd^{3+}>Cr^{3+}>Pr^{3}$

for divalent and trivalent cations used as their chlorides.

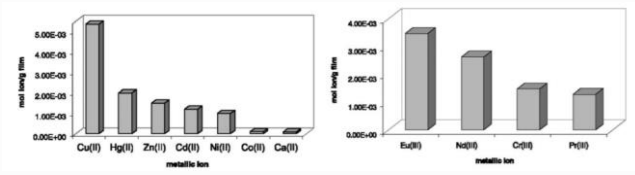

Figure 17 Ion selectivity of chitosan amount (moles) for divalent and trivalent cations fixed per g of film. Taken from reference.(Rhazi et al., 2002)

The effect of the nature of the anion was separately demonstrated (Rhazi et al., 2002; Mitani et al., 1991) : e.g., sulfate increases the fixation on swollen chitosan beads. In another study, chitosan powder was dispersed in a silver nitrate solution or used to fill a column to adsorb mercuric ions from a chloride solution. (Peniche-Covas et al., 1992) The conditions for using chitosan (50 mesh particles of chitosan or chemically crosslinked beads of chitosan) play a large role in the adsorption. This is also true for the kinetics of retention. (Ruiz et al., 2002; Annachhatre & Chandrachang, 1996) Mixing chitosan powder in 1.5M ferric chloride yields the formation of the complex of chitosan with Fe^{3+}. The solid formed was washed, dried and investigated. (Nieto et al., 1992) These authors obtained an intramolecular water-soluble chitosan–Fe(III) complex and determined that one $Fe3^{+}$ interacts with two chitosan residues, three molecules of water and one chloride ion. The general formula given is $[Fe(H_2O)_3(GlcN)_2Cl^-]\ 2Cl^-.\ H_2O$, where GlcN represents the glucosamine moiety. An aqueous solution of polymer and ferric chloride mixed in stoichiometric proportions yields formation of the complex. One Fe^{3+} interacts two $–NH_2$ groups and four molecules of oxygen from which at least one water molecule. The remaining N and O are part of the two saccharide units of chitosan (Fe^{3+} are hexa or penta coordinated). (Bhatia & Ravi, 2000).

Chitosan extracted from tendon was immersed in solutions of various salts of transition metals and further submitted to X-ray investigation. (Ogawa et al., 1992) The ratio of glucosamine to copper (II) was 2:1. The crystal structure of $CuCl_2$/chitosan was different from that in complexes formed with other salts. Several derivatives of chitosan have been prepared in view of enhancing the formation of complexes. (Gomez-Guillen et al., 1992; Chiessi et al., 1993; Hall & Yalpani, 1984; Muzzarelli et al., 1982a,b; Muzzarelli, 1989) In one study, the same order of ionic selectivity for divalent cations, as given above, (Rhazi et al., 2002) was found by calorimetric measurements with N-carboxymethyl chitosan. (Muzzarelli, 1989)

Chitosan, as a polyelectrolyte, forms electrostatic complexes under acidic conditions. Two different types of complexes are considered: electrostatic complexes with an oppositely charged surfactant (SPEC) and polyelectrolyte complexes (PEC).

10.2. Complexes with surfactants.

The general behavior of polyelectrolytes is demonstrated with chitosan and sodium dodecyl sulfate (SDS). An electrostatic complex forms in the presence of a low DA chitosan involving cooperative stacking of surfactant alkyl chains. The association forms a micellar system that precipitates out. The addition of tiny amounts of surfactant generates interesting interfacial properties. Surface tension measurements detect a critical aggregation concentration (c.a.c.) around 100-fold smaller than the c.m.c. of the surfactant alone. (Desbrieres et al., 1997; Babak et al., 2002). The cooperativity of the observed interaction depends directly on the charge density of the chitosan (in fact, it depends on the distance between two adjacent ionic sites) as shown for carboxymethyl chitin in the presence of tetra-decyltrimethylammonium bromide (TTAB). (Desbrieres & Rinaudo, 1999)

There is the formation of a capsule when a chitosan solution is dropped into an SDS surfactant solution; a chitosan gel layer (characterized by an ordered nanostructure) cross-linked by charged surfactant micelles is formed in the interfacial film. (Babak et al., 2000abc) Such a structure can encapsulate enzymes. (Babak et al., 2001) This type of electrostatic complex has been examined by calorimetry. The strong affinity and its dependence on the excess of external salt confirm the electrostatic mechanism. (Prado et al., 2004;

Thongngam & McClements, 2004; Thongngam & McClements, 2005) This electrostatic interaction can be compared with covalent analogs obtained by grafting alkyl chains on a chitosan backbone (see below for a description of these derivatives). The interfacial properties of the chitosan-derived polymer surfactant display a relatively low surface tension activity but interesting bulk properties. The role of sulfated N-acyl chitosan (S–Cn–Chitosan) in a lipid membrane compare with that of SDS. This SDS dissociates the membrane, whereas the polymer stabilizes the membrane, and even increases its rigidity, suggesting low toxicity in bio-organisms. In solution, when the alkyl chain in S–Cn–chitosan is longer than ten units, the polymers form more stable micelles than those formed by the same alkyl chain surfactant alone. (Nonaka et al., 2002) Interactions of this kind are relevant to the field of food chemistry, involving specific interactions of chitosans with phospholipids and bile acids. (Thongngam & McClements 2005)

10.3. Complexes with oppositely charged polymers (macromolecules, polyanions, DNA)

Mixing oppositely charged polyelectrolytes results in the formation of a polyelectrolyte complex (PEC) based on electrostatic interactions depending upon pH and external salt concentration. (Kabanov, 2003; Kabanov & Zezin, 1984) Developments of new biomaterials production and novel biomedical applications use electrostatic complexes involving natural biopolymers (Jeong et al., 2017; Volodkin et al., 2007) Strong electrostatic interactions between oppositely charged systems takes place at the interface. They can stabilize or destabilize liposomes for drug release (Volodkin et al., 2007; ial et al., 2005) or for the formation of lipoplexes especially with DNA (Serikawa et al., 2000; Stephan et al., 1996; Bochichio et al., 2015) or to stabilize a colloidal dispersion. (Domard et al., 1989; Pefferkorn, 1995) It has been demonstrated that liposomes are stabilized by chitosan adsorption against osmotic or pH shocks and that chitosan is adsorbed flat on the surface. (Quemeneur et al., 2010)

Layer by layer formation between oppositely charged polyelectrolytes is an important development of polyelectrolyte complexes. It was applied to form capsules by deposition on liposomes and their stabilization (Fukui & Fujimoti, 2009; Angelini et al., 2008), to stabilize the biological activity of peptide hormones or to coat blood vessel using chitosan and hyaluronan.

Literature cites many electrostatic PEC between chitosan and synthetic or natural charged polymers : e.g. polyacrylic acid, sodium salt (PAA), carboxymethylcellulose (Peniche & Arguelles-Monal, 2001; Arguelles-Monal & Peniche,1988), xanthan, carrageenan, alginate (extracted from brown algae), pectin, heparin, hyaluronan (HA) (Rusu-Balaita et al., 2003; Vasiliu et al., 2005) sulfated cellulose, dextran sulfate, N-acylated chitosan/ chondroitin sulfate. (Kubota & Kikuchi, 1998; Goycoolea et al., 2000) The electrostatic interaction has been discussed in relation to the stiffness of the backbone and nature of the ionic groups involved. Especially with alginate or HA, a pH-dependent complex occurs, whose stability depends on the ionic strength. The complex formation was investigated in dilute solution by potentiometry following changes in pH and conductivity to determine the fraction of ion pairs ($–COO^-+NH_3–$) formed, depending on the experimental conditions.(Rusu-Balaita et al., 2003; Arguelles-Monal et al., 2000) The interaction between chitosan and alginate gives an electrostatic complex which, so far, has been used mostly for biological applications.

The main applications of these electrostatic complexes are antithrombogenic materials, controlled release systems, encapsulation of drugs, immobilization of enzymes and cells, and gene carriers. Among the examples, are the applications of alginate/chitosan complexes. One aspect of these complexes deals with the preparation, layer-by-layer (successively, one layer of polyanion–one layer of polycation), of polyelectrolyte capsules or films based on charged biocompatible polysaccharides or chitosan/synthetic PEC. (Vasiliu et al., 2005; Zang et al., 2005; Berth et al., 2002) In the case of chitosan (Zang et al., 2005), PAA is used to form the capsules, then the chitosan is cross-linked, and the PAA is re-dissolved. Such chitosan capsules are more stable than in the absence of chemical crosslinking; they are pH-sensitive, swell at low pH, and shrink at high pH. Calcium alginate gel-stabilized by complexation with galactosylated chitosan (a water-soluble derivative) yields a porous gel (sponges). (Chung et al., 2002) Dropwise addition of Na–alginate to chitosan–$CaCl_2$ solution produces a complex in the form of beads. These beads differ from Ca-alginate beads in exhibiting maximum swelling at pH 9 (Lee et al., 1997) Oligo-chitosans, low molecular weight chitosans, were also complexed with alginates to form capsules with controlled permeability. (Bartkowiak & Hungeler, 1999; 2000; Bartkowiak et al., 2000)

10.4. Non –viral vectors for gene therapy.

In the areas of bioactive systems, the attention was focused on cationic polymers able to compact DNA and to be used as non-viral vectors for gene delivery. (Kabanov & Kabanov, 1995; Richardson et al., 1999; Mao et al., 2001; Strand et al., 2005; Buschmann et al., 2013; MacLaughlin et al., 1998; Vijayanathan et al., 2002; Leong et al., 1998; Luc & Saltzman, 2000;Tiera et al., 2006; Santos-Carballal et al., 2018) The mechanism is described in the schematic picture (Figure 18). The polycationic entities usually examined are polylysine, polyethyleneimine (PEI) and chitosan. Low immunogenicity, biocompatibility and minimal cytotoxicity of chitosan are actually recognized and proposed to develop a better alternative to viral or lipid vectors.

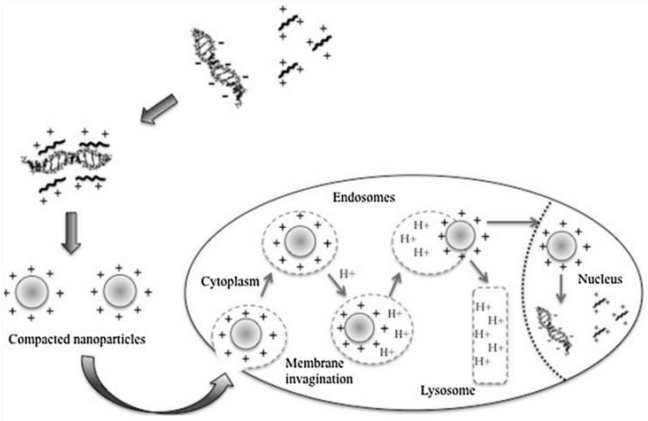

Figure 18. Schematic representation of DNA/ polycation complex in gene transfer mechanism (Adapted with permission from Vijayanathan et al., 2002) Taken from Bravo-Anaya et al., 2016

The first description of chitosan as a possible carrier for gene therapy was reported in 1995. (Mumper et al., 1995) Chitosan is a pseudo-natural polysaccharide exhibiting properties as biocompatibility, biodegradability, muco-adhesion, and non-toxicity. Besides of their immunogenicity, chitosan molecules condense efficiently with DNA forming polyplexes that prevent degradation by DNAses or within the serum. (Richardson et al., 1999; Leong et al., 1998; Hill et al., 2001; Koping-Hoggard et al., 2001). These electrostatic complexes occur reversibly without alteration of the DNA double helix conformation, as tested with low molar mass chitosan (MW=40 000;

degree of acetylation (DA) = 1%). (Prashabth et al., 2007; Liu et al., 2005) Electrostatic interactions between the protonated chitosan -NH_2 and the charged phosphate groups of DNA drive the formation of Chitosan-DNA complexes. (Bravo-Anaya et al., 2016; 2019) The parameters that control the formation of complexes and influence the particles size and stability include chitosan molecular weight (MW; N being the number of totals -NH_2), plasmid concentration (characterized by the phosphate content P) and the charge ratio (N+/P-). (Strand et al., 2005; MacLaughlin et al., 1998; Alatorre-Meda et al., 2001; Amaduzzi et al. 2014; Bordi et al., 2014; Ma et al., 2009; Alatorre-Melda et al., 2009; Lavertu et al., 2006; Howard et al., 2006) All these works demonstrated that the size of the formed particles decreases when the molar mass of chitosan decreases but that the stability of the complex increased when the MW increased. Furthermore, the size of the complex increases when plasmid concentration increases. Besides, higher molecular weight chitosans associate more strongly with plasmids. Those complexes are more stable to salt and serum challenge. (MacLaughlin et al., 1998)

Chitosan characteristics are most important in complex formation since they control the condensation and their stability. Many characteristics of chitosan have been previously discussed in the literature. (Rinaudo, 2006) and summarized as follows : (1) chitosan molar masses MW (partial depolymerization with sodium nitrite generates samples with different MW). (MacLaughlin et al., 1998) (ii) chitosan degrees of acetylation (DA usually between 0 and 0.3) to be soluble in acidic conditions) (iii) their acetyl groups distribution along the chains (usually not examined in complex formation).

Several articles deal with the influence of MW and DA of chitosan on complex formation with DNA. (Strand et al., 2005; Buschmann et al., 2013; Bordi et al., 2014; Ma et al., 2009; Amaduzzi Alatorre-Meda et al., 2009; Lavertu et al., 2006) In these works, the main techniques used are electrophoretic mobility (and zeta potential), Dynamic Light Scattering (DLS), Atomic Force Microscopy (AFM), potentiometry or microcalorimetry. Since chitosan is a weak base with a pK_0=6.5, pH is an essential factor to control the degree of protonation of the amino groups; the positive fraction of charge (-NH^{3+}) being the factor which controls the complex formation with the highly negatively charged DNA. (Strand et al., 2005; Sato et al., 2001) The higher the degree of chitosan protonation, the stronger is the stability of complex formed with DNA and the condensation of the complex. (Strand et al. 2005) A minimum of 6 to 9 monomeric units is necessary to complex DNA. However, the stability of the complex tends to be weak, dissociating at pH> 6.5 or in salt excess. (Strand et al., 2005) The stability of the complex depends on pH, N+/P- charge ratio, and salt concentration *in vitro*.

The most important parameter after chitosan characteristics is the ratio N/P (or chitosan units/phosphate units). For progressive additions of chitosan at a pH lower than 6.5 to a dilute DNA solution, N+/P- increases while the complex forms. The complex is negatively charged, up to an isoelectric point followed by the charge inversion. The N+/P- charge ratio at null charge is usually found around the charge stoichiometry when only the protonated fraction is considered. (Bravo-Anaya et al., 2016) For gene delivery, it is important to use a positive complex (N+/P- >1) in the nanometric range of particle diameters able to interact with the negatively charged cell membrane. This is a requirement to the entrance in the cell through endocytosis and pinocytosis to allow transfection. (Mao et al., 2001; Strand et al., 2005; Buschmann et al., 2013; Amaduzzi et al., 2014; Lavertu et al., 2006) It was claimed that N/P molar ratio=3 gives the highest transfection activity in serum using a MW=70 000 chitosan. Compared with other polycations often proposed for gene therapy, chitosan has a lower toxicity than polylysine. After 96 hours, this polycation was ten times more efficient than PEI. (Erbacher et al., 1998)

Another important and delicate point is the evaluation of the transfection efficiency of chitosan/DNA systems in vivo. The stability of the complex depends on several factors such as chitosan characteristics (DA and MW), local pH and salt composition, charge ratio of chitosan to DNA (N+/P-) playing on complex stability. It also depends on the cell type, nanoparticle size, enzyme and protein interactions, and interactions with membranes. It was reported that the transfection efficiency at pH 6.5 was higher than at pH 7.4. (Nimesh et al., 2010) At pH=8, the complex was fairly insoluble and did not penetrate the membrane. (Erbacher et al., 1998) Nevertheless, the transfection efficiency based on DNA/chitosan complex is not yet fully understood. The control of the different steps of the mechanistic pathway for gene transfection is required. It includes the collapse of extended DNA chains into compact nanometric particles. This process, known as DNA condensation, has received considerable attention for the production of gene delivery vehicles (Vijayanathan et al., 2002) and for this step chitosan is a good and adequate candidate. (Mao et al., 2001) Then, the positive particles of DNA compacted by polycations interact with the anionic proteoglycans at the cell surface and are transported by endocytosis. The cationic agents have a buffering capacity in the endosomal pH range (pH 4.5 to 7.5) inhibiting the degradation of DNA by lysosomal enzymes. (Buschmann et al., 2013; Richard et al., 2013) The mechanism was fully elucidated for PEI/DNA complexes. (Akinc, et al., 2005; Benjaminsen et al., 2013; Neuberg & Kichler, 2014)

Potentiometry demonstrated that the buffering capacity, or proton sponge effect, was larger for chitosan than for PEI for the same number of ionic sites.(Richard et al., 2013). The presence of an excess of free chitosan increased the osmotic pressure and destabilized the endosome. This releases DNA complex migrating into the nucleus where it can decondense after separation from the cationic delivery vehicle and can regulate gene expression (Richardson et al., 1999; Buschmann et al., 2013; Richard et al., 2013; Thibault et al., 2011; Ma, et al., 2010a,b). In fact, the last limiting step is the un-packaging of DNA from the complex after its localization in the nucleus. Labeled polylysine having different molecular weights that the complex formed dissociates more rapidly with lower molecular both *in vitro* and *in vivo*. (Howard et al., 2006)

The question of the stability of the complex in vivo is important but not yet solved: higher stability hampers the transfection efficiency. (Koping-Hoggard et al., 2001; Alatorre-Meda et al., 2001) There is a fine balance between extracellular DNA protection (better with high MW and lower DA) and ability of efficient intracellular unpacking (better with low MW) in order to get a large level of transfection. (Buschmann et al., 2013; Luo & Saltzman, 2000; Liu et al., 2005; Ma et al., 2009; Lavertu et al., 2006) In a previous work, DNA-chitosan complex stoichiometry, net charge, dimensions, conformation and thermal stability were determined and discussed (Bravo-Abaya et al., 2016) The isoelectric point of DNA/chitosan complexes is directly related to the protonation degree of chitosan. The electrostatic interactions between DNA and chitosan are the main phenomena taking place in the solution up to the stoichiometric charge ratio N+/P-=1. This work was completed using DNA concentration in the dilute regime, i.e., around 10 times lower than the average value of the overlap concentration C* (0.23 mg/mL) (Bravo-Anaya et al., 2016) to establish the mechanism of chitosan /DNA interaction in relation with the composition or ratio N/P since this point has been rarely covered. The ionic interaction between the negative phosphate sites and the positive -NH_3^+ from chitosan is essential for complex formation. The influence of chitosan protonation in

DNA/complexes stability, as well as chitosan DA (Figure 19) was proposed through the analyses of several physical-chemical and biophysical techniques, i.e., UV-Vis and DLS measurements, and by gel electrophoresis assays.

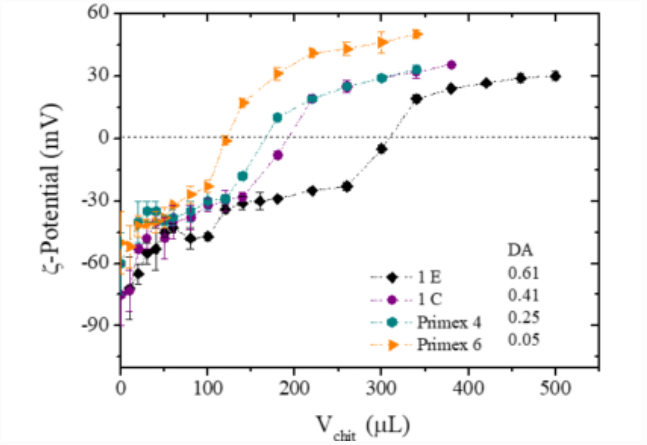

Figure 19. ζ-Potential as a function of volume of fully protonated chitosan with different DA added in 10 mL DNA solution for CDNA = 0.03 mg/mL (pH = 6.0) dissolved in water. (Bravo et al. 2019)

Chitosan samples were prepared in presence of slight excess of HCl and at a concentration of 1 mg/mL Two hypotheses may be suggested for complex formation: i) the complexation occurs randomly in solution, i.e chitosan associated on each DNA chains in the ionic ratio N+/P- ; ii) cooperative interactions with positively charged chitosan saturate a DNA chain, and the complementary DNA fraction remains free of chitosan as long as N+/P- ≤ 1. A remaining question concerns the case when N+/P- ≥1: when the complex becomes positively charged. Is there an excess of chitosan fixed on the complex to control this charge inversion? Gel electrophoresis was used to demonstrate that the fraction of fixed DNA equals the amount of positive chitosan charges added (Figure 20)

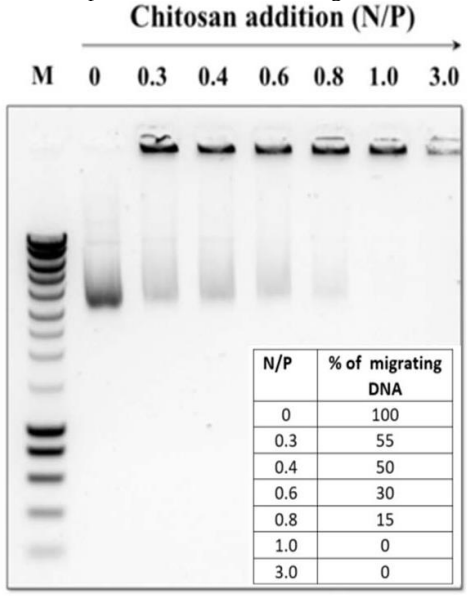

Figure 20. Gel electrophoresis showing the fraction of free DNA during complex formation by progressive addition of chitosan. (Bravo et al. 2019)

This means that complex forms by a cooperative electrostatic interaction. In the same work, using fluorescent chitosan it was shown that no free chitosan is left when N+/P- ≤1. In excess of chitosan, a small fraction is additionally fixed on the complex to turn the net charge to positive up to R=N/P =3. The excess of chitosan is essential in the buffering effect, as demonstrated by potentiometry (Figure 21)

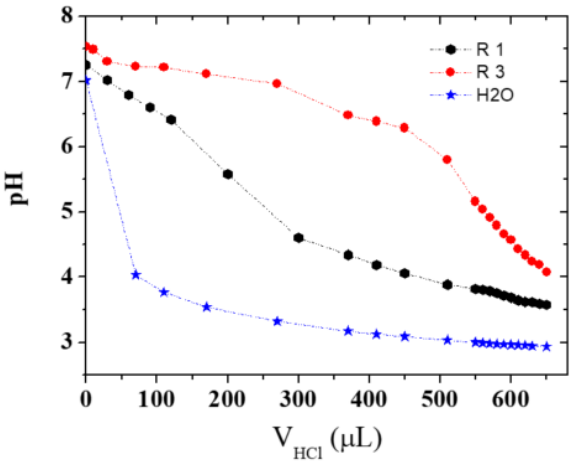

Figure 21. Demonstration of sponge effect related to excess of free chitosan. pH variation in excess of chitosan (R=3), at stoichiometric ratio (R=1) and in water for HCl addition. (Bravo et al. 2019)

The pH remains nearly constant when HCl is added to the system corresponding to R=3 i.e. in presence of free chitosan.

CHITIN AND CHITOSAN DERIVATIVES

11.1. Chitin Derivatives

Chitin displays properties such as biodegradability, and non-toxicicity, which form the basis of many applications in biomedicine, pharmacy, biotechnology, food industry, nanotechnology. (Arguelles-Monal et al., 2018)

Due to analogy between cellulose and chitin, reactions are often performed in similar experimental conditions. For highly acetylated chitin, reactions allowing to generate chitin derivatives involve the hydroxyl groups at C-3 and C-6 positions in accessible zones. Considering data from literature, it is necessary to precise that papers devoted to chitin derivatives are obtained in fact by chitosan modification (i.e chitin partially deacetylated). Then, due to their insolubility in many solvents (particularly chitin), chemical reactions are carried out under heterogeneous conditions. Using words from Kurita, "*reactions under heterogeneous conditions are usually accompanied by problems including poor extents of reaction, difficulty in regioselective substitution, structurally ununiformly products, and partial degradation due to severe reaction conditions*". (Kuirta, 2006)

11.1.1. Grafting on chitin. For grafting on chitin in most cases, radical polymerization has been used, although there is also a report of anionic ring-opening polymerization. (Arguelles-Monal et al. 2018) Acrylic monomers (especially acrylic acid) are among the most frequently grafted into chitin. Therefore, almost no-control over the macromolecular structure is attained, giving rise to a heterogeneous distribution of the grafting chains along the chitin backbone, and in some cases, only low degrees of grafting could be reached.

Chitin-g-polypyrole and chitin-g-polystyrene were obtained to increase the mechanical properties of initial chitin. Kurita *et al* investigate the influence of several conditions of the copolymerization reaction of acrylamide and acrylic acid onto chitin. (Kurita et al., 1991) They were able to obtain percentages of grafting above 200 % with enhanced solubility and hygroscopic behavior. (Kurita et al. 1991).

11.1.2. Chitin modification. The most important derivative of chitin is chitosan, which is obtained by (partial) deacetylation of chitin in the solid state under alkaline conditions (concentrated NaOH) or by enzymatic hydrolysis in the presence of a chitin deacetylase. Because of the semi-crystalline morphology of chitin, chitosans obtained by a solid-state reaction have a heterogeneous distribution of acetyl groups along the chains. β-chitin exhibits much higher reactivity for deacetylation than α-chitin. (Kurita et al., 1993) The examination of the influence of this acetyl distribution. (Aiba, 1991) show that the distribution, random or blockwise, is critical in controlling solution properties.

Reacetylation, up to 51%, of a highly deacetylated chitin in the presence of acetic anhydride gives a water-soluble derivative, whereas a heterogeneous product obtained by partial deacetylation of chitin is soluble only under acidic conditions, or even insoluble. NMR measurements have demonstrated that the distribution of acetyl groups must be random to achieve the higher water solubility around 50% acetylation. Homogeneously deacetylated samples were obtained recently by alkaline treatment of chitin under dissolved conditions. (Cho et al., 2000) On the other hand, the reacetylation of a highly deacetylated chitin was done (Maghami & Roberts, et al., 1988) and provided homogeneous samples used in SEC analysis discussed previously. (Brugnerotto et al. 2001) Toffey et al. transformed chitosan films cast from aqueous acetic acid into chitin by heat treatment. (Toffey et al. 1996; Toffey & Glaser, 1999)

After chitosan, the most studied derivative of chitin is carboxymethyl chitin (CM-chitin), a water-soluble anionic polymer. The carboxymethylation of chitin is performed similarly to that of cellulose; chitin is treated with monochloracetic acid in the presence of concentrated sodium hydroxide. The same method can be used for carboxymethylation of chitosan. (Muzzarelli, 1985; Hudson & Jenkins, 2003; Muzzarelli, 1985) The method for cellulose derivatization is also used to prepare hydroxypropyl chitin, a water-soluble derivative used for artificial lachrymal drops. (Wang et al., 1997; Park & Park, 2001) Other derivatives such as fluorinated chitin (Chow & Khor, 2001), N and O-sulfated chitin (Muzzarelli, 1985; Hudson & Jenkins, 2003; Muzzarelli, 1985; Tokura & Itoyama, 1994;Tokura et al., 1992), diethylaminoethyl chitin (Kurita et al., 1990), phosphoryl chitin (Andrew et al., 1998), mercapto chitin (Yoshino et al., 1992) and chitin carbamates (Vincendon, 1992) have been described in the literature. Modification of chitin is also often performed via water-soluble derivatives of chitin (mainly CM-chitin). The same type of chemical modifications (etherification and esterification) as for cellulose can be performed on the available C-6 and C-3 –OH groups of chitin. (Rinaudo & Reguant, 2002) Chitin can be used in blends with natural or synthetic polymers; it can be crosslinked by the same reactants as used for cellulose (epichlorohydrin, glutaraldehyde, etc.) or grafted in the presence of ceric salt (Ren et al., 1993) or after selective modification. (Kurita & Inoue, 1989) The use of new solvents such as ionic liquids offer routes to further chemical modifications.

11.1.3. Depolymerization. Chitin is partially degraded by acid to obtain a series of oligochitins. (Kurita et al., 1993) These oligomers, as well as those derived from chitosan, are recognized for their bioactivity: including anti-tumor, bactericidal and fungicidal activity, eliciting chitinase regulating plant growth. They are used in testing for lysozyme activity. They are also used as active starting blocks to be grafted on protein and lipids to obtain analogs of glycoproteins and glycolipids

11.2. Chitosan Derivatives

11.2.1. O- and N-Carboxymethylchitosans. Carboxymethylchitosan (CM-chitosan) is the most thoroughly explored derivative of chitosan; it is an amphoteric polymer, whose solubility depends on pH. Controlled reaction conditions (with sodium monochloracetate in the presence of NaOH), yields O- and N-carboxymethylation. NMR establishes the yield of substituents on the three positions. (Rinaudo et al., 1992 a; b; Le Dung et al., 1994) This reaction extends the range of pH (pH>7) in which chitosan is water-soluble, but a phase separation due to the balance between positive and negative charges on the polymer takes places in the range: 2.5<pH<6.5. Most impressive is the preparation of N-carboxymethyl chitosan by reaction with glyoxylic acid in the presence of a reducing agent.(Le Dung et al., 1994) ^{1}H and ^{13}C NMR.established the distribution of monosubstituted (–NH–CH_2COOH) and disubstituted (–N (–$CH_2COOH)_2$) groups. Disubstitution is easily obtained, giving a derivative for ion complexation. Specific oxidation of the C-6 position hydroxyl group was conducted using the TEMPO reactant on chitin to produce a chitin-based hyaluronic acid analog. (Muzzarelli et al., 1999) This derivative is water soluble in a wide range of pH, but only when prepared from fully acetylated chitin.

11.2.2. Chitosan 6-O Sulfate. This derivative is an anticoagulant; it was first prepared as an O- sulfated derivative (Terbojevich et al., 1989) and as N-sulfated chitosan. (Hoe & Perlin, 1997)

11.2.3. N-methylene phosphonic chitosans. These anionic derivatives display some amphoteric character; they were synthesized under various conditions and proved to have excellent complexing efficiency for cations such as Ca^{2+}, and those of transition metals (Cu (II), Cd (II), Zn (II), etc.). (Heras et al., 2000; Wojcik, 2003) The complexation provides protection against corrosion for metal surfaces. (Wojcik, 2003) These derivatives were also modified and grafted with alkyl chains to obtain amphiphilic properties that have potential applications in cosmetics. (Ramos et al., 2003)

11.2.4. Trimethyl chitosan ammonium. This cationic derivative is water-soluble over all the available pH range. It is obtained by quaternization of chitosan (Domard et al., 1987) with methyl iodide in sodium hydroxide under controlled conditions. NMR provided its full characterization. (Le Dung et al., 1994; Domard et al., 1987*)* Under all conditions tested, there appears a large decrease of molecular weight during this reaction. These polymers show good flocculating properties with kaolin dispersions, suggesting applications to papermaking. (Domard et al., 1989) The other quaternized derivatives display antistatic properties. (Suzuki et al., 2000)

11.2.5. Carbohydrate branched chitosans. Carbohydrates can be grafted on the chitosan backbone at the C-2 position by reductive alkylation. For that purpose, disaccharides (cellobiose, lactose, etc.) having a reducing end group, are introduced, in the presence of a reductant, on chitosan in the open-chain form. (Hall & Yalpani, 1980) These derivatives are water soluble. A previous record mentioned galactosylated chitosan. (Chung et al., 2002) Carbohydrates can also be introduced without ring opening on the C-6 position. (Holme & Hall, 1992) These derivatives are useful as they interact with the corresponding specific lectins and thus could be used for drug targeting. (Morimoto et al., 2002) A particular case is the grafting of a cyclic oligosaccharide, cyclodextrin.

11.2.6. Chitosan-grafted copolymers. One of the most explored derivatives is poly(ethylene glycol)- grafted chitosan, which has the advantage of being water soluble, depending on the degree of grafting. Higher molecular weight PEG at low DS give higher solubility than low molecular weight PEG. (Morimoto et al., 2002) PEG can be also be introduced by reductive amination of chitosan using PEG-aldehyde. (Harris et al., 1984) Polypeptides have been grafted by reaction with N-carboxy anhydrides of amino acids to develop new biomaterials. (Alba et al., 1986), but the degree of polymerization of the grafted chains cited in this work remains low (DP ~5.9–6.6). Chitosan-g-N-isopropylacrylamide (NIPAm) water-soluble copolymers were synthesized. (Recillas et al., 2009). These polymers consist of LCST materials based on the properties of the poly NIPAm blocks. They display a fully reversible thermoresponsive behavior in aqueous solutions. The copolymers prepared were characterized by a LCST between 19 and 20 °C, when dissolved in 10% aqueous acetic acid.

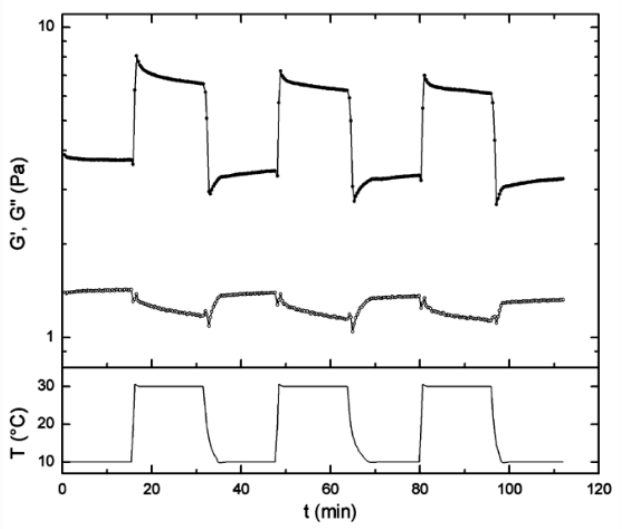

Figure 22. The thermoresponsive behaviour of Chitosan-g-N-isopropylacrylamide (NIPAm) water-soluble copolymers. Variations in mechanical moduli, G' (●) and G'' (○), for 1% (w/w) solution in 10% aqueous acetic acid (sample C3, grafting %23.9)) to stepwise periodic changes in temperature between 10 and 30 °C (ω) 1 rad s^{-1}; γ = 5%). (Recillas et al., 2009)

11.2.7. Alkylated chitosans. Alkylated chitosans are very important as amphiphilic polymers based on polysaccharides. The first derivative having these characteristics was a C-10-alkyl glycoside branched chitosan with a high degree of substitution (DS) around 1.5, which gelled when heated over 50°C. (Holme & Hall, 1991) Another approach was used for selective N- and O-palmitoylation giving a derivative with two or three long alkyl chains per monomeric unit. This reaction involved the protection and deprotection of the C-6 position. (Kohgo et al., 1992) Highly substituted derivatives with low regularity were prepared using carboxylic anhydrides with different chain lengths on CM-chitosan. They were insoluble in water and their biodegradability decreased. (Hirano et al., 1992) Using the reductive amination, a series of amphiphilic derivatives were produced with different chain lengths (carbon chain from 3 to 14) and controlled DS (usually lower than 10% to maintain water solubility in acidic conditions). (Desbrieres et al., 1996; Rinaudo et al., 2005)

11.2.8. Cyclodextrin-linked chitosans. The cyclic oligosaccharides, namely α–, β–, γ-cyclodextrins (CD), are important because of their ability to encapsulate hydrophobic molecules in their toroidal hydrophobic cavity, whose selectivity depends on the number of glucose units (respectively 6, 7, 8 D-glucose units). (Eli et al., 1999; Rekharsky & Inoue, 1998; Kriz et al., 2003) For various applications, it is interesting to graft the cyclodextrin on a polymeric backbone such as a biocompatible polysaccharide. A synthesis of α- and β-cyclodextrin-chitosans with relatively high degree of substitution has been described. (Sakairi et al., 1999) The authors found that these new derivatives had the ability to differentially recognize and retain certain guest compounds based on their molecular shapes and structures. They proposed to use these polymers as supports for reverse-phase adsorption or as adsorbents in controlled release systems. A β-cyclodextrin with a specific modification on one of the –OH groups on its small side was grafted to chitosan by reductive amination. At a DS lower than 10%, these derivatives are water soluble in acidic conditions with loose inter-chain interactions. (Auzely & Rinaudo, 2003; Auzely & Rinaudo, 2001)The grafted cyclodextrin has the same association constant as the free CD with small hydrophobic molecules such as adamantane. (Auzely & Rinaudo, 2001) This modified chitosan should be adaptable for drug delivery. When these CD-chitosans were mixed with chitosan grafted with adamantane (AD), the specific recognition led to a self-assembled gel. (Auzely & Rinaudo, 2002) This physical gel is stabilized by specific CD/AD linkages in a dynamic mechanism with a relaxation time depending on polymer concentration, temperature, and the presence of excess free CD or AD. (Charlot et al., 2003)

SOME APPLICATIONS OF CHITIN AND CHITOSAN

Firstly, it is necessary to underline that, the polymer used is named chitin, in literature, when it is the deacetylated form considered i.e. chitosan. It is therefore difficult to separate from those of chitosan.

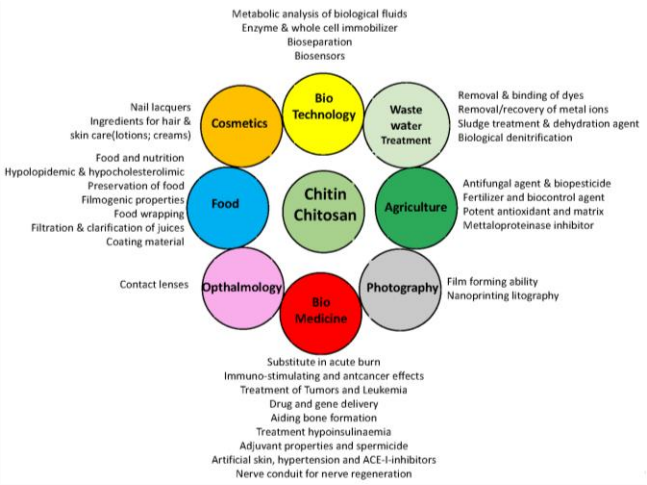

Figure 23. summarizes the main properties of chitin and chitosan and potential biomedical and other applications that they imply.

The high interest in medical applications of chitosan and some of its derivatives are readily recognized. The cationic character of chitosan is unique: it is the only pseudo-natural cationic polymer. Its film-forming properties and biological activity invite new applications.

12.1. Chitin-based Materials

Chitin has low toxicity and is inert in the gastrointestinal tract of mammals. It is biodegradable, owing to the presence of chitinases widely distributed in nature and present in bacteria, fungi, and plants as well as in the digestive systems of many animals. Chitinases are involved in host defense against bacterial invasion. Lysozymes from egg white, fig and papaya plants, degrade chitin and bacterial cell walls. A certain degree of deacetylation is required to allow the hydrolysis of chitin. (Sashiva et al., 1990; Varum et al., 1997)

Chitin has been used to prepare an affinity chromatography column to isolate lectins and determine their structure.(Datta et al., 1984). Chitin and 6-O-carboxymethyl chitin activates peritoneal macrophages *in vivo*. They suppress the growth of tumor cells in mice and stimulate nonspecific host resistance against *Escherichia coli* infection. Chitin also accelerates wound healing. (Hudson et al., 2003)

Chitin is widely used to immobilize enzymes and whole cells. Enzyme immobilization has applications in the food industry, such as clarification of fruit juices and processing of milk when α- and β-amylases or invertases are grafted onto chitin. Because of its biodegradability, nontoxicity, physiological inertness, antibacterial properties, hydrophilicity, gel-forming properties and affinity for proteins, chitin has found applications in many areas other than food such as in biosensors. One of these sensors was described for polar vapour detection. (Bouvree et al., 2009)

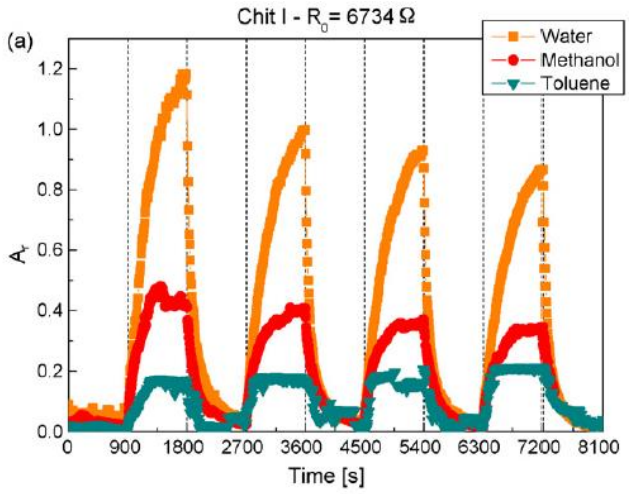

Figure 24. Responses to water, methanol and toluene of (a) Chit-CNP, initial resistance R_0 =6734 (Mw~500000; DA=0.2) enlever les a (Bouvree et al., 2009)

Chitin-based materials are also used for the treatment of industrial pollutants and adsorb silver thiosulfate complexes (Songkroah et al., 2004) and actinides. (Songkroah et al., 2004) Chitin can be processed in the form of films and fibers. (Austin et al., 1977; Hirano, 2001) The chitin fibers, obtained by wet spinning of chitin dissolved in a 14% NaOH solution, can also result of blending with cellulose (Hirano, 2001; Hirano & Midorikawa, 1998) or silk. (Hirano et al. 1990) They are non-allergic, deodorizing, antibacterial and moisture controlling. (Yoshino et al., 1992) Regenerated chitin derivative fibers are used as binders in the papermaking process; addition of 10% n-isobutyl chitin fiber improves the breaking strength of paper. (Tokura et al., 1982; Kobayashi et al., 1982) However, the main development of chitin film and fiber is in medical and pharmaceutical applications as wound-dressing material. (Yusof et al; 2003; Hudson 1998; 1999; Rathke et al., 1994) and controlled drug release. (Kanke et al., 1989; Kato et al., 2003)

Chitin is also used as an excipient and drug carrier in film, gel or powder form for applications involving mucoadhesive property. Another exciting application is in hydroxyapatite–chitin–chitosan composite bone-filling material. It forms a self-hardening paste for guided tissue regeneration in the treatment of periodontal bony defects.(Ito et al., 1998) Chitin was O-acetylated to prepare gels which are still hydrolyzed by an enzyme such as hen egg white lysozyme. (Hirano et al., 1989; Zhang et al., 1994) CM-chitin was selectively modified to obtain antitumor drug conjugates. (Ouchi et al., 1992) For example, 5-fluorouracil, which has marked antitumor activity and the D-glucose analog of muramyl-L-alanyl-isoglutamine, responsible for immuno-adjuvant activity were grafted on CM-chitin using a specific spacer and an ester bond.

Chitin oligomers with DP = 5 is active in controlling the photosynthesis of maize and soybeans. (Khan et al., 2002) Considering the original properties of chitin especially for biomedical applications, the processes from solutions under different morphologies is interesting to consider (i.e., beads, fibers, films...).

The development of a new solvent allows chitin chains to rapidly self-assemble into nanofibers in NaOH/urea aqueous solution by a thermally induced method. (Zhang et al., 2015) Then, the chitin solution is emulsified into liquid microspheres in isooctane with the surfactants Tween-85 and Span 85 under vigorous stirring at 0 °C. Then, a rapid increase of temperature up to 60°C induces the formation of chitin nanofibers, forming structured microspheres within 2 minutes (with average diameters from 15 to 65 μm). Cells can adhere to the chitin microspheres and exhibit a high attachment efficiency. Then, the novel elastic nitrogen-doped carbon microspheres were obtained by pyrolyzing the chitin microspheres. (Duan et al., 2018) Those microspheres are excellent support of ultra-small Pd clusters and used as a catalyst. The same solvent is proposed to obtain films, fibers, hydrogels, and aerogels and those materials are well described. (Duan et al., 2018)

Ionic liquids were also used to spun chitin fibers with excellent mechanical characteristics: from commercial chitin, Young modulus equals 4.7 GPa and from shrimp shell 8.6 GPa with a failure strain (%) of 5 and 3.3 respectively. (Quin et al., 2010) Films were also prepared. (King et al., 2017) Chitosan-polylactic acid blend with different weight ratios were spun to produce composite fibers. From the Ionic liquid solution, the mechanical properties of chitin fibers are Young modulus 4.2 ± 0.2 GPa, and strain % equals 3.0 ± 0.2. The presence of PLA increases the performance slightly. (Shamshine, 2018)

12.2. Chitosan-based Materials

Chitosan is used to prepare hydrogels, films, fibers or sponges and a large number of applications relate to the biomedical field to which the biocompatibility offered by chitosan is essential. There is a rich literature devoted to the uses of chitosan; the following examples provide an overview of some promising applications. Chitosan is much easier to process than chitin, but the stability of chitosan materials is generally lower, owing to their more hydrophilic character and, especially, pH sensitivity. To control both their mechanical and chemical properties, various cross-linking techniques, often adapted from the cellulose world, are used, as mentioned previously for chitin.

Pure chitosan nanofibers were produced by electrospinning. A porous mat is obtained with fibers having 80-220 nm as diameter. (Garcia et al., 2018; Mengistu Lemma et al. 2018) Due to the low spinnability of chitosan alone, a water soluble polymer PEO (MW≥ $1x10^6$) was blended with chitosan solution in 0.5M acetic acid.

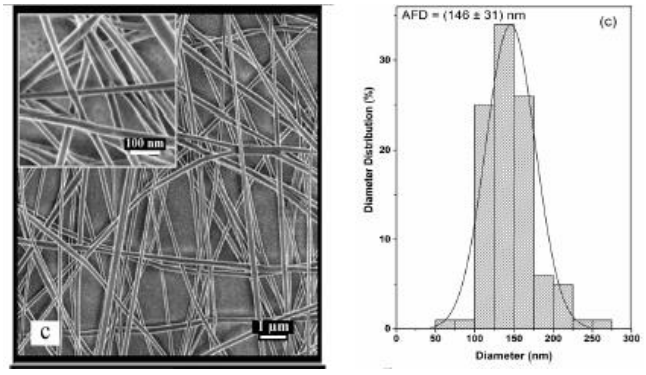

Figure 25. Nanofibers obtained using a blend chitosan/PEO 70/30 in acetic acid. Average diameter equals 146 nm (Mengistu Lemma et al., 2016)

Such mat, after extraction of PEO, becomes a stable biomaterial over pH=7 with good mechanical properties and hydrophily (around 4g/g of dried material). It is well adapted for soft tissue engineering and wound healing. The advantage of electrospinning technique is to obtain nanoscale fibers with high surface area to volume ratio.

Chitosan may be crosslinked by reagents such epichlorohydrin, diisocyanate (Weish & Price, 2003) or 1, 4-butanediol diglycidyl ether. (Roy et al., 1998) Blend of starch and chitosan can undergo specific crosslinking: starch was oxidized to produce a poly-aldehyde that reacts with the $–NH_2$ group of chitosan in the presence of a reducing agent. (Baran et al., 2004) Many chitosan hydrogels are obtained by treatment with multivalent anions : the case of glycerolphosphate is mentioned above (Chenite et al., 2000), but oxalic acid has also been used (Zhane et al., 1994; Hirano et al., 1990; Yamagushi et al., 1978) as well as tripolyphosphate. (Desai & Park, 2005; Lee et al., 1998) Blends and composites have been prepared in the way mentioned previously for chitin. (Hirano, 2001) Other systems are proposed in the literature: chitosan/polyamide 6. (Ko et al., 1997) chitosan/cellulose fibers (Hosohawa et al., 1990), chitosan/cellulose using a common solvent (Hasegawa et al., 1994), chitosan/polyethylene glycol (Mucha et al., 1999) chitosan/polyvinylpyrrolidone and chitosan/polyvinyl alcohol. (Abou-Alad, 2005) Carbon nanotubes may reinforce chitosan film; this composite exhibits a large increase of the tensile modulus with the incorporation of only 0.8% of multiwalled carbon nanotubes. (Wang et al., 2005) The advantage of chitosan in such materials is not only its biodegradability and its antibacterial activity, but also the hydrophilicity introduced by the addition of the polar groups able to form secondary interactions (–OH and $–NH_2$ groups involved in H bonds with other polymers).

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