Functional Characterization of Chitin and Chitosan

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Abstract: Chitin and its deacetylated derivative chitosan are natural polymers composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitin is insoluble in aqueous media while chitosan is soluble in acidic conditions due to the free protonable amino groups present in the D-glucosamine units. Due to their natural origin, both chitin and chitosan can not be defined as a unique chemical structure but as a family of polymers which present a high variability in their chemical and physical properties. This variability is related not only to the origin of the samples but also to their method of preparation. Chitin and chitosan are used in fields as different as food, biomedicine and agriculture, among others. The success of chitin and chitosan in each of these specific applications is directly related to deep research into their physicochemical properties. In recent years, several reviews covering different aspects of the applications of chitin and chitosan have been published. However, these reviews have not taken into account the key role of the physicochemical properties of chitin and chitosan in their possible applications. The aim of this review is to highlight the relationship between the physicochemical properties of the polymers and their behaviour. A functional characterization of chitin and chitosan regarding some biological properties and some specific applications (drug delivery, tissue engineering, functional food, food preservative, biocatalyst immobilization, wastewater treatment, molecular imprinting and metal nanocomposites) is presented. The molecular mechanism of the biological properties such as biocompatibility, mucoadhesion, permeation enhancing effect, anticholesterolemic, and antimicrobial has been updated.

Keywords: Chitin, chitosan, molecular weight, deacetylation degree, crystallinity, functional characterization.

1. INTRODUCTION

Among the novel families of biological macromolecules, whose relevance is becoming increasingly evident, are chitin and its main derivative, chitosan. Potential and usual applications of chitin, chitosan and their derivatives are estimated to be more than 200 [1]. This wide range of applications includes biomedicine, food, biotechnology, agriculture and cosmetics, among others. The importance of chitin and chitosan in the last years is evident in Table **1**.

Chitin and chitosan are described as a family of linear polysaccharides consisting of varying amounts of β (1→4) linked residues of N-acetyl-2 amino-2-deoxy-D-glucose (denoted in this review as **A** residues) and 2-amino-2-deoxy-D-glucose residues (denoted in this review as **D** residues). Chitin samples have a low amount of **D** units and hence the polymer is insoluble in acidic aqueous media (Fig. **1a**). On the other hand, the amount of **D** units in chitosan samples is high enough to allow the polymer to dissolve in acidic aqueous media. Some authors consider that chitosan is the polymer with at least 60% of **D** residues [2]. Chitin is the second most abundant natural polymer in nature after cellulose and it is found in the structure of a wide number of invertebrates (crustaceans' exoskeleton, insects' cuticles) and the cell walls of fungi, among others. On the other hand, chitosan only occurs naturally in some fungi (*Mucoraceae*) [3].

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Chitosan can also be prepared by cleavage of N-acetyl groups of the chitin **A** residues. This reaction is rarely conducted to full completion; hence chitosan polymeric chain is generally described as a copolymeric structure comprised of D-glucosamine (**D** residues) along with N-acetyl residues (Fig. **1b**).

The fine structure of chitosan is defined by the overall or bulk content of D-hexosamine residues as well as their distribution along the polymeric chain. The molar fraction of residual **A** groups in chitosan is expressed as a degree of N-acetylation (DA) or fraction of acetylation (F_a). The molar fraction of **D** residues, deacetylation degree (DD), is also very frequently used.

In contrast to chitin, the presence of free amine groups along the chitosan chain allows this macromolecule to dissolve in diluted aqueous acidic solvents due to the protonation of these groups, rendering the corresponding chitosan salt in solution. So, there are important experimental variables that should be taken into account when working with chitosan solutions such as the nature of the salt counterion, degree of acetylation, Mw, pH, ionic strength and the addition of a non-aqueous solvent.

The aim of the present review is to present a state-of-the-art study of the relationship between the physico-chemical properties of these two polymers and their biological activities, as well as their applications. Since this aim is very ambitious, due to the extension of the topic, chitin and chitosan derivatives are not considered.

The review has been divided into the following sections: the first part is devoted to the preparation, characterization,

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Table 1. Number of Scientific Publications Related to Chitin and Chitosan. Source: Scopus. Publication Year After 2000

Search	Reviews	Articles	Patents
Chitin and not chitosan	182	2741	9064
Chitosan and not chitin	401	5959	20041
Chitin and chitosan	119	2040	11804

effects of the preparation process on the properties of chitin and chitosan and regulatory aspects. The second part covers the main biological properties of the polymers and relates these properties to the physicochemical characteristics. Finally, several applications of both polymers are reviewed emphasizing the effect of the polymers' characteristics on these applications.

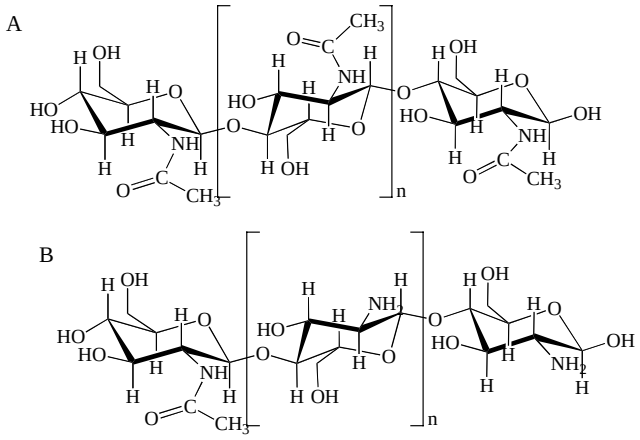


Fig. (1). Chemical structure of 100% acetylated chitin (**A**) and chitosan (**B**).

2. METHODS OF PREPARATION

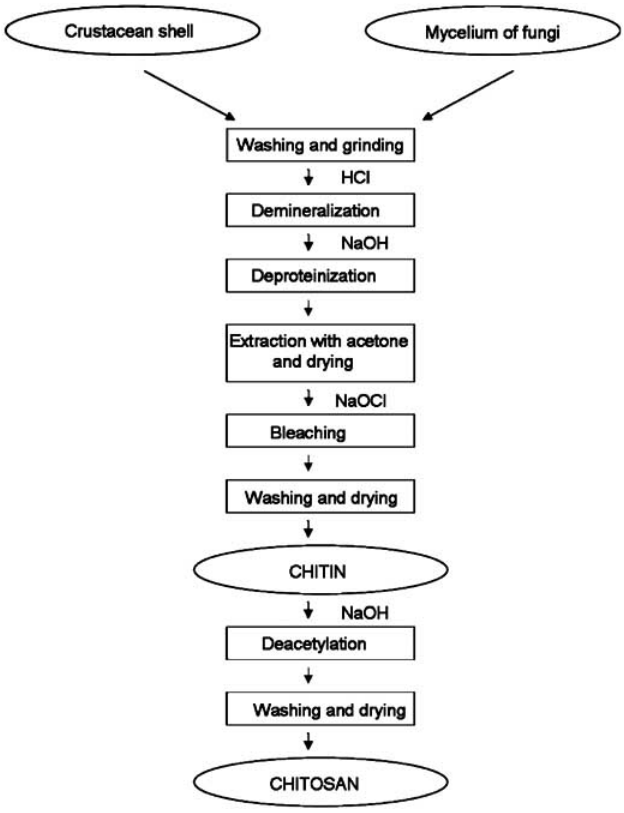
A schematic representation of the processes to prepare chitin and chitosan from raw material is shown in Scheme **1**.

2.1. Chitin Extraction

As mentioned above, chitin is present within numerous taxonomic groups. However, commercial chitins are usually isolated from marine crustaceans, mainly because a large amount of waste is available as a by-product of food processing. In this case, α-chitin is produced while squid pens are used to produce β-chitin.

The structure of α-chitin has been investigated more extensively than that of either the β- or γ- form, because it is the most common polymorphic form. Very few studies have been carried out on γ- chitin. It has been suggested that γ-chitin may be a distorted version of either α- or β-chitin rather than a true third polymorphic form [3].

In α –chitin, the chains are arranged in sheets or stacks, the chains in any one sheet having the same direction or 'sense'. In β-chitin, adjacent sheets along the *c* axis have the same direction; the sheets are parallel, while in α-chitin adjacent sheets along the *c* axis have the opposite direction, they are antiparallel .In γ- chitin, every third sheet has the opposite direction to the two preceding sheets [3]. A schematic representation of the three structures is shown in Fig. (**2**).



Scheme 1. Preparation of chitin and chitosan from raw material.

Crustacean shells consist of 30-40% proteins, 30-50% calcium carbonate, and 20-30% chitin and also contain pigments of a lipidic nature such as carotenoids (astaxanthin, astathin, canthaxanthin, lutein and β-carotene). These proportions vary with species and with season. On the other hand, β-chitin is associated with a higher protein content but lower carbonate concentration. Chitin is extracted by acid treatment to dissolve the calcium carbonate followed by alkaline extraction to dissolve the proteins and by a depigmentation step to obtain a colourless product mainly by removing the astaxantine [4].

2.2. Chitin Deacetylation

Chitosan is prepared by hydrolysis of acetamide groups of chitin. This is normally conducted by severe alkaline hydrolysis treatment due to the resistance of such groups imposed by the *trans* arrangement of the C2-C3 substituents in the sugar ring [5]. Thermal treatments of chitin under strong aqueous alkali are usually needed to give partially deacetylated chitin (DA lower than 30%), regarded as chitosan. Usually, sodium or potassium hydroxides are used at a concentration of 30-50% w/v at high temperature (100ºC).

In general, two major different methods of preparing chitosan from chitin with varying degree of acetylation are

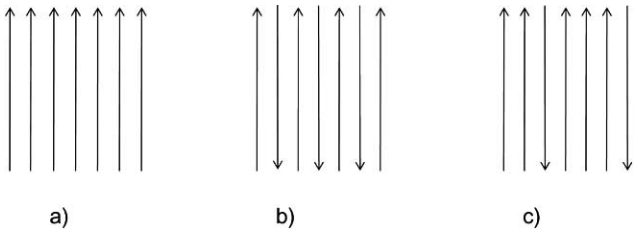


Fig. (2). Three polymorphic configurations of Chitin (**A**) α-chitin, (**B**) ß-chitin and (**C**) γ-Chitin.

known. These are the heterogeneous deacetylation of solid chitin and the homogeneous deacetylation of pre-swollen chitin under vacuum (by reducing pressure) in an aqueous medium. Heterogeneous deacetylation, which is the preferred industrial treatment, involves preferential reaction in the amorphous regions of the polymer, leaving almost intact the intractable crystalline native regions in the parent chitin. Alternatively, homogeneous modification is conducted by use of moderately concentrated alkali (13% w/w) acting on pre-swollen chitin to improve the interaction with the alkali and left to react at 25-40ºC for 12-24 hours.

In both, heterogeneous or homogeneous conditions, the deacetylation reaction involves the use of concentrated alkali solutions and long processing times which can vary depending on the heterogeneous or homogeneous conditions from 1 to nearly 80 hours. Factors that affect the extent of deacetylation include concentration of the alkali, previous treatment, particle size and density of chitin. The last two factors affect penetration rate of the alkali into the amorphous region and to some extent also into the crystalline regions of the polymer, needed for the hydrolysis to take place. In practice, the maximal DD that can be achieved in a single alkaline treatment is about 75-85% [3]. In general, during deacetylation, conditions must be the proper ones to deacetylate, in a reasonable time, the chitin to yield a chitosan soluble in diluted acetic acid.

Thiophenol and $NaBH_4$ have been used as oxygen scavenger and reducing agents, respectively, thus effectively resulting in a product of greater viscosity [6]. Also, treatments with concentrated NaOH in the presence of water-miscible diluents such as 2-propanol, 2-methyl-2-propanol, polyethylene glycol dimethyl ether, acetone or paraffin oil have enabled the volume of concentrated NaOH required to be reduced by at least 85%. Several alternative processing methods have also been developed to reduce the long processing times and large amounts of alkali typically needed to deacetylate chitin to an acid-soluble derivative. Examples of these include the use of successive alkali treatments using thiophenol in DMSO [7]; thermo-mechanical processes using a cascade reactor operated under low alkali concentration [8]; flash treatment under saturated steam [9]; use of microwave dielectric heating [10]; and intermittent water washing [11].

There is evidence that in certain bacteria and fungi, enzymatic deacetylation can take place [12]. Deacetylases have been isolated from various types of fungi, namely *Mucor rouxii*, *Aspergillus nidulans* and *Colletotrichum lindemuthianium*. However, the activity of these deacetylases is severely limited by the insolubility of the chitin substrate. There have been some attempts to use amorphous chitin of high DA as a substrate for the deacetylase enzyme, however no acid-soluble chitosan could be isolated and characterized [13]. The lack of solubility of chitinous substrates with high DA in aqueous solvents still represents a practical limitation for the preparation of chitosan using the chitin deacetylase system, a process which so far has been achieved *in vivo* [14].

2.3. Chitosan Depolymerization

The main limitations in the use of chitosan in several applications are its high viscosity and low solubility at neutral pH. Low molecular weight (Mw) chitosans and oligomers can be prepared by hydrolysis of the polymer chains. For some specific applications, these smaller molecules have been found to be much more useful. Chitosan depolymerization can be carried out chemically, enzymatically or physically. Chemical depolymerization (Fig. **3**) is mainly carried out by acid hydrolysis using HCl or by oxidative reaction using HNO_2 and H_2O_2 [15]. It has been found to be specific in the sense that HNO_2 attacks the amino group of D-units, with subsequent cleavage of the adjacent glycosidic linkage. In the case of enzymatic depolymerization, low molecular weight chitosan with high water solubility were produced by several enzymes such as chitinase, chitosanase, gluconase and some proteases. Non-specific enzymes including lysozyme, cellulase, lipase, amylase and pectinase that are capable of depolymerizing chitosan are known [16]. In this way, regioselective depolymerization under mild conditions is allowed. Physical depolymerization yielding dimers, trimers and tetramers has been carried out by radiation (Co-60 gamma rays) but low yields have been achieved [17].

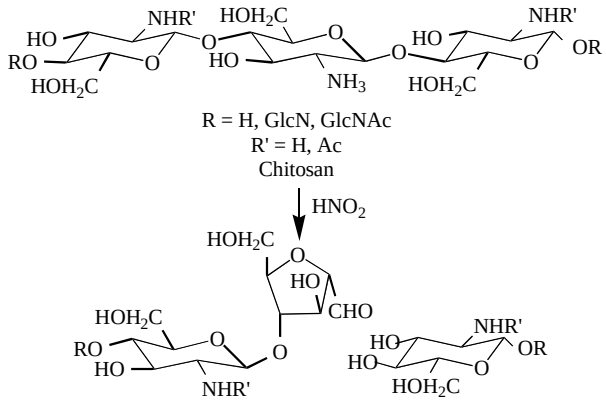


Fig. (3). Chemical depolymerization of chitosan.

2.4. Influence of the Preparation Methods on the Physicochemical Characteristics

The preparation method is a factor that affects the sample characteristics. Early studies have clearly demonstrated that specific characteristics of these products (Mw, DD) depend on the process conditions.

Typically commercial chitins are prepared by a first step of deproteinisation followed by a second step of demineralization. In these conditions a "collapsed chitin", in which the native structure of the chitin is lost, is extracted. On the other hand, "compacted chitin", in which the native chain and fibrous structures are intact and stabilized, is extracted when demineralization occurred in the first step. Another way to damage chitin structure was found to be even brief exposure to bleaching agents [18].

The DA value of the bulk molecules depends directly on the process conditions. Early studies by Kurita and coworkers showed that chitosan produced under homogeneous conditions presented broad X-ray diffraction patterns, which was interpreted as a consequence of a more randomly distributed fine structure in terms of A and D groups [19,20]. It has become evident that the overriding factor regarding the fine structure of chitosan is the chemical polydispersion of the DA value [21].

During chitosan deacetylation, the degradation of the polymeric chain takes place. At the same time, the crystallinity of chitosan can be damaged by using harsh reaction conditions [22]. Taking these two facts into account, the reaction conditions must be controlled when preparing chitosan [23]. Our findings have shown that the proper conditions to deacetylate chitin avoiding high degradation involve using heterogeneous conditions with NaOH 75% (w/v) and a temperature of 110ºC [24]. The type of crustacean and the chitin isolation process are also factors that affect chitosan quality [25].

3. METHODS OF CHARACTERIZATION

As will be shown in this review, chitin and chitosan characteristics have a great effect on their properties and hence on their possible applications. In fact, not every chitin or chitosan sample can be used for the same applications. That is why a complete characterization of the samples is mandatory.

Three crystalline forms are known for chitin: α-, β- and γ-chitins. Chitosan is also crystalline and shows polymorphism depending on its physical state. Depending on the origin of the polymer and its treatment during extraction from raw resources, the residual crystallinity may vary considerably. Crystallinity is maximal for both chitin (i.e. 0% deacetylated) and fully deacetylated chitosan (i.e. 100%).

Rinaudo has reported in a recent review that the origin of chitin influences not only its crystallinity and purity but also its polymer chain arrangement, and hence its properties [26]. It has also been reported that the surface area of the material is related to the source (i.e., crab>lobster >shrimp).

The main parameters affecting the polymer properties are DD, Mw, polydispersity and crystallinity. For applications related to human consumption such as food and medical applications, the purity (ash content), the moisture and the content of heavy metals, endotoxin and proteins must be determined.

It has been reported that the DD is one of the most important chemical characteristics, [27] which could influence the performance of chitosan in many of its applications [28]. The influence of average Mw on the viscosity development of aqueous solutions plays a significant role in the biochemical and biopharmacological significance of chitosan [29]. It is important to note that due to its low solubility chitin Mw is not easily determined.

As is shown in Table **2**, various methods have been reported for the determination of chitin and chitosan characteristics [30-45]. Different results are obtained when using methods based on different principles. Therefore, it is important to indicate the characterization method. Today, even the best characterized chitosans available in the market are usually described only with regard to their average degree of acetylation and their average degree of polymerization (DP), their ash content and the absence of contaminating bacteria, in some cases also indicating the polydispersity index. In addition to the above criteria, the distribution of the acetyl groups along the linear backbone of the chitosan molecules may be of crucial importance in defining the interactions with the biological systems [46]. For further information about preparation of chitin and chitosan, characterization and

Table 2. Physicochemical Characteristics of Chitin and Chitosan and the Determination Methods

Physicochemical Characteristics	Determination Methods
DD	Infrared spectroscopy [30,31,35] First derivative UV-spectrophotometry [32, 33] Nuclear magnetic resonance spectroscopy (^{1}HNMR) and (^{13} CNMR) [34-37] Conductometric titration [37] Potentiometric titration [38] Differential scanning calorimetry [39]
Average Mw and/or Mw distribution	Viscosimetry [40] Gel Permeation chromatography [41] Light scattering [42]
Crystallinity	X-ray Diffraction [3, 43]
Moisture content	Gravimetric analysis [44]
Ash content	Gravimetric analysis [44]
Protein	Bradford method [45]

DD: deacetylation degree.

chemistry Rinaudo and Kurita's reviews are recommended [26,47].

4. REGULATORY ASPECTS

Chitosan has been approved as functional food in some Asian countries (Japan, Korea) during the last decade. The inclusion of chitin and chitosan was considered in 2003 by the Codex Alimentarius Commission but it is not currently listed in the General Standard for Food Additives nor has it been authorized as a food ingredient in the EU. Although several studies have shown that this compound is not toxic, no long-term studies of human safety have been reported.

In the field of medical applications, chitosan has not been approved yet by the FDA. However, The American Society of Testing Materials (ASTM F04 division IV) is making a concerned effort to establish standard guidelines for tissue-engineered medical products (TEMPs). The F2103 guide covers the evaluation of chitosan salts suitable for use in medical applications considering aspects such as control of protein content and, hence, potential for hypersensitivity, endotoxin content, and total bioburden [44]. The F2260-03 guide covers the determination of DD while the WK965 guide covers the determination of Mw of chitosan and chitosan salts [48,49].

A derivative of chitosan (*chitosan hydrochloride*) has been included in the European Pharmacopoeia in 2002 [50]. This monograph includes tests for heavy metal as contaminats but bioburden, sterility and bacterial endotoxins are not addressed. Taking into account that purity, which is quantified as the remaining ashes, proteins, insolubles and also the bio-burden (microbes, yeasts and moulds, endotoxins,...), is vital particularly for high value products, a more detailed characterization is needed. Further information regarding this topic is found in reference [51].

5. BIOLOGICAL PROPERTIES OF CHITIN AND CHITOSAN

Chitin and chitosan are currently receiving a great deal of interest as regards medical and pharmaceutical applications because they have interesting properties that make them suitable for use in the biomedical field, such as biocompatibility, biodegradability and non toxicity. Moreover, other properties such as analgesic, antitumor, hemostatic, hypocholesterolemic, antimicrobian, and antioxidant properties have also been reported [1,52,53].

A deeper understanding of the mechanism of these properties makes it necessary for chitosan to be well characterized and purified from accompanying compounds [54]. In addition, chitins and chitosans derivatized in a variety of fashions can be used to prove molecular hypothesis for the biological activity. Since the majority of the biological properties are related to the cationic behaviour of chitosan, the parameter with a higher effect is the DD. However, in some cases, the Mw has a predominant role.

In addition to the DD and Mw, other properties such as chain conformation, solubility or degree of substitution have been considered. Chitosans produced by heterogenous deacetylation, with a block arrangement of acetylated and deacetylated units, have a tendency to form aggregates in aqueous solutions [55]. Extensive aggregation and intermolecular interactions may reduce available sites on the chitosan molecule. This may account for some of the differences between reported effects of chitosan, especially if the authors did not pay close attention to the preparation of chitosan dispersions or if the preparation procedure in these studies was different [56]. The relationship between some chitin and chitosan biological properties and their physicochemical characteristics are shown in Table **3**.

Table 3. Relationship Between Chitin and Chitosan Biological Properties and their Characteristics

Property	Characteristic
Biodegradability	DD, distribution of acetyl groups, Mw
Biocompatibility	DD
Mucoadhesion	DD, Mw (only chitosan)
Hemostatic	DD, Mw
Analgesic	DD
Adsorption enhancer	DD (only Chitosan)
Antimicrobian	Mw
Anticholesterolemic	DD, Mw, viscosity
Antioxidant	DD, Mw

DD: deacetylation degree.
Mw: molecular weight.

5.1. Biodegradability

Chitin and chitosan are absent from mammals but they can be degraded *in vivo* by several proteases (lysozyme, papain, pepsin…). Their biodegradation leads to the release of non-toxic oligosaccharides of variable length which can be subsequently incorporated to glycosaminoglycans and glycoproteins, to metabolic pathways or be excreted [57].

Lysozyme, a non-specific protease present in all mammalian tissues, seems to play a degradation role on chitin and chitosan. The degradation kinetics seem to be inversely related to the degree of crystallinity which is controlled mainly by the degree of deacetylation. Moreover, the distribution of acetyl groups also affects biodegradability since the absence of acetyl groups or their homogeneous distribution (random rather than block) results in very low rates of enzymatic degradation [2,58].

Finally, several studies reported that the length of the chains (Mw) also affects the degradation rate [59-61]. The understanding and control of the degradation rate of chitin and chitosan-based devices is of great interest since degradation is essential in many small and large molecule release applications and in functional tissue regeneration applications. Ideally, the rate of scaffold degradation should mirror the rate of new tissue formation or be adequate for the controlled release of bioactive molecules. Thus, it is important to understand and control both the mechanism and the rate by which each material is degraded.

The degradation rate also affects the biocompatibility since very fast rates of degradation will produce an accumulation of the amino sugars and produce an inflammatory response. Chitosan samples with low DD induce an acute inflammatory response while chitosan samples with high DD

induce a minimal response due to the low degradation rate. Degradation has been shown to increase as DD decreases [62-64]. Kofuji *et al.* investigated the enzymatic behaviours of various chitosans by observing changes in the viscosity of chitosan solution in the presence of lysozyme [65]. They found that chitosan with a low DD tended to be degraded more rapidly. However, other authors reported that differences in degradation are due to variations in the distribution of acetamide groups in the chitosan molecule [2,66]. This occurs due to differences in deacetylation conditions which influences viscosity of the chitosan solution by changing the inter- or intra-molecular repulsion forces [64]. Therefore, It can be concluded that it is impossible to estimate biodegradation rate from the DD alone.

5.2. Biocompatibility

Both chitin and chitosan show very good compatibility but this property depends on the characteristics of the sample (natural source, method of preparation, Mw and DD). Due to its higher versatility and biological properties the majority of the assays have been carried out on chitosan samples.

Although the gastrointestinal enzymes can partially degrade both chitin and chitosan, when both polymers are orally administered they are not absorbed. For this reason, they are considered as not bioavailable. Chitosan shows a LD_{50} of around 16g/kg, very similar to the salt and glucose values in assays carried out on mice [67]. Toxicity of chitosan is reported to depend on DD. Schipper *et al.* reported that chitosans with DD higher than 35% showed low toxicity, while a DD under 35% caused dose dependant toxicity. On the other hand, Mw of chitosan did not influence toxicity [68] .

Chitosan presents higher cytocompatibility *in vitro* than chitin. The cytocompatibility of chitosan has been proved *in vitro* with myocardial, endothelial and epithellial cells, fibroblast, hepatocytes, condrocytes and keratinocytes [69]. This property seems to be related to the DD of the samples. When the positive charge of the polymer increases, the interactions between chitosan and the cells increase too, due to the presence of free amino groups. The adhesion and proliferation of keratinocytes and fibroblasts on several chitosan films with different DDs depend on both, DD and cell type. In both cells, the percentage of cell adhesion was strongly dependent of the DD, increasing with this parameter. The type of cell was a factor that also affected the adhesion, being more favourable for fibroblasts which exhibit a more negative charge surface than for keratinocytes. On the other hand, the proliferation decreased considerably by increasing the DD.

Residual proteins in chitin and chitosan could cause allergic reactions such as hypersensitivity. The protein content in a sample depends on the source of the sample and, especially, on the method of preparation.

5.3. Haemostatic

It has been reported that chitosan, as well as sulphated chitosan oligomers, presents anticoagulant activity tested *in vitro* [70]. The anticoagulant activity of chitosan seems to be related to its positive charge since red blood cells' membranes are negatively charged and chitin is less effective than chitosan [71, 72]. Chitosan Mw also affects the binding or agglutination of red blood cells [73]. In a recent paper, a comparative study has been carried out among solid-state chitosan and chitosan acetic acid physiological saline solution. Several chitosan samples with Mw from 2000 to 400 kDa and DD from 90 to 70% were tested. It was found that solid-state chitosan and chitosan acetic acid physiological saline solution followed different haemostatic mechanisms. When blood was mixed with chitosan acetic acid physiological saline solution, the erythrocytes aggregated and they were deformed. The DD, especially a high DD, in the chitosan acetic acid physiological saline solution, had a significant effect on the unusual aggregation and deformation of erythrocytes, compared with the effect of Mw within a range between 10^5 and 10^6. However, this phenomenon could not be observed in solid-state chitosan soliquoid. Solid-state chitosan with a high DD bound more platelets and was more haemostatic [74].

5.4. Analgesic Effect

Several authors have reported that both chitin and chitosan show analgesic effects [75-77]. Okamoto *et al.* have studied the analgesic effect of chitin and chitosan on inflammatory pain due to intraperitoneal administration of acetic acid and have proposed a mechanism for this analgesic effect [78]. These authors found that chitosan showed a greater effect than chitin. This difference was explained by the different action mechanism of the two polymers. The results suggested that the main analgesic effect of chitosan is the absorption of proton ions released in the inflammatory area.

Due to its polycationic nature, the free primary amino groups of chitosan can protonate in the presence of proton ions and the reduction in the pH is the main cause of the analgesic effect. On the other hand, chitin was also able to slightly absorb the proton ions but the concentration needed to show the same effect as chitosan was lower than expected. From experimental data, it was concluded that the analgesic effect was due to the absorption of bradykinin, one of the main substances related to pain.

5.5. Antitumor Activity

An antitumor activity of chitosan has been claimed by inhibition of the growth of tumor cells mainly due to an immune stimulation effect. However, this property is very controversial [73].

Jeon and Kim have found that chitosan oligomers possess antitumor activities tested both *in vitro* and *in vivo* [79]. Studies carried out using mice that had ingested low-Mw chitosan revealed significant antimetastatic effects of chitosan against Lewis lung carcinoma. Partially deacetylated chitin as well as chitin with a carboxymethyl group have also been effective to demote tumor progression [80]. The suggested mechanism involves immunostimulating effects of chitin and its carboxymethyl derivatives via stimulation of cytolytic T-lymphocytes. This activity increases with smaller molecular sizes and it is suggested that they have immunostimulating effects that activate peritoneal macrophages and stimulate non-specific host resistance. However, higher Mw oligomers have also exhibited antitumor activity. The same mechanism has been suggested for their activity via increased production of lymphokines by activated lymphocytes [81].

Ueno *et al.* studied the effect of chitosan on tumor growth and metastasis. The activation of macrophages by chitosan is suggested to mediate its antitumor effects *in vivo*, while its angiogenic inducing properties may be the harmful effects of chitosan, such as promotion of tumor growth and invasion [82].

5.6. Mucoadhesion

Several factors affect chitosan mucoadhesion, such as physiological variables and the physicochemical properties of chitosan. The mucus is composed of a glycoprotein called mucin, which is rich in negative charges since it has sialic acid residues. In the stomach, chitosan is positively charged due to the acidic environment and, therefore, it can interact with mucin by electrostatic forces. The extent of this union depends on the amount of sialic acid present in the mucin and on the Mw and DD of chitosan. It has been found that when the Mw of chitosan increases, the penetration in the mucin layer also increases and hence the mucoadhesion is stronger [83]. On the other hand, a higher DD leads to an increase in charge density of the molecule and the adhesive properties become more relevant [84].

Huang *et al.* evaluated the effects of Mw and DD on the cellular uptake and *in vitro* cytotoxicity of chitosan molecules and nanoparticles [59]. They found that the binding affinity and uptake capacity of chitosan nanoparticles decreased when decreasing polymer Mw and degree of deacetylation. The effect of the degree of deacetylation was greater than the effect of Mw because of its effect on the zeta potential of the nanoparticles. However, the uptake of chitosan molecules was less dependent on Mw and degree of deacetylation.

El-Kamel *et al.* developed mucoadhesive micromatricial chitosan/poly(ε-caprolactone) films for the treatment of periodontal diseases [85]. These authors found that films containing different Mw chitosans had different forces of adhesion but statistical analysis revealed that there was no significant difference in bioadhesion force between the films. On the contrary, Roldo *et al.* showed that the maximal detachment force of medium Mw chitosan was higher than that of both low and high Mw chitosans [86].

5.7. Permeation Enhancing Effect

It has been reported that chitosan acts as a permeation enhancer by opening epithelial tight junctions [87, 88]. The mechanism underlying this behaviour is based on the interaction of positively charged chitosan and the cell membrane resulting in a reorganization of the tight junction-associated proteins [89].

Schipper *et al.* investigated the effect of chitosan structural characteristics (Mw and DD) on their absorption enhancing properties *in vitro* (Caco-2 cell monolayers), using chitosan hydrochloride salts at pH 5.5 [68]. It was found that the capacity of chitosan to improve mannitol transport is dependent on Mw and the DD; accordingly, while chitosans with a high DD were efficient as permeation enhancers at low and high Mw, those with low degrees of deacetylation were efficient only at high Mws. Subsequently published articles in this field agree that > 80% deacetylation affords the greatest promoter effect on cells in culture [89,90].

Soane *et al.* investigated the effect on mucociliary transport velocity of five different types of chitosan with varying Mws and degrees of deacetylation. The five types of chitosan tested were shown to have no toxic effect on the frog palate clearance mechanism [91]. The cilia beat frequency in guinea pigs after nasal administration of chitosan solution was also studied for 28 days and none of the chitosans used showed any effect on the cilia frequency, which suggests that using various types of chitosan for nasal delivery applications is not harmful.

5.8. Anticholesterolemic

There are several proposed mechanisms for cholesterol reduction by chitosan. The latest findings in this field consider more than one hypothesis. The entrapment caused by a viscous polysaccharide solution is thought to reduce the absorption of fat and cholesterol in the diet. On the other hand, the presence of the amino group in its structure determines the electrostatic force between chitosan and anion substances, such as fatty acids and bile acids. Muzzarelli *et al.* propose a spontaneous formation of insoluble chitosan salts from bile acids whose hydrophobic nature should permit the collection of cholesterol and lipids via hydrophobic interaction [92]. A commercial food grade chitosan of DD 87 and average Mw of 150 kDa was used to demonstrate this theory.

The interaction between chitosan and anionic surface-active materials (phospholipids, bile acids) depends on its three types of reactive functional groups: the amino group at the C2 position and primary and secondary hydroxyl groups at the C-3 and C-6 positions, respectively. Thongngam *et al.* have demonstrated the formation of micelle-like clusters within the chitosan structure in its interactions with a model bile salt [93,94]. Another mechanism accounts for the adsorption of chitosan to the surface of the emulsified lipid and the formation of a protective coating that might prevent the lipase/co-lipase from adsorbing to the droplet surfaces and gaining access to the lipids inside the droplets [95].

Although great effort has been made to make a correlation between the physicochemical characteristics of chitosan and its fat-binding capacity, only some significant relationships have been demonstrated. No *et al.* used six commercially available chitosans with varying physicochemical characteristics and showed that the fat binding capacity was negatively correlated to the bulk density in a significant way whereas it showed a trend to positively correlate with the Mw [96]. The same group studied the fat binding capacity of five chitosans of increasing Mw (range 500-800 kDa) prepared by different depolymerization times, keeping a similar DD, and found that the sample showing significant higher activity was the one with the second lowest Mw [97].

In another study, a chitosan sample was submitted to degradation with irradiation and sonolysis, and five decreasing Mw where produced in the range 25-400 kDa. Samples showed a trend to increase the fat-binding activity with decreasing Mw using a biopharmaceutical model of digestive track [98]. Different experimental designs have been used with the aim of mimicking the reactions taking place in the stomach and duodenum. A digestive chemical model has been used to study the interaction between chitosans of different viscosity and DD and sunflower oil. Although a negative correlation was found between the percentage of en-

trapped oil and increasing oil addition, no significant differences where found in chitosan behaviour according to its characteristics [99]. Another *in vitro* human digestion model was used to check the adsorption of chitosan to the fat droplets. It was observed that the high Mw chitosan adsorbed to the droplet more strongly than the low Mw. The reasons proposed for this phenomenon were the different conformations of the chitosan molecule, with cationic loops and tails in the high Mw and its higher surface activity [100].

A recent contribution has examined and compared eleven chitosan preparations for their *in vitro* fat-binding capacity, potency to bind individual bile acids, DD, solution viscosity, and swelling volume. It was noted that the chitosan sample having the strongest binding capacity against a selected bile acid did not necessarily exhibit the strongest binding capacity against other bile acids. No correlation was detected between individual bile acid-binding capacity and any other tested physico-chemical properties of chitosan. These data suggested that Mw, as reflected by solution viscosity, DD, or swelling capacity might not be used to predict the bile acid-binding capacity of chitosan [101].

5.9. Antimicrobial Activity

The antimicrobial activity of chitin, chitosan, and their derivatives against different groups of microorganisms, such as bacteria, yeast, and fungi, has received considerable attention in recent years. Two main mechanisms have been suggested as the cause of the inhibition of microbial cells by chitosan. The interaction with anionic groups on the cell surface, due to its polycationic nature, causes the formation of an impermeable layer around the cell, which prevents the transport of essential solutes. It has been demonstrated by electron microscopy that the site of action is the outer membrane of gram negative bacteria. The permeabilizing effect has been observed at slightly acidic conditions in which chitosan is protonated, but this permeabilizing effect of chitosan is reversible [102].

The second mechanism involves the inhibition of the RNA and protein synthesis by permeation into the cell nucleus. Liu *et al.* have observed labelled chitosan oligomers with Mw from 8 to 5 kDa inside the *E. coli* cell and they showed good antibacterial activities [103]. In this case the Mw is the decisive property (Table **4**).

Table 4. Influence of Chitosan DD and Mw on Antimicrobial Activity

Physico-Chemical Property	Effect on Antimicrobial Activity
↑ DD	↑ electrostatic binding to membrane
	↑ permeabilizing effect
↑ Mw	↓ permeation into the cell nucleus

DD: deacetylation degree.

Mw: molecular weight.

Other mechanisms have also been proposed. Chitosan may inhibit microbial growth by acting as a chelating agent rendering metals, trace elements or essential nutrients unavailable for the organism to grow at the normal rate. Chitosan is also able to interact with flocculate proteins, but this action is highly pH-dependent [104]. Several authors have proposed that the antimicrobial action of chitosan against filamentous fungi could be explained by a more direct disturbance of membrane function [105]. However, it is not clear whether the antimicrobial activity of chitosan is caused by growth inhibition or cell death.

Antibacterial activities were found to increase in the order of *N*,*O*-CM-chitosan, chitosan, and *O*-CM-chitosan. The first product, where amino and hydroxyl groups have been substituted by carboxymethyl groups, contains fewer amino residues. In the case of O-CM-chitosan, its number of amino groups is not changed. Moreover, its carboxyl group may have reacted with the amino groups intra- or intermolecularly and charged these groups. The authors concluded that the antibacterial activities of chitosan and carboxymethylated derivatives depend on the effective number of $-NH_3^+$ groups [103].

Several studies prove that an increase in the positive charge of chitosan makes it bind to bacterial cell walls more strongly [106]. The relationship between Mw, number of charges and antimicrobial activity has been pointed out by Kim *et al.* [107]. They showed that O-CM chitosan derived from degraded chitosan was more effective than plain chitosan. This was attributed to the interaction of the COOH group with the NH_2 group intra-or intermolecularly to impart a charge, the number of $-NH_3$ groups becoming larger. In the case of native chitosan, an excessive concentration of amino groups on O-CM chitosan promotes a structure that involves cross-linking through strong intramolecular hydrogen bonding, where the number of amino groups that are available to attach bacterial surfaces is reduced.

In contrast, some authors have not found a clear relationship between the degree of deacetylation and antimicrobial activity. These authors suggest that the antimicrobial activity of chitosan is dependent on both the chitosan and the microorganism used [108, 109]. Park *et al.* studied the antimicrobial activity of hetero-chitosans and hetero-COs with different degrees of deacetylation and Mws against three Gram-negative bacteria and five Gram-positive bacteria and found that the 75% deacetylated chitosan showed more effective antimicrobial activity compared with that of 90% and 50% deacetylated chitosan [110].

5.10. Antioxidative Activity

Chitosan has shown a significant scavenging capacity against different radical species, the results being comparable to those obtained with commercial antioxidants. Samples prepared from crab shell chitin with DD of 90, 75 and 50% where evaluated on the basis of their abilities to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide radical and alkyl radical. The results revealed that chitosan with higher DD exhibited the highest scavenging activity [111].

On the other hand, chitosans of different size as well as their sulphate derivatives were assayed against superoxide and hydroxyl radicals. A negative correlation was found between chitosan Mw and activity (Table **5**). The chitosan sulphated derivatives presented a stronger scavenging effect on peroxide radicals but the chitosan of lowest Mw showed more considerable ferrous ion-chelating potency than others [112]. The chelation of metal ions is one of the reasons why chitosan may be considered as a potential natural antioxidant

for stabilizing lipid containing foods to prolong shelf life. Chitosans may retard lipid oxidation by chelating ferrous ions present in the system, thus eliminating their prooxidant activity or their conversion to ferric ion [113].

Table 5. Influence of Chitosan DD and Mw on Antioxidative Activity

Physico-Chemical Property	Effect on Antioxidant Activity
↑ DD	↑ scavenging effect
↑ Mw	↓ radical scavenging effect ↓ ion-chelating potency

DD: deacetylation degree.

Mw: molecular weight.

This activity has been also studied in chitooligosaccharides (COS). Chitobiose and chitotriose have proved to be more potent than three reference compounds (aminoguanidine, pyridoxamine and trolox) in scavenging hydroxyl radicals while glucosamine and the corresponding N-acetylchito-oligosaccharides did not show any capacity [114]. Electron spin resonance spectrometry has been used to follow the scavenging activity of chitooligosaccharide mixtures fractionated by ultrafiltration. This activity was shown to be dependent on the Mw, the fraction 1-3 kDa having the highest radical scavenging effect [115]. When the DD was considered, a correlation between scavenging activity over all tested free radicals with the increment of deacetylation values of COS was found. Therefore, it has been pointed out that the free amino groups in the hetero COS play an important role in free radical scavenging activity, probably by forming stable macromolecule radicals [116]. This capacity of oligosaccharides has been further assayed *in vivo*. Yang *et al.* assayed two different Mw COS (1.1 and 0.5 kDa) against H_20_2 released from polymorphonuclear leukocytes stimulated by phorbol-12-myristate-13-acetate in rats [117]. They found that the radical scavenging capacity was higher for the first COS.

6. BIOMEDICAL APPLICATIONS OF CHITIN AND CHITOSAN

Due to its high biocompatibility, chitosan has been employed in drug delivery systems, implantable and injectable systems such as orthopaedic and periodontal composites, wound healing management and scaffolds for tissue regeneration [118,119].

6.1. Wound Healing

Chitin and chitosan activate immunocytes and inflammatory cells such as PMN, macrophage, fibroblasts and angioendothelial cells. These effects are related to the DD of the samples, chitin presenting a weaker effect than chitosan [82].

Chitosan oligomers have also exhibited wound-healing properties, it is suggested that their wound-healing properties are due to their ability to stimulate fibroblast production by affecting the fibroblast growth factor. Subsequent collagen production further facilitates the formation of connective tissue [120].

Recently, the effects of chitin and chitosan oligomers and monomers on wound healing have been studied [121]. This study shows that in addition to chitin and chitosan, their oligomers and monomers enhance wound healing acceleration. Wound break strength and collagenase activity of the chitosan group (D-glucosamine (GlcN), chito-oligosaccharide (COS), chitosan) were higher than the chitin group (N-acetyl-D-glucosamine (GlcNAc), chiti-oligosaccharide (NA-COS), chitin). Collagen fibres run perpendicular to the incisional line in the oligosaccharide group (NACOS, COS) and many activated fibroblasts were observed in the histological studies around the wound in the chitosan groups. The break strength was stronger and more activated fibroblasts were observed at higher DD.

The potential use of chitin oligosaccharides (DP2, DP3, DP4, DP5 and DP7) in wound healing as well as their capacity against chronic bowel disease has been studied. For the first time, a mucin-stimulating effect of chitin oligomers DP3 and DP5 has been observed in an *ex-vivo* model [122]. The wound healing effect of chitin and chitosan oligomers and monomers is of great interest because *in vivo* lysozyme degrades chitin and chitosan to these smaller molecules.

6.2. Drug Delivery Systems

An important application of chitosans in industry is the development of drug delivery systems such as nanoparticles, hydrogels, microspheres, films and tablets (Fig. **4**). As a result of its cationic character, chitosan is able to react with polyanions giving rise to polyelectrolyte complexes [123-124]. Pharmaceutical applications include nasal, ocular, oral, parenteral and transdermal drug delivery. Three main characteristics of chitosan to be considered are: Mw, degree of acetylation and purity. When chitosan chains become shorter (low Mw chitosan), it can be dissolved directly in water, which is particularly useful for specific applications in biomedical or cosmetic fields, when pH should stay at around 7.0.

In drug delivery, the selection of an ideal type of chitosan with certain characteristics is useful for developing sustained drug delivery systems, prolonging the duration of drug activity, improving therapeutic efficiency and reducing side effects. Kofuji *et al.* suggested that the physicochemical characteristics of chitosan are important for the selection of the appropriate chitosan as a material for drug delivery vehicles [65]. Investigations have indicated that DD and Mw of chitosan have significantly affected the role of chitosan in therapeutic and intelligent drug delivery systems [125, 126].

Mi *et al.* studied the gelation properties of microspheres cross-linked with glutaraldehyde as it had significant effect on drug incorporation [127]. Microspheres prepared with a high Mw chitosan gelled faster than those prepared with a low Mw because they have different activation energies of gelation. Chitosan with short chains have higher activation energy and need more time to interact with the other chains and to gelate with glutaraldehyde.

Gupta and Jabrail studied the effect of degree of deacetylation and cross-linking on physical characteristics, swelling and release behaviour of centchroman loaded chitosan microspheres [128]. The DD controls the degree of crystallinity and hydrophobicity in chitosan due to variations in hydrophobic interactions which control the loading and release characteristics of chitosan matrices. The DD also controls the degree of cross-linking of chitosan in the presence of any

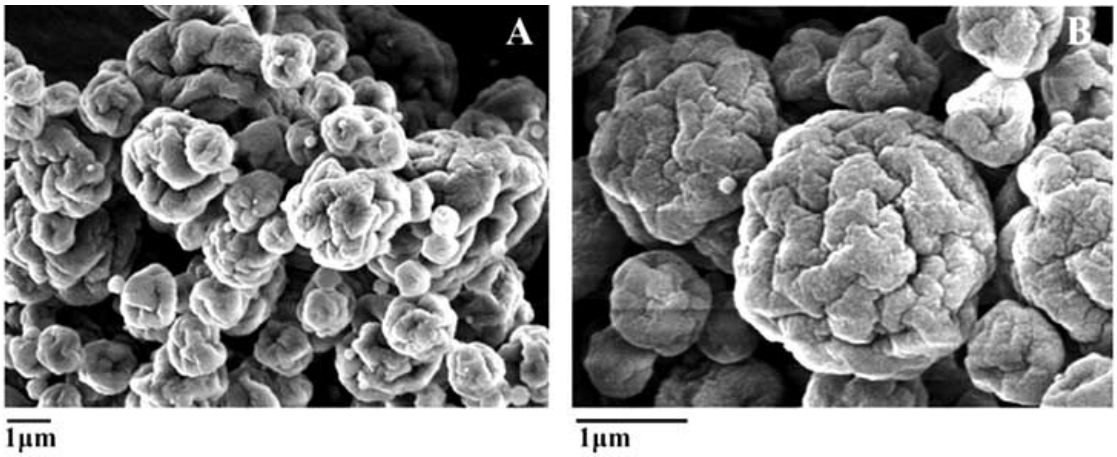


Fig. (4). (**A**) High Mw Chitosan (640 kDa) microspheres crosslinked with 0.2% TPP obtained by spray-drying. (**B**) Detail of the microspheres.

suitable cross-linker. The higher the DD is, the higher the number of free amino groups and therefore the degree of covalent cross-linking increases [129]. When analyzing the influence of cross-linking degree and degree of deacetylation on size and morphology of the microspheres, these authors reported that the size and the surface roughness decreased on increasing the degree of cross-linking and the degree of deacetylation. Zhang *et al.* also reported that a high degree of chitosan deacetylation and narrow polymer Mw distribution were shown to be critical for the control of particle size distribution [130].

A higher degree of cross-linking and a higher DD in chitosan increase the compactness of matrices and its hydrophobicity, thus controlling the degree of swelling and diffusivity of the drug entrapped in chitosan matrixes. It was observed that a DD between 48-62% promotes maximal loading capacity, due to the size of the cross-links and pores formed. Regarding the release properties, a very low DD can induce burst release [128].

In another study with chitosan microspheres loaded with centchroman and crosslinked with glutaraldehyde, Gupta and Jabrail observed that the lower Mw of chitosan employed, the lower sphericity of the microspheres obtained and these microspheres were larger in size than those prepared with medium-high Mw chitosan due to the poorer molecular packing and crosslinking [131]. These results are in agreement with those presented by Desai and Park, who studied the influence of Mw of chitosan on chitosan-TPP microspheres prepared by spray-drying [132]. They observed that an increase of Mw also produced more spherical microspheres, with greater size homogeneity and a smoother surface. In addition they found that an increase in molecular weight gave bigger microspheres as a result. Gupta and Jabrail in their study also found that microspheres prepared with high Mw chitosan presented a very low degree of swelling and a high degree of crosslinking, thus, those microspheres prepared with medium Mw chitosan that lead to less strong intermolecular interactions being more appropriate for sustained release [131]. These results were also in agreement with Desai and Park who observed that the release rate of vitamin C was much lower as the Mw of chitosan used for preparing microspheres increased [132]. They studied the release kinetics and found that it followed Fick's law of diffusion.

Low Mw chitosan leads to poor retention of centchroman in microspheres due to a high degree of swelling and a fragile network structure. The microspheres with medium Mw chitosan showed an optimum loading efficiency [131]. Microspheres with medium Mw chitosan are more efficient in releasing the centchroman in a controlled manner in comparison to low and high Mw chitosan microspheres. The initial burst release of centchroman from microspheres with different Mws and different degrees of deacetylation of chitosan varied linearly with the square root of the release time indicating a diffusion-controlled release of centchroman from these microspheres ($n = 0.5$). However, the release of centchroman in the controlled stage of drug release was anomalous [133]. The initial slope of these curves was used to calculate the diffusion coefficient (D) for centchroman from chitosan microspheres. The value of the diffusion coefficient for centchroman from microspheres decreased on increasing the Mw of chitosan, and decreased on increasing the DD in chitosan. This clearly indicates that the release of centchroman from these microspheres is diffusion controlled and the variation in the diffusion coefficient (D) of centchroman on varying the Mw and degree of deacetylation in chitosan is due to the variations in the structure of microspheres [131]. The influence of chitosan DD and Mw on the microspheres properties prepared as matrix for drug delivery is shown in Table **6**.

Desai and Park in the study of the influence of chitosan Mw on chitosan-TPP microspheres found that it does not affect the spray drying yield [132]. However, it has influence on some parameters of the microspheres that have already been commented on. In addition they studied the influence on zeta potential and observed some differences that were not very significant.

Chiou *et al.* investigated the effect of post-coating PLLA microspheres with different chitosans on the initial burst and controlling the drug release of the microspheres [134]. Without chitosan, 20% lidocaine was released within the first hour and the time of 50% release was 25 hours. This period was extended to 90 hours after coating with chitosan. They observed that when applying chitosan of the same Mw, the

efficacy of reducing the initial burst of drug release was higher for a lower degree of deacetylation. With chitosan in acetic acid solution, coating the microspheres with high Mw and high viscosity could most effectively reduce the initial burst and control drug release of PLLA microspheres. The study indicated that manipulating the viscosity of the chitosan solution was the most important factor in contributing to controlling the drug release of chitosan post-coated PLLA microspheres.

Table 6. Influence of Chitosan DD and Mw on Microspheres Properties

Physico-Chemical Property	Effect on Microsphere Properties
↑ DD	↑ covalent crosslinking
	↓ size
	↓ surface roughness
	↓ swelling
	↑ compactness and hydrophobicity
	↓ loading capacity
	↓ burst release
↑ Mw	↑ sphericity
	↑ morphology homogeneity
	↑ crosslinking
	↓ swelling
	↓ release rate
	↓ diffusion coefficient (D)

DD: deacetylation degree.

Mw: molecular weight.

Kofuji *et al.* studied the relationship between physicochemical characteristics and functional properties of chitosan such as the ability to form spherical gel, control of drug release from chitosan gel and biodegradation of chitosan [65]. They found that the formation of spherical chitosan gels in aqueous amino acid solution or aqueous solution containing metal ions was affected mainly by viscosity of the chitosan solution. High concentration of chitosan species with a high Mw could not be used to prepare chitosan spherical gel due to its high viscosity and the use of very low concentration of chitosan did not result in instantaneous spherical gel formation because the diffusion of chitosan within the preparative medium was too rapid. The degree of deacetylation also had an effect on spherical gel formation in the case of gelation of chitosan by chelation with metal ions. Chitosan with high degree of deacetylation was able to form spherical gel by chelation due to higher availability of amino groups that chelated with metal ions better than chitosan of low DD. Only in the case of chelation with metal ions was the extent of deacetylation related to drug release.

El-Kamel *et al.* developed mucoadhesive micromatricial chitosan/poly(ε-caprolactone) films for the treatment of periodontal diseases [85]. They examined the effect of different molar masses of chitosan on morphology of microparticles trapped in the films, water absorption, *in vitro* bioadhesion, mechanical properties and *in vitro* drug release. The mean size of entrapped caprolactone particles was higher in films containing higher Mw chitosan. These authors attributed this to the increased viscosity of the chitosan solution as the Mw increased. After studying water absorption capacity, results revealed that there was no statistically significant difference in percentage water uptake with different Mw chitosans. This result was in agreement with Roldo *et al.* [86], who found no correlation between the Mw of chitosan and its swelling behaviour.

The mechanical properties of films with different Mw chitosans were also measured by El- Kamel *et al.* The tensile strength (TS), the percentage elongation at break (% EB) and the elastic modulus (EM) are important parameters to indicate the strength and elasticity of the film [85]. They found that medium Mw chitosan films had highest values for TS and EM, followed by high Mw and low Mw chitosan films. On the other hand, the highest % EB was obtained for low Mw chitosan films, followed by high and medium Mw chitosan films.

With regard to *in vitro* release studies, they found that the amount of drug released from prepared films was similar for films that contained low and medium Mw chitosan and lower for the ones prepared with high Mw chitosan. This behaviour was predictable, taking into account the direct relationship between the molar mass of chitosan and the viscosity of its solution. By increasing the viscosity of the polymer, the diffusion of the drug through the formed gel layer into the release medium was retarded [135]. The high polymer viscosity may also affect the size of particles formed by reducing the homogenization efficiency, leading to the formation of larger PCL microparticles, as indicated by the particle-size analysis studies. Therefore, the exposed surface area is reduced and the release of the entrapped drug is decreased.

6.3. Gene Delivery

Due to its positive charge, chitosan has the ability to interact with negative molecules such as DNA. This property was used for the first time to prepare a non-viral vector for a gene delivery system by Mumper in 1995 [136]. The use of chitosan as non-viral vector for gene delivery offers several advantages compared to viral vectors. Mainly, chitosan does not produce endogenous recombination, oncogenic effects or immunological reactions [137]. Moreover, chitosan/pDNA complexes can be easily prepared at low cost.

The Mw of chitosan is a key parameter in the preparation of chitosan/pDNA complexes since transfection efficiency correlates strongly with chitosan Mw. High molecular weight chitosan renders very stable complexes but the transfection efficiency is very low. To improve transfection efficiency, recent studies have examined the use of low Mw chitosans [138-146] and oligomers [147-149] in gene delivery vectors. It appears that a fine balance must be achieved between extracellular DNA protection (better with high Mw) versus efficient intracellular unpackaging (better with low Mw) in order to obtain high levels of transfection. Lavertu *et al.* studied several combinations of Mw and DA of chitosan finding two combinations of high transfection efficiency using a chitosan of 10 kDa and DD of 92 and 80%, respectively [150].

Kiang *et al.* studied the effect of the degree of chitosan deacetylation on the efficiency of gene transfection in chito-

san-DNA nanoparticles [151]. Highly deacetylated chitosan (above 80%) releases DNA very slowly. They suggest that the use of chitosan with a DD below 80% may facilitate the release of DNA since it lowers the charge density, may increase steric hindrance in complexing with DNA, and is known to accelerate degradation rate. They reported an increase in luciferase expression when the degree of deacetylation was decreased from 90% to 70%. Formulations with 62% and 70% deacetylation led to luciferase transgenic expression two orders of magnitude higher than chitosan with 90% deacetylation.

6.4. Tissue Engineering

Recent studies in regenerative tissue engineering suggest the use of scaffolds to support and organize damaged tissue because three-dimensional matrices provide a more favourable ambient for cellular behaviour. Due to their low immunogenic activity, controlled biodegradability and porous structure, chitosan scaffolds are promising materials for the design of tissue engineered systems [152-154].

Tığlı *et al.* studied the influence of DD on some structural and biological properties of chitosan scaffolds for cell culture and tissue engineering [155]. They observed that chitosan scaffolds with low DD (75-85%) displayed a more regular structure and the pores were fairly uniform and parallel with a polygonal cross section. The lateral pore connectivity was much lower than for scaffolds with high deacetylation degrees (>85%). It is known that the microstructure such as pore size, shape and distribution, has prominent influence on cell intrusion, proliferation and function in tissue engineering. Swelling studies were also performed but no relationship was found between DD and swelling ratio. Mechanical testing of chitosan scaffolds showed that mechanical strength was higher with higher DD. Biodegradability of the scaffolds also depends on the DD. Cell attachment studies on the scaffolds showed that higher DD favoured cell adhesion.

Other authors also reported that a lower degree of acetylation favoured cell adhesion [69,156]. The viability of fibroblasts on chitosan scaffolds with different DD was evaluated. A significant increase in cell number was observed on >85% deacetylated chitosan scaffolds. A high proliferation trend was suggested when compared to low deacetylated chitosan scaffolds.

Chitin and chitosan tubes for nerve regeneration were prepared by Freier *et al*. [157]. The compressive strength of these tubes was found to increase with decreasing acetylation. Both chitin and chitosan support adhesion and differentiation of primary chick dorsal root ganglion neurons *in vitro*, with significantly enhanced neurite outgrowth on chitosan than on chitin films. The effect of DA on the cell adhesion and biodegradation of chitin and chitosan films was studied to find the most suitable conditions for cell compatibility and optimum biodegradation [158].

Injectable thermosetting chitosan hydrogels are attractive systems for drug delivery and tissue engineering that combine biodegradability, biocompatibility and the ability to form *in situ* gel-like implants. Thermally-induced gelation relies advantageously on biopolymer secondary interactions, avoiding potentially toxic polymerization reactions that may occur with *in situ* polymerizing formulations. Besides β-glycerophosphate [159], other molecules such as 1,3-propanediol, 1,2-propanediol as well as glycerol, mannitol or polyoses such as trehalose have been reported to induce the thermogelation of chitosan [160].

Schuetz *et al*. studied the effect of the Mw of chitosan on the properties of the thermosetting chitosan hydrogels during storage and sterilization by autoclaving [160]. The autoclaving process produced a reduction of the Mw of the chitosan samples which was affected by the initial Mw of the sample. The authors concluded that chitosans exhibiting highly reduced Mw when autoclaved might not be adapted to this sterilization method in specific applications where maximal mechanical performance is essential for implant function. With regard to the freeze storage, low Mw chitosan thermogels or those prepared with low enough concentration might be kept frozen for prolonged storage.

Porous scaffolds were prepared by freeze-drying a solution of collagen and chitosan, followed by cross-linking by dehydrothermal treatment. The effect of the chitosan Mw and the blending ratio was studied. The lysozyme biodegradation test demonstrated that the presence of chitosan, especially the high-molecular-weight species, could significantly prolong the biodegradation of collagen/chitosan scaffolds. *In vitro* culture of L929 mouse connective tissue fibroblast evidenced that low-molecular-weight chitosan was more effective for promoting and accelerating cell proliferation, particularly for scaffolds containing 30% (w/w) chitosan. The results elucidated that the blends of collagen with low-Mw chitosan have a high potential to be applied as new materials for skin-tissue engineering [161].

Apart from the aforementioned characteristics, which are specific for each application, there is a degree of consensus regarding general characteristics that must be present in chitosan samples to be used in the field of biomedical applications (Table **7**) [162, 163].

7. FOOD APPLICATIONS OF CHITOSAN

Chitosan offers a wide range of unique applications in the food industry, including preservation of foods from microbial deterioration, formation of biodegradable films, and recovery of material from food processing discards. Moreover, it can act as a dietary fibre and as a functional food ingredient.

7.1. Dietary Ingredient

Chitosan has been used in multiple nutritional supplement products due to its ability to bind fat. The *in vivo* studies are intended to demonstrate a significant reduction in the body weight gain or the plasma lipid content of humans or animals.

Recently, Liu *et al.* have reported that rats fed diets containing the highest deacetylated chitosan significantly lowered plasma cholesterol and LDL-C, and increased HDL-C level [164]. Chitosan with high Mw limited the body weight gain of adult rats significantly. When the DD and particle size were considered, chitosan with higher Mw also exhibited better cholesterol-binding capacity *in vitro*. These results indicated that the viscosity in the upper gastrointestinal tract was not the major factor influencing the hypocholesterolaemic effect of chitosan. Nonetheless, they concluded that

Table 7. Characterization of Chitosan for Medical Application [162, 163]

Variable Under Study	Appropiate Characteristic
Moisture Content	<10%
Ash content	<0,2%
Protein content	<0,3%
Insolubility	<0,1%
Turbidity	Cs 1%w/v in AcOH 1%v/v <15 NTU
Viscosity	<5 cps
DD	70-100%
Heavy metal content	As<10ppm, Pb<10ppm
Bioburden, aerobic bacteria plus fungi	(<100CFU; abscence of pathogens)
Organoleptic properties	No taste, no smell

DD: deacetylation degree.
Cs: chitosan.
NTU: Nefelometric turbidity unit.
CFU: Colony forming units.

when the particle is finer, and DD and Mw are relatively high, the effect is better.

Zeng *et al.* studied the *in vivo* absorption phenomena of different Mw chitosan in mice and found that absorption of chitosan increased with the decrease of Mw and the increase of water–solubility [165]. Chitosan with very high Mw was very difficult to absorb and enter the blood. Chitooligomers were easily degraded into much smaller molecules, quickly absorbed and distributed to other places.

Sumiyoshi and Kimura examined the effects of various water-soluble low Mw chitosans (average Mw: 21, 46 and 130 kDa) on pancreatic lipase activity, the 46 kDa chitosan being the most effective in the inhibition of this enzyme [166]. This chitosan prevented increases in body weight; various white adipose tissue weights and liver lipids (cholesterol and triacylglycerol) in mice fed a high fat diet, and further increased the faecal bile acid and fat. This group had previously reported that water- insoluble, high Mw chitosan (650 kDa), which is the minimal size of that approved by the Japanese Ministry of Health, Labour and Welfare as functional food, prevented the increases in bodyweight and white adipose tissue weights, hyperlipidaemia and fatty liver induced by feeding the high-fat diet for 9 weeks, by inhibiting the intestinal absorption of dietary fat [167].

The effect of differences in the viscosity of chitosan preparations on plasma lipoprotein cholesterol and the lipid peroxidation status in rats has been studied. The serum cholesterol-lowering action of chitosan was reported to be independent of its viscosity. However, a comparison of the liver lipid-lowering and lipid oxidation effects of chitosan samples with different viscosity showed that the total liver lipid and cholesterol-lowering action of chitosan was greater for the high-viscosity samples when the DD of the preparations were comparable [168].

The effects of chitosan properties on fat binding and fat metabolism are shown in Table **8**.

7.2. Food Preservative

Chitosans have been identified as versatile biopolymers of natural origin for food preservation due to their antimicrobial action against food spoilage microorganisms and antioxidant properties. The pH-dependent solubility allows them to be formed into various shapes (beads, films and membranes) using aqueous processing [169].

The results of the experiments indicate that, in general, low (5-27 kDa) and medium (48-78 kDa) Mw chitosans and high DD 85-98% effectively suppress the growth of both gram-positive and gram-negative bacteria [106,170]. A study of chitosan obtained from cuticles of housefly larvae points to the fact that the antibacterial effect of chitosan decreases with increase in Mw; in this case chitosans with Mw ranging from 21 to 44 kDa were more effective than chitosans of 8 and 476 kDa [171].

However, very often the most effective Mw of chitosan varies with the microorganism tested. In the case of *Candida kruisei*, chitosan apparently cannot bind to the surface of the cell wall of the fungus and penetrate inside. However, this effect is apparently species-specific, because another *Candida* species, *C. albicans*, was highly sensitive to all chitosans tested [106]. Liu *et al.* showed that at the high (200, 500 and 1000 ppm) and low (20 ppm) concentrations, the antibacterial activity of chitosan had no relationship to the Mw. However, at the middle concentration from 50 to 100 ppm, with the decrease of Mw, antibacterial activities increased [172].

Higher DD are related with better results. Tsai *et al.* compared the antimicrobial activities of chitin and chitosan obtained by chemical and biological treatments of shrimp shell. The MICs, which were in the range of 50-200 ppm, became smaller with increasing DD [56].

7.2.1. Food Emulsions

The antimicrobial properties of chitosan in a liquid medium will be poorly represented in complex food systems where the interaction of chitosans with other components may modulate their activity [109]. Chitosan solubility in aqueous acetic acid and its location at the interface are excellent predispositions for its application as antimicrobial agent in food emulsions [173]. Despite the fact that emulsions contain large concentrations of oil that do not support growth, these emulsions may contain spilage and pathogenic micro-

Table 8. Influence of Chitosan DD and Mw on Fat Binding and Fat Metabolism

Physico-Chemical Property	Effect *In Vitro*	Effect *In Vivo*
↑ DD	↑ electrostatic force between chitosan and fatty and bile acid	↓ plasma cholesterol ↓ LDL ↑ HDL
↑ Mw	↑ adsorption to lipid droplets ↓ adsorption to droplet surface of lipase	↓ body weight gain ↓ adsorption and blood distribution ↓ liver total lipid and cholesterol

DD: deacetylation degree.
Mw: molecular weight.
LDL: low density lipoprotein.
HDL: high density lipoprotein.

organisms in the non-lipid phase. Mayonnaise has been choosen as a model system where three target microorganisms have been inoculated. The most effective Mw of chitosan had been shown to vary with the microorganism tested. Viable cell counts decreased significantly without chitosans, although its addition markedly reduced the viable cell counts as compared with those of controls [109].

Studies of the effect of solubility of chitosan revealed that the water insoluble chitosan exhibited the antimicrobial effect, whereas water-soluble chitosan itself had no significant antimicrobial effect against both bacteria and yeast [170]. However, Chung *et al.* have reported the metal-ion chelating capacity and antibacterial activity of a chitosan-glucosamine derivative prepared by the Maillard reaction. This derivative appeared to be more effective than other chitosans or chitosans derivatives as a natural bactericidal agent [174].

The Maillard reaction has been used to develop biofunctional biopolymers as food preservatives with broad antimicrobial effects. Chitosans of different degrees of polymerization were mixed with lysozyme [175] and gluten peptides [176] and conjugated through this reaction. The results demonstrate that high Mw chitosan conjugates were very effective in improving the bactericidal activity of proteins or peptides compared to low Mw chitosan conjugates. It has been shown that the Maillard reaction can be successfully employed to generate products from β-lactoglobulin and chitosan, which exhibit improved bactericidal properties with respect to β-lactoglobulin alone [177].

7.2.2. Aqueous Systems

Apple juice has been used as an aqueous model system to study the antioxidative activity of chitosans with different Mws. Low Mw chitosan exhibited stronger scavenging activity than medium or high Mw and ascorbic acid, which was used as a positive scavenger. However the authors conclude that *in vivo* antioxidant activity and the various antioxidant mechanisms must be further investigated [178].

7.2.3. Solid Matrix Systems

The iron bound to fish tissue proteins such as myoglobin, haemoglobin, ferritin and transferrin may be released during storage and cooking, thus activating oxygen and initiating lipid oxidation. Kim and Thomas have examined chitosans of different Mw as antioxidative agents in salmon based on the measurement of 2-thiobarbituric acid-reactive substances (TBARS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity [179]. The 30 kDa chitosan showed the highest scavenging activity compared to 90 and 120 kDa chitosan. The increase in concentration of 30kDa chitosan resulted in the increase of total amino groups responsible for scavenging more radicals.

Fatty (herring) and lean (cod) fishes have been used as model systems to assay the antioxidant activity of 3 chitosans prepared with different deacetylation times of the same sample. The lowest viscosity chitosan presented the highest antioxidant effect. This was attributed to the lower chelation by high viscosity (high Mw) chitosan, as the intramolecular electric repulsive forces would increase the hydrodynamic volume by extended chain conformation. However, the DD was pointed to as another factor that may be involved in the chelation ability of chitosans [108-181].

7.2.4. Edible Films and Coatings

Coatings can retard ripening and water loss and reduce decay but they may also alter the flavour. Semi-permeable coatings such as chitosan may create a modified atmosphere similar to the controlled atmosphere used in storage, but at a lower cost [182]. Although many studies on chitosan coating have been published, very few of them consider the influence of the physicochemical properties on their activity.

Shortfin squid chitosan and shrimp chitosan membranes were tested for water vapour apparent permeability, swelling in water, and mechanical properties, in order to evaluate the effect of the acetylation degree and Mw of chitosan on these properties. The results indicated that decreasing the number of the bulky acetyl groups led to more intermolecular interactions among polymer chains, namely hydrogen-bonding, resulting in a tightening of the polymer network. Therefore, lower water vapour permeability and swelling in water are found, and the mechanical properties are improved.

Decreasing the molecular mass of the chitosan chains, without any significant change in DD, led to membranes with lower water vapour permeability and swelling in water. This significant effect is probably related to the increasing excluded volume effects with increasing molecular mass, contributing to the formation of a more effective packing of polymer chains within the membrane matrix and a lower degree of interstitial space in the case of those membrane networks formed from lower Mw chitosan chains. However, this decrease impairs mechanical properties as a result of decreasing entanglement density and crosslinking degree and formation of a looser network [183].

The use of chitosan coating as a protective barrier to extend the storability of many fruits and vegetables has been widely documented [184-186]. The latest reports consider the low Mw chitosan. The effect of coating Murcott tangor with low and high Mw chitosan has been investigated. This study showed that low Mw chitosan retarded ripening, water loss and decay. The coated samples exhibited greater antifungal resistance than thiabendazole [178]. Similar results were obtained with the low Mw with sliced red pitayas [187].

Jeon *et al.* compared the preservative efficacy of different viscosity chitosans in coated herring and Atlantic cod. This study demonstrated the potential of chitosan as a preservative coating in reducing or preventing moisture loss, lipid oxidation, and microbial growth, the higher viscosity (360 cP) chitosan exerting a better preservative effect in both fish model system s [188]. The effect of the DD and the Mw has been reported in the preservation of fish fillets. Presoaking the samples with a high DD chitosan solution extended the shelf life from 5 to 9 days. Chitosan showed stronger activity against bacteria rather than against fungi [56].

The coating of eggs for shelf life improvement with chitosan has attracted much interest in recent years. Chitosan may offer a protective barrier against the transfer of carbon dioxide and moisture through the eggshell. This barrier was also effective in keeping a high Haugh unit and yolk index, whose decrease is caused mainly by diffusion of water from the albumen. Only one of the groups involved in these studies has reported the comparison of different Mw chitosans. The results showed that lower Mw chitosan presented the strongest bactericidal effect and best kept the internal quality of eggs [189].

7.3. Emulsifying Agent

Chitosan produces w/o/w emulsions without adding any surfactant, because this biopolymer is composed of a mixture of molecules with different DD: some less deacetylated molecules may stabilize the water droplets inside the oil drops, while the hydrophilic ones stabilize the oil drops in the multiple emulsion formed [190].

The influence of the DD of chitosan on the emulsification of sunflower oil has been studied in HCl solutions. The resulting distribution was unimodal at low DD (75%) and high DD (95%) for all used concentrations. At intermediate DD, distribution was unimodal only when the most concentrated solution was used. When chitosan concentration increased, emulsion viscosity as a function of time was more stable [191].

Laplante *et al.* studied the emulsion stabilizing properties of various chitosans in the presence of whey protein isolate (WPI) [192]. A low deacetylated chitosan seemed better for stabilisation of the emulsion. They reported that there is a positive correlation between the increase of stability against syneresis and the increase of viscosity of chitosan (Table **9**). The most unfavourable effect from a low Mw preparation on stability is mainly explained by a loss of interfacial coadsorption efficiency. When studying other variables such as pH, ionic strength and the WPI/chitosan ratio, a low deacetylated chitosan displayed better behaviour, showing the predominance of electrostatic interactions in the interfacial stabilisation.

Table 9. Influence of Chitosan DD and Viscosity on Emulsifying Activity

Physico-Chemical Property	Effect on Emulsifying Activity
↑ DD	↓ stabilization of emulsion
↑ Viscosity	↑ stability against syneresis

DD: deacetylation degree.

Emulsions stabilized by surfactant-chitosan membranes have been shown to have better stability to pH, ionic strength, thermal processing and freezing than emulsions stabilized by surfactant alone, which was attributed to the increase in electrostatic and steric repulsion between the droplets [96,193]. The influence of the molecular characteristics of chitosan on the properties of oil-in-water emulsions has been studied. The ζ- potential and mean diameter of the particles in the secondary emulsions was not strongly influenced by chitosan Mw; however, with the lowest DD (40%) the fraction of droplets that were aggregated was considerably lower [194].

Table **9** summarized the effect of chitosan Mw and DD on emulsions.

8. APPLICATION OF CHITIN AND CHITOSAN IN BIOCATALYSIS

Immobilization was firstly defined as the process in which the enzyme is confined in a definite position thus rendering an insoluble form which retains the catalytic activity and can be reused several times. Later, this definition was extended to be defined as the process which includes not only enzymes but also cells, organelles and so on. There are four principal methods available for immobilizing enzymes and cells: adsorption, covalent binding, entrapment/membrane confinement and cross-linking [195].

Chitin and chitosan have been widely used as supports for enzyme and cell immobilization due to their appropriate characteristics. The extraction of chitin from shells and the subsequent deacetylation of chitin to produce chitosan is a relatively low cost process. Moreover, the raw material is a by-product of the seafood processing industry, the production of chitin and chitosan being a means to reduce these waste products. Both supports present appropriate density, mechanical stability and rigidity.

Chitin has been used mainly for the immobilization of enzymes and to a lesser extent to immobilize cells. On the other hand due to its higher versatility, chitosan has been used to immobilize not only enzymes but also cells, mainly by entrapment and membrane confinement. Table **10** summarized the use of chitin and chitosan for cell immobilization [196-206].

Several studies covering the field of the immobilization of enzymes on chitin and chitosan have been published [207-209] but the effect of chitin and chitosan characteristics is not issued. In general, not many specific studies related to the effect of the properties of chitin and chitosan on this field are found in the literature. The vast majority of the research focuses on the method of immobilization and its effect on the properties of the biocatalyst without taking into account that chitin and chitosan samples are available with a wide range

Table 10. Examples of the Use of Chitin and Chitosan as Support for Immobilization of Cells

Cells	Method	Support	Ref
Rhodococcus sp	Adsorption	Chitin	[196]
E. coli	Adsorption	Chitosan	[197]
Several microorganism	Entrapment	B-Chitin gels	[198]
Agrobacterium radiobacter	Covalent binding	Chitin and chitosan powders	[199]
Pseudomonas sp	Adsorption	Chitosan –agar-alginate beads	[200]
Pseudomonas putida	Membrane confinement	Chitosan beads croslinked with TPP	[201]
Several microorganism	Membrane confinement	Chitosan beads	[202]
Saccharomices cerevisiae	Membrane confinement	Chitosan- carboxymethil celulose	[203]
Saccharomyces cerevisiae	Covalent binding	Globular chitosans activated with glutaraldehyde	[204]
Hepatocyte spheroids and hepatocytes	Membrane confinement	Alginate-chitosan polyelectrolyte complex	[205]
Sporidiobolus salmonicolor	Membrane confinement	Chitosan capsules crosslinked with gloxal and diso-dium sulphate	[206]

of Mw and DD. In this part of the review we intend to show the relationship between chitin and chitosan properties and the behaviour of the immobilized biocatalyst.

8.1. Immobilization of Enzymes and Cells on Chitin and Chitosan (Adsorption and Covalent Binding)

In immobilization by adsorption, the enzymes or cells are bound to the carrier material via reversible surface interactions. The forces involved are van der Waals forces, ionic and H-bonding interaction as well as hydrophobic forces. Due to the low amount of free amino groups on its surface, chitin can be considered a neutral support and there is a low possibility of interaction through H-bonding or ionic forces. As the amount of free amino groups increase the ionic and H-bonding forces achieve more relevance. When attaching to non-charged supports such as chitin, proteins showed their maximal adsorption at their isoelectric point [210]. On the other hand, when the support is charged the maximal interaction occurs when both the enzyme and the support exhibit opposite charge. Chitosan is a positively charged polymer at pH lower than its pKa (6.4), in these conditions negatively charged proteins bind easily to chitosan.

The adsorption of cells on chitin and chitosan has also been studied. Strand et al studied the effect of the chemical composition and Mw of chitosan on the adsorption and flocculation of *E. coli* cells. These authors found that adsorption increases with the pH but decreases with the Mw of chitosan. Chitosan samples with a high DD showed the best adsorption behaviour [197].

The covalent immobilization involves the formation of a covalent bond between the enzyme/cell and the support material. The bond is formed between functional groups on the carrier and the enzyme (amino, carboxyl, hydroxyl and sulphydryl groups). Through chemical modification, functional groups on the carriers can be altered to create new interactions between the enzyme and the support. In chitin and chitosan the most usual process for the immobilization of the enzymes and cells is the immobilization through a bifunctional dialdehyde (most frequently glutaraldehyde). The density of free amino groups on the surface of the support will determine the amount of interaction points between the enzyme and the support. When using chitin as support, the number of free amino groups on the support will be low and then each enzyme molecule may be attached to the support in a single point. On the other hand, when chitosan is used as support each enzyme may be linked to the support through several residues. In these conditions a multipoint immobilization is achieved (Fig. (**5**)).

In our laboratory, a crude cell extract from *Agrobacterium radiobacter*, containing D-hydantoinase and D-carboamylase activities to produce D-amino acids, was immobilized on different chitin and chitosan samples by adsorption, covalent binding and membrane confinement [211-213]. When the extract was immobilized by adsorption, the results showed that the activity of the immobilized derivatives on chitin were around 2-fold higher than the ones immobilized on chitosan (Table **11**). On the other hand, when the extract was immobilized by covalent binding on chitosan no activity was detected. In this particular case, the extract immobilized on chitin showed half of the activity immobilized by adsorption.

These results showed the effect of the DD on the biocatalyst activity. When the biocatalyst was immobilized by adsorption on chitosan, the low activity was related to the pI of the catalytic enzymes of the extract. The pIs of D-

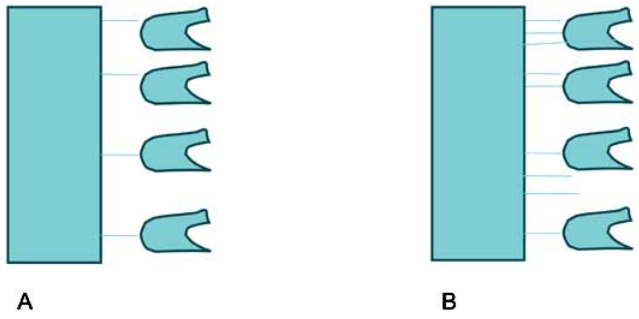


Fig. (5). Covalent immobilization of enzymes on chitin/chitosan. **a**) Low free amino group density (Single point immobilization) and **b**) high free amino group density (multipoint immobilization).

Table 11. Chitin and Chitosan as Support for the Immobilization of a Crude Cell Extract by Adsorption and Covalent Binding

Sample	Source	DA	Ash, %	Crystallinity	p-HPG Yield, %[1]	p-HPG Yield, %[2]
Chitin	Shrimp (*Ploeticus mülleri*)	0.96	0.33	α	18	8.1
Chitin	Shrimp (*Penaeus caramote*)	0.95	0.62	α	14	8.0
Chitin	Lobster (*Palinurus vulgaris*)	0.96	12.25	α	0	0
Chitin	Squid (*Dosidicus Gigas*)	0.91	0.48	β	16	----
Chitosan	Shrimp(*Penaeus caramote*)	0.33	0.22	----	9	0
Chitosan	Lobster(*Palinurus vulgaris*)	0.61	8.25	----	0	0
Chitosan	Crab (*Paralomis granulosa*)	0.16	0.89	----	9	0
Chitosan	Crab *Commercial*	0.09	0.15	----	10	0

[1] Immobilization by adsorption, [2] Immobilization by covalent binding.

DA: acetylation degree.

p-HPG: p-hydroxyphenylglycine.

hydantoinase and D-carboamylase from *Agrobacterium* have been reported to be 6.5 and 5.5 [214,215]. These values are close to the pKa of chitosan and thus the interaction between the enzymes and the support are not favoured. In the case of covalent immobilization, the absence of the activity can be related to conformational changes due to the multipoint immobilization as well as irreversible interactions between catalytic residues of the enzyme and the support [213].

A new green bean (*Phaseolus vulgaris*) tissue homogenate-based biosensor was developed for the square-wave voltammetric determination of caffeic acid in white wine. The biosensor was constructed by immobilization of green bean tissue homogenate, as a source of peroxidase, in a chemically crosslinked chitin matrix with epichlorohydrin and glutaraldehyde that was incorporated in a carbon paste electrode. Two samples of chitin, a commercial one from Sigma and a homemade one from squid pens were tested. The squid pen samples were chosen as the most suitable due to a higher DD (91.6 *vs* 86.8) [216].

Another property of the samples that shows a great effect on the activity is the ash content of the supports. When the extract was immobilized on lobster chitin and chitosan no activity was detected. Synowiecki et al. studied the immobilization of α-amylase on several chitin samples by adsorption and covalent binding; in both cases, the enzyme immobilized on supports with high ash content showed lower activity [217]. On the other hand, we have used lobster chitin samples with high ash content to immobilize tyrosinase and α-chymotripsin and in both cases, the biocatalyst showed good catalytic properties [218,219]. In this context, it is clear that a deeper study of the effect of the ash content should be conducted to clarify these results.

Chitin crystallinity is another aspect to keep in mind when choosing the best chitin for a catalytic purpose. Since β-chitin is a more hydrated structure than α-chitin, chitin samples with high β-structure content are more hydrophilic supports. Moreover, due to much weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains, β-chitins are more reactive than α-chitins [220]. Enzymes are able to catalyze reactions not only in aqueous media but also in organic media. In these processes the water content in the microenvironment of the enzymes is basic to the catalytic process.

When α-chymotrypsin was immobilized by covalent binding on several chitin samples the derivative immobilized on squid chitin (β-chitin) showed the highest hydrolytic activity in organic media due to the higher water content in the microenvironment of the enzyme [219]. Due to the higher porosity of krill chitin (β-chitin) when compared to other sources, this chitin was selected as the most appropriate support for modification with carbon disulfide [221]. This derivative has both positively charged amino groups and negatively charged dithiocarbamino groups showing better catalytic properties than control chitin when several enzymes were immobilized by adsorption. Squid pen chitin (β-chitin) and crab chitin have been used to prepare thin films to immobilize several oxidases to prepare a biosensor. The structure of the β-chitin sample was more favourable than the α-chitin sample [222].

Laccase has been conjugated with chitosan of several Mw with a carbodiimide reaction. The effect of the Mw of chitosan on the activity loss during conjugation was studied. Small chitosan molecules gave high residual activity. The conjugated laccase exhibited a high stability in the following repeated phase changes and had the same temperature and pH profile as those of free laccase. Compared to free laccase, the conjugated laccase had a similar affinity (K_m), but reduced turnover (k_{cat}) that was adversely affected by the increase of the molecular mass of chitosan [223].

The source of the support also affects the properties of the biocatalyst. A lipase has been immobilized on chitosan films prepared from mycelia of *S. racemosum* and from a crustacean source using glutaraldehyde as a bifunctional agent. The lipase immobilized on the crustacean chitosan showed higher activity that the one immobilized on the mycelia sample. However, after four cycles the remaining activity of the lipase immobilized on mycelia chitosan was higher [224].

8.2. Entrapment of Biocatalysts on Chitosan and Chitosan Derived Systems

In encapsulation and entrapment the enzyme or cells are free in solution, but restricted in movement by the lattice

structure of a gel. The porosity of the gel lattice is controlled to ensure that the structure is compact enough to prevent leakage of the enzymes or cells, while allowing free movement of substrate, cofactors, products and nutrients when necessary.

Alsarra et al. have studied the effect of the Mw and DD of chitosan on the a*c*tivity of lipase encapsulated in chitosan-TPP capsules [225]. These authors found that chitosan with high Mw and DD improve lipase loading and lessen the release of the entrapped lipase. Moreover, they developed equations which can predict the effect of the Mw and DD on entrapment efficiency and other parameters of interest.

Alginate-chitosan polyelectrolyte complexes can be prepared by different methodologies. Among them, the one-step procedure and two-step procedures have been used to encapsulate proteins, mainly with the purpose of preparing drug delivery systems. However, this method can also be used to encapsulate enzymes with a catalytic purpose [226, 227]. In our laboratory, a crude cell extract from *Agrobacterium rb* has been encapsulated by using both methods. The effect of the Mw and DD of chitosan on the properties of the biocatalyst was studied. The amount of encapsulated protein was around 60% and did not depend on the chitosan characteristics. In all cases, a negligible release was observed so the biocatalyst can be used in both long term process and in batch. The effect of the chitosan characteristics on the mechanical stability of the capsules is shown in Table **12**. It is clear that the stability of the capsules is greatly dependent on the Mw of chitosan being unaffected by the DD (Fig. (**6**)).

The ability of the biocatalyst to produce p-hydroxyphenylglycine was also tested. As can be seen in Table **13**, the DD of chitosan negatively affects the production of the amino acid while the Mw showed a lighter effect on it.

Alginate capsules coated with chitosan have also been used to protect urease from α-chymotrypsin and other proteases. The effect of the Mw of chitosan on the retention activity of urease after treatment with α-chymotrypsin was studied. The best results were observed with the medium Mw chitosan with a retention activity of 48% while the low and high Mw showed a lower retention of 20 and 25%, respectively. Activity retention of urease encapsulated in alginate coated capsules with medium Mw chitosan after treatment with trypsin and proteinase K was 44 and 73% respectively [228].

Table 12. Mechanical Stability of the Alginate-Chitosan PEC Capsules Loaded with Extract. Burst Assay (N=100)

CHITOSAN Mw, kDa	DD	Stability, (%)[1]	Stability, (%)[2]
816	0.90	30	100
495	0.90	100	100
75	0.90	0	100
563	0.70	100	100

[1] One step procedure. [2] two step procedure.
DD: deacetylation degree.
Mw: molecular weight.
PEC: polyelectrolyte complex.

Table 13. Effect of Chitosan Characteristics on the Production of p-HPG

CHITOSAN Mw,	DD	p-HPG Yield,(%)[1]	p-HPG Yield,(%)[2]
816	0.90	68	73
495	0.90	68	63
75	0.90	63	60
563	0.70	38	30

[1] One step procedure. [2] Two steps procedure.
p-HPG: p- hydroxiphenylglycine.

Alginate mixed chitosan capsules have been used to immobilize carbonic anhydrase (Mw 30 kDa) for a biocatalytic purpose. The effect of the Mw of chitosan on the enzyme retention was studied. The best enzyme retention was achieved with the medium Mw chitosan [229]. Tannase has been encapsulated in chitosan crosslinked TTP capsules as well as in alginate coated chitosan capsules for the hydroly-

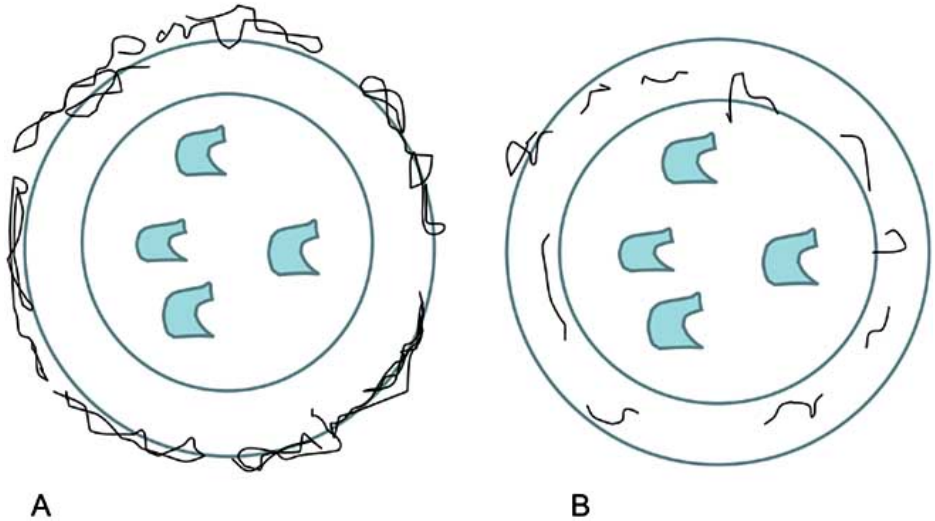


Fig. (6). Alginate-chitosan polyelectrolyte complexes. **a**) High molecular weight chitosan piles on the capsule surface **b**) Low Molecular weight chitosan gets into the alginate network.

sis of tea tannins [230]. A high Mw and a low Mw chitosan were used. The release of the enzyme from the alginate-chitosan capsules was studied. After three days, the enzyme encapsulated using the high Mw chitosan showed a higher release than the small one (83% *vs* 28%).

9. WASTE WATER TREATMENT

The chemical contamination of water from a wide range of toxic products such as metals, aromatic molecules, dyes and so on, is a serious environmental problem owing to the latter's potential human toxicity. The use of low-cost polymers such as chitin and chitosan to remove water pollutants is of great interest [231].

Chitin and chitosan have been used to remove a great variety of water pollutants [232]. Guibal and co-workers have extensively studied the effect of chitosan properties on the adsorption of metals, dyes and organic compounds among others [233-235]. The use of chitosan based material to remove anionic dyes has been recently reviewed by Crini and Badot [236]. This work includes a specific mention of the effect of chitosan characteristics on the adsorption process.

The coagulation-flocculation process as well as the adsorption process depends on the DD, chitosan being more efficient than chitin to remove metal ions [233, 237], PCBs [238] and anionic dyes [232]. On the other hand, chitin was more efficient than chitosan in removing polycyclic aromatic hydrocarbons from petrochemical wastewater [239]. When comparing chitosan samples with different DD for the adsorption of an azo dye the sample with the higher DD showed the highest efficiency [240]. However, in some cases this rule does not work.

Guibal and coworkers have suggested that rather than the deacetylation degree the parameter that controls these processes is the accessibility of the amino groups to water and pollutants [233-235]. This accessibility is mainly controlled by the crystallinity of the samples, which depends on the origin of the sample as well as the treatment during extraction.

Zhang and Schiewer have also studied the effect of the deacetylation degree and crystallinity of chitin and chitosan samples (DD from 10 to 90%) on the adsorption of As (V) [241]. Their results also support the role of crystallinity in the adsorption of As (V). Wong *et al.* studied the adsorption of five acidic dyes on chitin and chitosan samples [242]. They suggested that the adsorption mechanism involves a one-step process of dyes complexing with the free amino groups. When varying the DD from 52% to 97% a decrease in the dye adsorption capacity was observed suggesting that more amorphization may cause changes in the internal structure of chitosan and reduce the adsorption capacity.

Moreover, Kurita *et al.* found that metal adsorption rate for samples with the same DD was higher when homogeneous hydrolysis was used to prepare the chitosan samples, which indicates that the distribution of the acetyl groups along the chains also affects the adsorption process [243].

Huang *et al.* have found that the coagulant efficiency of chitosan also depends on the Mw of the sample. Samples with the same Mw increase their coagulant efficiency by increasing DD but when the Mw of the sample was increased a reduction of the coagulant efficiency was reported even when DD was higher (86 *vs* 77%) [244]. Bought *et al.* compared several chitosan samples with different Mw and viscosity and found that higher values of Mw were predictive of greater effectiveness for coagulation but the effectiveness did not correlate linearly with Mw [245].

Chitosan has been used as adsorbent, coagulant and bactericide in the treatment of aquaculture wastewater. The higher the DD, the higher the removal of organic compounds, inorganic nutrients (nitrates and phosphates) and pathogen was. On the other hand, the higher Mw chitosan showed a higher reduction of turbidity, suspended solids, BOD and COD while the lower Mw was very effective in the reduction of inorganic nutrients [174,246].

The effectiveness of chitosan in coagulating and flocculating organic suspensions at pH close to neutrality and low ionic strength (distilled water) is improved by using high DD and low Mw chitosan samples [234]. On the other hand, the effect of DD and Mw decreased at acidic pH and higher ionic strength (tap water), the effectiveness of the process being almost unaffected by chitosan conditions. When concentrated suspensions of bentonite were adsorbed on chitosan in either tap water or demineralized water at either pH 5 or 7, efficiency was higher when the Mw of the chitosan samples was higher, though efficiency tended to level off when the Mw of chitosan exceeded 10 kDa [235].

Alginate-chitosan polyelectrolyte complexes using different Mw and DD chitosan samples were used to recover soluble proteins from surimi wash water. Protein recovery from aqueous processing streams by chitosan–alginate complexes has been proposed to occur by mechanical entrapment and electrostatic interaction with the complex. The effect of DD and Mw of chitosan was tested. However, no correlation was found [247].

Apart from DD and Mw, the purity (protein and pigment content) of the samples is another aspect that affects the effectiveness of chitinous materials in the field of waste water treatment since it affects the accessibility of the free amino groups to the pollutants. From these results, it can be concluded that the choice of a chitinous sample for a specific pollutant will depend on several factors, such as the type of pollutant, polymer characteristics (mainly DD, crystallinity and Mw) and the characteristics of the water (pH, ionic strength). However, as a general rule chitosan is preferred over chitin in the vast majority of applications. Moreover, chitosan samples with low crystallinity and high DD are generally preferred.

10. NEW APPLICATIONS OF CHITIN AND CHITOSAN

Apart from the aforementioned applications of chitin and chitosan which have been developed in recent decades, new applications that have not been previously reviewed have appeared in the last few years. Although not much information can be found with regard to these applications we have tried to update the knowledge on the relationship between physicochemical properties and function.

10.1. Imprinted Chitosan-Based Matrixes

Molecularly imprinted polymer (MIP) represents a new class of materials that have artificially created receptor struc-

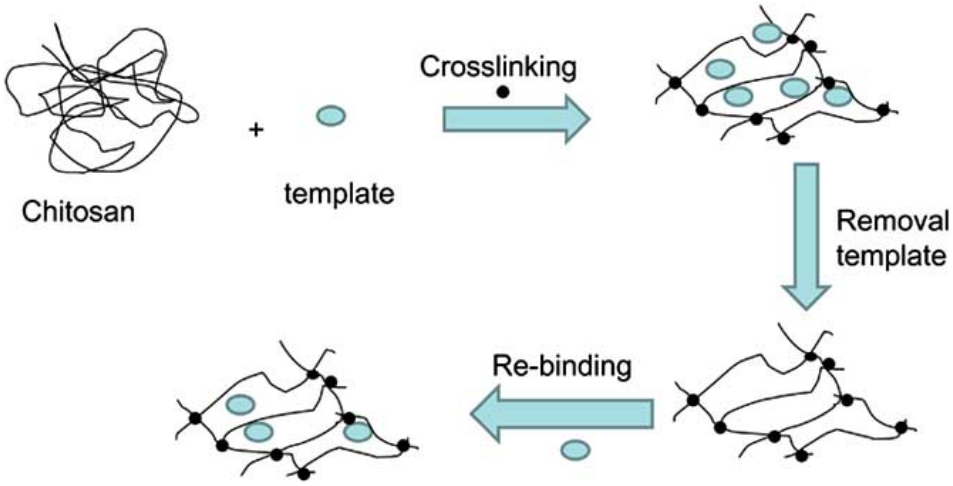


Fig. (7). Schematic representation of imprinted chitosan-based matrix preparation.

tures. This potential technology is a method for making selective binding sites in synthetic polymers by using a molecular template. MIPs have steric and chemical memory toward the template and hence could be used to rebind it (Fig. (**7**)).

As can be seen in Table **14**, chitosan imprinted matrixes have been prepared with different purposes by using different chitosan samples [248-265].

In general, samples with DD ≥90% and wide range of Mw have been used to prepare chitosan imprinted materials. As far as we know, there is only one paper studying the effect of chitosan Mw (92, 47 and 29 kDa) on the rebinding properties of molecular imprinted chitosan matrixes [255]. In this case, the sample with 47 kDa achieved the best rebinding capacities.

It is well known that the number of recognition sites in MIPs increases with network density. The crosslinking den-

Table 14. Preparation of MIP Chitosan with Different Purposes

Matrix	CS Characteristics	Crosslinker	Template	Application	REF
Cs hydrogels	DD 94%,30 kDa	Glu	DBT	Fuel desulfurization	[248]
Cs hydrogel	DD 90%, 78 kDa	Genipin	O-xylene	Waste water treatment	[249]
CS	Non data	ECH, GLU	PFOS	Waste water treatment	[250]
Cs	DD 90%, 521 ml/g	ECH	Hemoglobin	Protein binding	[251, 252]
Cs, binary xilosanes	DD 98% $6*10^4$g/mol	ECH, form	BSA	Protein binding	[253]
CS , nylon membranes	DD 98%, $1*10^5$g/mol	GPTMS	PEG	Protein binding	[254]
CS, acrylamide	DD 90%, 92,47,29 kDa	MBA	BSA	Protein binding	[255]
CS, acrylamide	DD 90%, 504 kDa	MBA	Hb	Protein binding	[256]
Cs TiO2	DD 90%	ECH	Ni, Cu	Metal Recovery and photodegradation	[257]
Cs fungi mycelium	DD 90%	ECH	Ni	Metal recovery	[258]
Cs, fungi mycelium, TiO2	DD 90%	ECH	Ni	Metal Recovery and photodegradation	[259]
CS resin	DD 90%	ECH	Ni	Metal recovery	[260]
Cs silica gel	DD 98%, $8*10^5$g/mol	GPTMS	PEG, sucrose	Metal recovery	[261]
Cs , fungi mycelium, TiO2	CS 90%	ECH	Ni	Organic compound degradation and metal recovery	[262]
Cs silica gel	DD 98%, $6*10^4$	GPTMS	Cd	Cd recovery	[263]
CS, acid metacrilic	DD 90%, 521ml/mg	ECH	quercetin	Flavonoids recovery	[264]
Cs	DD 92%	GPTMS	L-Phe	Quiral resolution	[265]

ECH: epichlorohydrin GLU: Glutaraldehyde GPTSM γ-glycidoxypropyltrimethoxysilane; MBA: metilenbisacrilamida; Form: Formaldehyde DBT: Dibenzothiophene sulfone. PFOS: Perfluorooctane sulfonate. BSA: bovine serum albumin. L-Phe : L-phenylalanin. PEG: Polyethylene glycol.

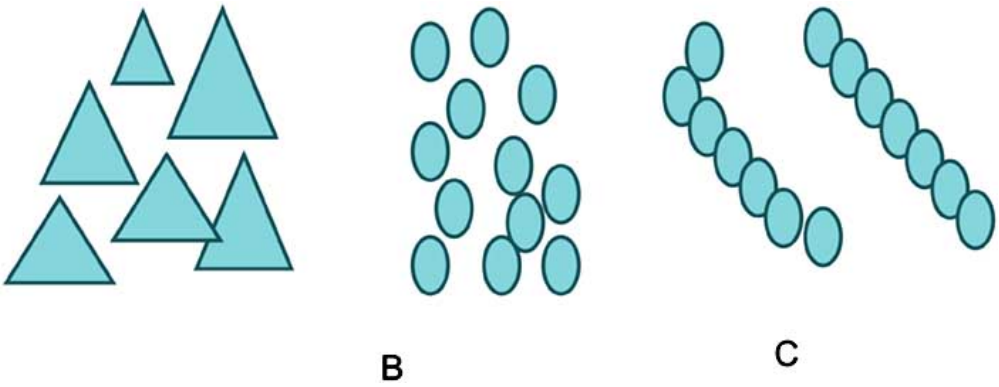


Fig. (8). Effect of chitosan molecular weight on the morphology of gold nanoparticles. (**A**) nanoplates (1000kDa), (**B**) single nanoparticles (50kDa) and (**C**) 2D chains (5 kDa).

sity is influenced by various parameters being favoured by high DD and Mw. Since crystallinity and surface area control the accessibility of the free amino groups to the chemical reagents, these parameters are expected to have an impact on the behaviour of the matrixes.

10.2. Chitosan-Metal Nanocomposites

As mentioned above, chitosan has high affinity for metal ions. This property has been used to prepare metal-chitosan nanocomposites with potential applications in several fields, such as biomedicine, catalyst, electronics, non-linear optics and so on.

Traditionally, metal ions were reduced by chemical agents, UV-light in the presence of chitosan which stabilized and controlled the size of the nanoparticles due to the presence of free amino groups [266]. Taking into account that some sugars are able to reduce metal ions, chitosan has been used to reduce the metal ions "in situ" without using other reductants. Gold nanoparticles have been prepared in acidic media in the presence of chitosan at 55ºC [267]. Twu *et al*. prepared silver nanoparticles using chitosan (1240 kDa, DD=87%) suspensions in basic medium at high temperature and suggested that low Mw chitosan degradation products may supply electrons and function as a reducing agent [268]. Murugadoss and Chattopadhyay have also prepared a silver-chitosan nanocomposite in basic medium at high temperature using a high Mw chitosan from Sigma (DD=75%, no data on Mw) [269]. A more exhaustive characterization of the system was carried out and these authors propose that free amino groups as well as hydroxyl groups are involved in Ag+ reduction. Moreover, Ag nanoparticles are adsorbed on the surface of the polymer due to the free amino groups and prevent further aggregation controlling the size of the nanoparticle.

Although the aforementioned authors indicated in their studies that the silver reduction only occurred in a basic medium, Wei and Qian have prepared silver and gold chitosan nanocomposites in acidic medium by using a chitosan sample with DD higher than 85% and viscosity higher than 200.000 cps. [270]. These authors suggested that free amino groups are bound to Ag nanoparticles during the reduction and the reduction of metal is due to oxidation of hydroxyl groups. Due to a not proper characterization of the samples a direct comparison is not possible; but it seems that the reduction of silver ions in basic media depends on the Mw of chitosan samples. Silver nanoparticles have also been prepared by γ-radiation of a chitosan solution in acidic media. The authors have suggested that the stabilization of Ag nanoparticles is due to the presence of depolymerized chitosan due to radiation [271].

Recently, Wu *et al*. have prepared gold nanoplates by reducing gold with a chitosan sample with a Mw of 50 kDa [272]. These authors found that by increasing the chitosan (Mw 1000 kDa), the final gold products were mainly composed of spherical gold nanoparticles and a small portion of sub-micro-sized gold nanoplates which indicates that chitosan Mw controls the morphology of the system. Moreover, Yang *et al*. have prepared gold 2D nanoparticles chains by using a low Mw chitosan (5000 Da) [273]. All these results point to a key role of chitosan characteristics, namely DD and Mw, in the synthesis of metal nanoparticles as well as in their morphology (Fig. (**8**)).

11. CONCLUSION

As we have shown in this review, chitin and chitosan present a great variety of properties, allowing them to have a large number of applications, but, at the same time, the very complex behaviour of these polymers is difficult to control.

These polymers have an intrinsic variability due to their natural origin. Moreover, depending on the manufacturing process, the properties of samples from the same source change. In general, a poor characterization of the polymers is carried out which makes it very difficult to compare results and to establish relationships between the physiological behaviour of chitin and chitosan and their properties. However, from data in the literature it is possible to give general recommendations regarding the properties of chitin and chitosan for a specific application, this being the main contribution of this review to the field of study of chitin and chitosan. These recommendations are shown in Table **15**.

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Table 15. General Recommendations for the Use of Chitin and Chitosan in Several Applications

Application	General Recommendations	
Wound healing	High DD chitosan preferred over chitin Low Mw samples (oligomers)	
Drug delivery systems	High DD High Mw	
Gene Delivery	DD ≤ 80 Low Mw (around 10 kDa)	
Scaffolds (tissue engineering)	DD around 85 (good proliferation and structure) High Mw (prolonged biodegradation)	
Cell immobilization	Chitosan preferred over chitin (high DD)	
Enzyme immobilization	Depend on the enzyme, immobilization method and reaction media Low ash content β-chitin preferred over α-chitin in organic reaction media	
	Adsorption	Chitin for neutral or positively charged proteins Chitosan for negatively charged proteins. High DD
	Covalent	Chitosan for multipoint immobilization. High DD Chitin or chitosan with low DD for single point immobilization
	Encapsulation	Chitosan-TPP High Mw, high DD better retention Chitosan-Alginate PECs Medium Mw better stability
Dietary ingredient	High DD; high Mw (viscosity) Fine particle	
Food preservative	High DD Medium-low Mw (5-80 kDa)	
Emulsifying agent	Low DD for emulsion stability High viscosity	
Waste water treatment	Depend on pollutant and water conditions (pH, ionic strenght) In general, chitosan preferred over chitin. High DD Low cristallinity	
Molecular imprinting	Not yet tested High DD is expected to improve crosslinking In general, low Mw chitosan is used	
Metal reduction	Metal reduction depends on chitosan characteristics (not yet fully tested) High DD and low Mw seems to stabilize the nanoparticles	
	Clear relationship between morphology and Mw	Low Mw chitosan 2D chains Medium Mw chitosan: single nanoparticles High Mw chitosan: nanoplates

REFERENCES

[1] Kumar MNVR. A review of chitin and chitosan applications. React Funct Polym 2000; 46: 1-27.

[2] Aiba S. Studies on chitosan: 4. Lysozymic hydrolysis of partially N-acetylated chitosans. I J Biol Macromol 1992; 14(4): 225-8.

[3] Roberts G. Chitin Chemistry. London: Macmillan 1998.

[4] Acosta N, Jimenez C, Borau V, Heras A. Extraction and characterization of chitin from crustaceans. Biomass Bioenerg 1993; 5(2): 145-53.

[5] Horton D, Lineback DR. N-deacetylation, chitodan from chitin. In: Whistler RL, Wolfson ML, Eds. Methods in Carbohydrate Chemistry. New York: Academic Press 1965: 403.

[6] Batista I, Roberts GAF. A novel facile technique for deacetylating chitin. Makromol Chem 1990; 191: 435-43.

[7] Domard A, Rinadudo M. Preparation and characterization of fully deacetylated chitosan. I J Biol Macromol 1983; 5: 49-52.

[8] Pelletier A, Lemire I, Sygusch J, Chornet E, Overend RP. Chitin/chitosan transformation by thermo-machano-chemical treatment including characterization by enzymatic depolymerization. Biotechnol Bioeng 1990; 36: 310-5.

[9] Focher B, Beltrane PL, Naggi A, Torri G. Alkaline N-deacetylation of chitin enhanced by flash treatments. Reaction kinetics and structure modifications. Carbohydr Polym 1990; 12: 405-18.

[10] Goycoolea FM, Higuera-Ciapara I, Hernandez JL, J., García KD. Preparation of chitosan from squid (Loligo spp) pen by a microwave-accelerated thermochemical process. In: Domard A, Roberts GAF, Vårum KM, Eds. Advances in Chitin Science. Lyon: Jacques André 1998: 78-83.

[11] Mima S, Miya M, Iwamoto R, Yoshikawa S. Highly deacetylated chitosan and its properties. J Appl Polym Sci 1983; 28: 1909-17.

[12] Gooday GW, Prosser JI, Hillman K, Cross MG. Mineralization of Chitin in an estuarine sediment: The importance of the Chitosan pathway. Biochem Syst Ecol 1991; 19(5): 395-400.

[13] Martinou A, Bouriotis V, Stokke BT, Varum KM. Mode of action of chitin deacetylase from Mucor Rouxii on a fully water-soluble, higly acetylated chitosan. In: Domard A, Roberts GAF, Vårum KM, eds. Advances in Chitin Science. Lyon: Jacques André Publisher 1998: 194-202.

[14] Martinou A, Bouriotis V, Stokke BT, Varum KM. Mode of action of chitin deacetylase from Mucor rouxii on partially N-acetylated chitosans. Carbohydr Res 1998; 311(1-2): 71-8.

[15] Prashanth R, Tharanathan R. Chitin/chitosan: modifications and their unlimited application potential- an overview. Trends Food Sci Tech 2007; 18: 117-31.

[16] Muzzarelli RAA. Depolymerization of chitins and chitosans with hemicellulase, lysozyme, papain and lipase In: Muzzarelli RAA, G PM, Eds. Chitin Handbook. Grottamore: European Chitin Society 1977: 153-65.

[17] Choi W, Ahn K, Lee D, Byun M, Park H. Preparation of chitosan oligomers by irradiation. Polym Degrad Stabil 2002; 78: 533-38.

[18] Bade ML. Structure and isolation of native animal chitins. In: Goosen MFA, Ed. Applications of chitin and chitosan. Lancaster: Technomic Publishing 1997: 253-77.

[19] Sannan T, Kurita K, Iwakura Y. Studies on Chitin, 2: Effect of deacetylation on solubility. Makromol Chem 1976; 177: 3589-600.

[20] Kurita K, Sannan T, Iwakura Y. Studies on Chitin, 4. Evidence for formation of block and random copolymers of N-acetyl-d-glucosamine and d-glucosamine by hetero- and homogeneous hydrolyses. Makromol Chem 1977; 178: 3197- 202.

[21] Roberts G. Chitosan production routes and their role in determining the structure and properties of the product. In: Domard A, Roberts GAF, Vårum KM, Eds. Adv Chit Sci Lyon Jacques André 1998: 22-31.

[22] Rege PR, Block LH. Chitosan processing: influence of process parameters during acidic and alkaline hydrolysis and effect of the processing sequence on the resultant chitosan's properties. Carbohyd Res 1999; 321(3-4): 235-45.

[23] Sukwattanasinitt M, Zhu H, Sashiwa H, Aiba S. Utilization of commercial non-chitinase enzymes from fungi for preparation of 2-acetamido-2-deoxy--glucose from [beta]-chitin. Carbohydr Res 2002; 337(2): 133-7.

[24] Galed G, Diaz E, Heras A. Conditions of N-deacetylation on chitosan production from alpha chitin. Nat Prod Commun 2008; 3(4): 543-50.

[25] Galed G, Miralles B, Paños I, Santiago A, Heras A. N-Deacetylation and depolymerization reactions of chitin/chitosan: Influence of the source of chitin. Carbohydr Polym 2005; 62(4): 316-20.

[26] Rinaudo M. Chitin and chitosan: Properties and applications. Prog Polym Sci 2006; 31(7): 603-32.

[27] Li QD, Dunn ET, Grandmaison EW, Goosen MFA. Applications and Properties of Chitosan J Bioact Compat Polym 1992; 7: 370-97.

[28] Baxter A, Dillon M, Anthony Taylor KD, Roberts GAF. Improved method for i.r. determination of the degree of N-acetylation of chitosan. I J Biol Macromol 1992; 14(3): 166-9.

[29] Tharanathan RN, Kittur FS. Chitin: The Undisputed Biomolecule of Great Potential. Crit Rev Food Sci Nut 2003; 43(1): 61-87.

[30] Kassai M. A review of several reported procedures to determine the degree of N-acetylation for chitin and chitosan using infrared spectroscopy. Carbohydr Polym 2008; 71: 497-508.

[31] Brugnerotto J, Lizardi J, Goycoolea FM, Arguelles-Monal W, Desbrieres J, Rinaudo M. An infrared investigation in relation with chitin and chitosan characterization. Polymer 2001; 42(8): 3569-80.

[32] Muzzarelli RAA, Rocchetti R. Determination of the degree of acetylation of chitosans by first derivative ultraviolet spectrophotometry. Carbohydr Polym 1985; (6): 61-72.

[33] Wu T, Zivanovic S. Determination of the degree of acetylation (DA) of chitin and chitosan by an improved first derivative UV method. Carbohyd Polym 2008; 73: 248-53.

[34] Duarte ML, Ferreira MC, Marvao MR, Rocha J. Determination of the degree of acetylation of chitin materials by 13C CP/MAS NMR spectroscopy. I J Biol Macromol 2001; 28(5): 359-63.

[35] Van de Velde K, Kiekens P. Structure analysis and degree of substitution of chitin, chitosan and dibutyrylchitin by FT-IR spectroscopy and solid state 13C NMR. Carbohydr Polym 2004; 58: 409-16.

[36] Varum KM, Antohonsen MW, Grasdalen H, Smidsrod O. Determination of the degree of N-acetylation and the distribution of N-acetyl groups in partially N-deacetylated chitins (chitosans) by high-field n.m.r. spectroscopy. Carbohyd Res 1991; 211(1): 17-23.

[37] Raymond L, Morin FG, Marchessault RH. Degree of deacetylation of chitosan using conductometric titration and solid-state NMR. Carbohydr Res 1993; 246(1): 331-6.

[38] Jiang X, Chen L, Zhong W. A new linear potentiometric titration method for the determination of deacetylation degree of chitosan. Carbohydr Polym 2003; 54: 457-63.

[39] Guinesi L, Cavalheiro E. The use of DSC curves to determine the acetylation degree of chitin/chitosan samples. Termochim Acta 2006; 444: 128-33.

[40] Rinaudo M, Milas M, Le Dung P. Characterization of chitosan. Influence of ionic stregth and degree of acetylation on chain expansion. I J Biol Macromol 1993; 15: 281-5.

[41] Brugnerotto J, Desbrieres J, Roberts G, Rinaudo M. Characterization of chitosan by steric exclusion chromatography. Polymer 2001; 42(25): 09921-7.

[42] Terbojevich M, Cosani A. Molecular weight determination of Chitin and Chitosan. In: Muzzarelli RAA, Peter MG, Eds. Chitin handbook. Grotammare: European Chitin Society 1997: 87-101

[43] Yen M, Yang J, Mau J. Physicochemical characterization of chitin and chitosan from crab shells. Carbohydr Polym 2009; 75: 15-21.

[44] ASTM. F2103-01 Standard guide for characterization and testing of chitosan salts as starting materials intended for use in biomedical and tissue-engineered medical product applications. 2001.

[45] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

[46] Moerschbacher B, El Gueddari N. Bio-activity matrices for partially acetylated chitosan oligomers. Advances in Chitin Science Vol IX (CD): 2007; 10-23.

[47] Kurita K. Chitin and chitosan: functional biopolymer from marine crustaceans. Mar Biotechnol 2006; 8: 203-26.

[48] ASTM. F2260-03 Standard Test Method for Determining Degree of Deacetylation in Chitosan Salts by Proton Nuclear Magnetic Resonance (1H NMR) Spectroscopy 2003.

[49] ASTM. WK965 New Test Method for Determining the Molar Mass of Chitosan and Chitosan Salts by Size Exclusion Chromatography with Multi-angle Light Scattering Detection (SEC-MALS). 2003.

[50] European Pharmacopeia 2002; 1774-5.

[51] Rauh F, Dornish M. Chitosan. In: Guelcher S, Hollinger J, Eds. An introduction to biomaterials. Boca Raton: Taylor & Francis 2006.

[52] Koide SS. Chitin-chitosan: Properties, benefits and risks. Nut Res 1998; 18: 1091-101.

[53] Kumar MNVR, Muzarelli RAA, Muzarelli C, Sashiwa H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. Chem Rev 2004; 104: 6017-84.

[54] Muzzarelli RAA, Muzzarelli C. Chitosan chemistry: Relevance to the biomedical sciences. Adv Polym Sci 2005; 186: 151-209.

[55] Anthonsen M, Varum K, Hermansson A, Smidsrod O, Brant D. Aggregates in acidic solutions of chitosans detected by static laser light scattering. Carbohydr Polym 1994; 25: 13-23.

[56] Tsai G, Su W, Chen H, Pan C. Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation. Fisheries Sci 2002; 68: 170-7.

[57] Pangburn SH, Trescony PV, Heller J. Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. Biomaterials 1982; 3(2): 105-8.

[58] Francis S, Matthew JK, Howard WT. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials 2000; 21(24): 2589-98.

[59] Huang M, Khor E, Lim L. Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharm Res 2004; 21(2): 344-53.

[60] Tomihata K, Ikada Y. In vitro and in vivo degradation of films of chitins and its deacetylated derivatives. Biomaterials 1997; 18: 567-75.

[61] Zhang H, Neau SH. In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation. Biomaterials 2001; 22(12): 1653-8.

[62] Hirano S, Tsuchida H, Nagao N. N-acetylation in chitosan and the rate of its enzymic hydrolysis. Biomaterials 1989; 10(8): 574-6.

[63] Kurita K, Kaji Y, Mori T, Nishiyama Y. Enzymatic degradation of [beta]-chitin: susceptibility and the influence of deacetylation. Carbohydr Polym 2000; 42(1): 19-21.

[64] Sashiwa H, Saimoto H, Shigemasa Y, Ogawa R, Tokura S. Distribution of the acetamide group in partially deacetylated chitins. Carbohydr Polym 1991; 16(3): 291-6.

[65] Kofuji K, Qian CJ, Nishimura M, Sugiyama I, Murata Y, Kawashima S. Relationship between physicochemical characteristics and functional properties of chitosan. Eur Polym J 2005; 41(11): 2784-91.

[66] Shigemasa Y, Saito K, Sashiwa H, Saimoto H. Enzymatic degradation of chitins and partially deacetylated chitins. I J Biol Macromol 1994; 16(1): 43-9.

[67] Knapczk J, Krowczynski L, Pawlik B, Liber Z. Pharmaceutical dosage form in chitosan. In: Skjak-Braek G, Anthonsen T, Sanford P, Eds. Chitin and chitosan: Sources, Chemistry, Biochemistry, Physical properties and Applications. London: Elsevier Applied Science 1984: 665-9.

[68] Schipper NGM, Varum K, Artursson P. Chitosans as absorption enhancers for poorly absorbable drugs. 1: influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells. Pharm Res 1996; 13(11): 1686-92.

[69] Chatelet C, Damour O, Domard A. Influence of the degree of acetylation on some biological properties of chitosan films. Biomaterials 2001; 22(3): 261-8.

[70] Park PJ, Je JY, Jung WK, Ahn CB, Kim SK. Anticoagulant activity of heterochitosans and their oligosaccharide sulfates. Eur Food Res Technol 2004; 219: 529-33.

[71] Rao S, Sharma CP. Use of chitosan as biomaterial: Studies on its safety and hemostatic potential. J Biomed Mater Res 1997; 34: 21-8.

[72] Klokkevoid PR, Fukuyama H, Sung EC, Bertolami CN. The effects of chitosan (Poly-N-acetyl glucosamine) on lingual hemostasis in heparinized rabbits. Int J Oral Max Surg 1999; 57: 49-52.

[73] Kim SK, Rajapakse N. Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. Carbohydr Polym 2005; 62(4): 357-68.

[74] Jian Y, Feng T, Zheng W, Qing W, Yan-Jun Z, Shi-Qian C. Effect of Chitosan Molecular Weight and Deacetylation Degree on Hemostasis. J Biomed Mater Res B 2008; 84B: 131-7.

[75] Allan GG, Altman lC, Besinger R, Ghosh D, Hirabayasi Y, Neogi S. Biochemical applications of chitin and chitosan. In: Zikakis JP, Ed. Chitin, chitosan and related enzymes. Orland, F L: Academic Press 1984: 119-33.

[76] Ohshima Y, Nishino H, Yonekura Y, Kishimoto S, Wakabayasi S. Clinical application of chitin non-woven fabric as wound dressing. Eur J Plast Surg 1987; 10: 66-9.

[77] Okamoto Y, Minami Y, Matsuhashi A, *et al*. Application of polymeric N-acetyl-D-glucosamine (chitin) to veterinary practice. J Vet Med 1993; 55: 743-7.

[78] Okamoto Y, Kawakami K, Miyakate K, Morimoto M, Shigesama Y, Minami S. Analgesic effects of chitin and chitosan. Carbohydr Polym 2002; 49: 249-52.

[79] Jeon YJ, Kim SK. Antitumor activity of chitosan oligosaccharides produced in ultrafiltration membrance reactor system. J Microbiol Biotechnol 2002; 12(3): 503-7.

[80] Tsukada K, Matsumoto T, Aizawa K, *et al*. Antimetastatic and growth-inhibitory effects of N-acetylchitohexaose in mice bearing Lewis lung carcinoma. Jpn J Cancer Res 1990; 81(3): 259-65.

[81] Suzuki K, Tokoro A, Okawa Y. Effect of N-acetylchitooligosaccharides on activation of phagocytes. Microbiol Immunol 1986; 30(8): 777-87.

[82] Ueno H, Mori T, Fujinaga T. Topical formulations and wound healing applications of chitosan. Adv Drug Deliv Rev 2001; 52: 105-15.

[83] Lehr CM, Bouwstra J, Schacht E, Junginger H. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int J Pharm 1992; 78 43-8: 43-8.

[84] He P, Davis SS, Illum L. In vitro evaluation of the mucoadhesive properties of chitosan microspheres. Int J Pharm 1998; 166(1): 75-88.

[85] El-Kamel A, Ashri L, Alsarra I. Micromatricial metronidazolbenzoate film as a local mucoadhesive delivery system for treatment of periodontal diseases. AAPS PharmSciTech 2007; 8(3). article 75. Doi 10.1208/pt0803075.

[86] Roldo M, Hornof M, Caliceti P, Bernkop-Schnurch A. Mucoadhesive thiolated chitosans as platforms for oral controlled drug delivery: synthesis and in vitro evaluation. Eur J Pharm Biopharm 2004; 57(1): 115-21.

[87] Junginger H, Verhoef J. Macromolecules as safe penetration enhancers for hydrophilic drugs: a fiction?. Pharm Sci Techol Today 1998; 1(9): 370-6.

[88] Kotze A, Luessen H, Thanou M, *et al*. Chitosan and its derivatives as arbsorption enhancers for peptide drugs across mucosal epithelia. In: Mathiowitz E, Lehr CM, Eds. Bioadhesive Drug Delivery Systems. New York: Marcel Dekker Inc 1999; 341-85.

[89] Smith J, Wood E, Dornish M. Effect of chitosan on Epithelial Cell Tight Junctions. Pharm Res 2004; 21(1): 43-9.

[90] Schipper NGM, Hoogstraate J, deBoer A, Vårum KM, Artursson P. Chitosans as Absorption Enhancers for Poorly Absorbable Drugs 2: Mechanism of Absorption Enhancement. Pharm Res 1997; 14(7): 923-9.

[91] Soane RJ, Hinchcliffe M, Davis SS, Illum L. Clearance characteristics of chitosan based formulations in the sheep nasal cavity. Int J Pharm 2001; 217(1-2): 183-91.

[92] Muzzarelli R, Orlandini F, Pacetti D, *et al*. Chitosan taurocholate capacity to bind lipids and to undergo enzymatic hydrolysis: An in vitro model. Carbohydr Polym 2006; 66: 363-71.

[93] Thongngam M, McClements DJ. Characterization of Interactions between Chitosan and an Anionic Surfactant. J Agric Food Chem 2004; 52(4): 987-91.

[94] Thongngam M, McClements DJ. Isothermal titration calorimetry study of the interactions between chitosan and a bile salt (sodium taurocholate). Food Hydrocolloid 2005; 19(5): 813-9.

[95] Ogawa S, Decker E, McClements D. Influence of Environmental Conditions on the Stability of Oil in Water Emulsions Containing Droplets Stabilized by Lecithin-Chitosan Membranes. J Agric Food Chem 2003; 51: 5522-7.

[96] No HK, Lee KS, Meyers SP. Correlation Between Physicochemical Characteristics and Binding Capacities of Chitosan Products. J Food Sci 2000; 65(7): 1134-7.

[97] No HK, Lee SH, Park NY, Meyers SP. Comparison of Physicochemical, Binding, and Antibacterial Properties of Chitosans Prepared without and with Deproteinization Process. J Agric Food Chem 2003; 51(26): 7659-63.

[98] Czechowska-Biskup R, Rokita B, Ulanski P, Rosiak JM. Radiation-induced and sonochemical degradation of chitosan as a way to increase its fat-binding capacity. Nucl Instrum Meth Physics Res 2005; 236(1-4): 383-90.

[99] Rodriguez MS, Albertengo LE. Interaction between Chitosan and Oil under Stomach and Duodenal Digestive Chemical Conditions. Biosci, Biotechnol Biochem 2005; 69(11): 2057-62.

[100] Beysseriat M, Decker EA, McClements DJ. Preliminary study of the influence of dietary fiber on the properties of oil-in-water emulsions passing through an in vitro human digestion model. Food Hydrocolloid 2006; 20(6): 800-9.

[101] Zhou K, Xia W, Zhang C, Yu L. In vitro binding of bile acids and triglycerides by selected chitosan preparations and their physicochemical properties. LWT - Food Sci Technol 2006; 39(10): 1087-92.

[102] Helander I, Nurmiaho-Lassila E, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol 2001; 71: 235-44.

[103] Liu X, Yun L, Dong Z, Zhi L, Kang D. Antibacterial action of chitosan and carboxymethylated chitosan. J Appl Polym Sci 2001; 79(7): 1324-35.

[104] Roller S, Covill N. The antifungal properties of chitosan in laboratory media and apple juice. Int J of Food Microbiol 1999; 47(1-2): 67-77.

[105] Fang SW, Li CF, Shih DYC. Antifungal Activity of Chitosan and Its Preservative Effect on Low-Sugar Candied Kumquat. J Food Protec 1994; 57: 136-40.

[106] Gerasimenko DV, Avdienko ID, Bannikova GE, Zueva OY, Varlamov VP. Antibacterial Effects of Water-Soluble Low-Molecular-Weight Chitosans on Different Microorganisms. Appl Biochem Microbiol 2004; 40(3): 253-7.

[107] Kim K, Thomas R, Lee C, Park H. Antimicrobial Activity of native Chitosan, Degraded Chitosan, and O-Carboxymethylated Chitosan. J Food Protect 2003; 66: 1495-8.

[108] Chien P, Chou C. Antifungal activity of chitosan and its application to control post-harvest quality and fungal rotting of Tankan citrus fruit (Citrus tankan Hayata). J Sci Food Agric 2006; 86: 1964-9.

[109] Oh H, Kim Y, Chang E, Kim J. Antimicrobial Characteristics of Chitosans against Food Spoilage Microorganisms in Liquid Media and Mayonnaise. Biosci Biotechnol Biochem 2001; 65(11): 2378-83.

[110] Park PJ, Je JY, Byun HG, Moon SH Kim SK. Antimicrobial Activity of Hetero-Chitosans and Their Oligosaccharides with Different Molecular Weights. J Microbiol Biotechnol 2004; 14(2): 317-23.

[111] Park PJ, Je JY, Kim SK. Free radical scavenging activities of differently deacetylated chitosans using an ESR spectrometer. Carbohydr Polym 2004; 55(1): 17-22.

[112] Xing R, Liu S, Guo Z, *et al*. Relevance of molecular weight of chitosan and its derivatives and their antioxidant activities in vitro. Bioorg Med Chem 2005; 13(5): 1573-7.

[113] Peng C. Synthesis of crosslinked chitosan-crown ethers and evaluation of these products as adsorbents for metal ions. J Appl Polym Sci 1998; 70: 501-6.

[114] Chen AS, Taguchi T, Sakai K, Kikuchi K, Wang MW, Miwa I. Antioxidant Activities of Chitobiose and Chitotriose. Biol Pharm Bull 2003; 26(9): 1326-30.

[115] Park PJ, Je JY, Kim SK. Free Radical Scavenging Activity of Chitooligosaccharides by Electron Spin Resonance Spectrometry. J Agric Food Chem 2003; 51(16): 4624-7.

[116] Je JY, Park PJ, Kim SK. Free radical scavenging properties of hetero-chitooligosaccharides using an ESR spectroscopy. Food Chem Toxicol 2004; 42(3): 381-7.

[117] Yang Y, Shu R, Shao J, Xu G, Gu X. Radical scavenging activity of chitooligosaccharide with different molecular weights. Eur Food Res Technol 2006; 222(1): 36-40.

[118] Berger J, Reist M, Mayer JM, Felt O, Gurny R. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. Eur J Pharm Biopharm 2004; 57(1): 35-52.

[119] Muzzarelli RAA. Amphoteric Derivatives of Chitosan and their Biological Signifiance. In: G. Skjak-Braek TA, P. Sandford, Ed. London: Elsiever Applied Science 1989: 87.

[120] Howling GI, Dettmar PW, Goddard PA, Hampson FC, Dornish M, Wood EJ. The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro. Biomaterials 2001; 22(22): 2959-66.

[121] Minagawa T, Okamura Y, Shigemasa Y, Minami S, Okamoto Y. Effects of molecular weight and deacetylation degree of chitin/chitosan on wound healing. Carbohydr Polym 2007; 67: 640-4.

[122] Deters A, Petereit F, Schmidgall J, Hensel A. N-Acetyl-D-glucosamine oligosaccharides induce mucin secretion from colonic tissue and induce differetiation of human keratinocytes. J Pharm Pharmacol 2008; 60: 1-8.

[123] Acosta N, Aranaz I, Peniche C, Heras A. Tramadol release from a delivery system based on alginate- chitosan microcapsules. Macromol Biosci 2003; 3: 546- 51.

[124] Peniche C, Arguelles-Monal W, Peniche H, Acosta N. Chitosan: An atractive biocompatible polymer for microencapsulation. Macromol Biosci 2003; 3: 511-20.

[125] Illum L, Farraj NF, Davis SS. Chitosan as novel nasal delivery system for peptides drugs. Pharm Res 1994; 11: 1186-9.

[126] Jiang H, Park I, Shim N, *et al*. In vitro study of the immune stimulating activity of an athrophic rhinitis; vaccine associated to chitosan microspheres. Eur J Pharm Biopharm 2004; 58: 471-6.

[127] Mi FL, Kuan CY, Shyu SS, Lee ST, Chang SF. The study of gelation kinetics and chain-relaxation properties of glutaraldehyde-cross-linked chitosan gel and their effects on microspheres preparation and drug release. Carbohydr Polym 2000; 41(4): 389-96.

[128] Gupta KC, Jabrail FH. Effects of degree of deacetylation and cross-linking on physical characteristics, swelling and release behavior of chitosan microspheres. Carbohydr Polym 2006; 66: 43-54.

[129] Draget KI. Association phenomenon in highly acetylated chitosan gel. Polym Gels Netw 1996; 4: 143-51.

[130] Zhang H, Oh M, Allen C, Kumacheva E. Monodisperse chitosan nanoparticles for mucosal drug delivery Biomacromolecule 2004; 5(6): 2461-8.

[131] Gupta K, Jabrail F. Glutaraldehyde cross-linked chitosan microspheres for controlled release of centchroman. Carbohydr Res 2007; 342: 2244-52.

[132] Desai KG, Park HJ. Effect of manufacturing parameters on the characteristics of vitamin C encapsulated tripolyphosphate-chitosan microspheres prepared by spray-drying. J Microencapsul 2006; 23(1): 79-90.

[133] Siepmann J, Gopferich A. Mathematical modeling of controlled drug delivery. Adv Drug Deliver Rev 2001; 48: 137-8.

[134] Chiou SH, Wu WT, Huang YY, Chung TW. Effects of the characteristics of chitosan on controlling drug release of chitosan coated PLLA microspheres. J Microencapsul 2001; 18(5): 613-25.

[135] Lorenzo-Lamosa ML, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Design of microencapsulated chitosan microspheres for colonic drug delivery. J Control Release 1998 ; 52(1-2): 109-18.

[136] Mumper R, Wang J, Claspell J, Rolland AP. Novel polymeric condensing carriers for gene delivery. Proceedings International Symposium Controlled Release Bioactive Materials; 1995; 1995. p. 178-9.

[137] Ferber D. Gene therapy: safer and virus-free? Science 2001; 294: 1638-42.

[138] Romoren K, Pedersen S, Smistad G, Evensen O, Thu BJ. The influence of formulation variables on in vitro transfection efficiency and physicochemical properties of chitosan-based polyplexes. Inte J Pharm 2003; 261(1-2): 115-27.

[139] Ishii T, Okahata Y, Sato T. Mechanism of cell transfection with plasmid/chitosan complexes. Biochim Biophys Acta 2001; 1514(1): 51-64.

[140] Koping-Hoggard M, Tubulekas I, Guan H, Edwards K, Nilsson M, Varum KM. Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. Gene Ther 2001; 8(14): 1108-21.

[141] Lee M, Nah JW, Kwon Y, Koh JJ, Ko KS, Kim SW. Water-soluble and low molecular weight chitosan-based plasmid DNA delivery. Pharm Res 2001; 18(4): 427-31.

[142] Li X, Lee D, Chan A, Alpar H. Sustained expression in mammalian cells with DNA complexed with chitosan nanoparticles. Biochim Biophys Acta 2003; 1630(1): 7-18.

[143] MacLaughlin FC, Mumper RJ, Wang J, Tagliaferri JM, Gill I, Hinchcliffe M. Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery. J Control Release 1998; 56(1-3): 259-72.

[144] Park IK, Kim TH, Kim SI, Park YH, Kim WJ, Akaike T. Visualization of transfection of hepatocytes by galactosylated chitosan-graft-poly(ethylene glycol)/DNA complexes by confocal laser scanning microscopy. Int J Pharm 2003; 257(1-2): 103-10.

[145] Richardson SC, Kolbe HV, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. Int J Pharm 1999; 178(2): 231-43.

[146] Sato T, Ishii T, Okahata Y. In vitro gene delivery mediated by chitosan. effect of pH, serum, and molecular mass of chitosan on the transfection efficiency. Biomaterials 2001; 22(15): 2075-80.

[147] Koping-Hoggard M, Melnikova Y, Varum K, Lindman B, Artursson P. Relationship between the physical shape and the efficiency of oligomeric chitosan as a gene delivery system in vitro and in vivo. J Gene Med 2003; 5: 130-41.

[148] Koping-Hoggard M, Varum K, Issa M, *et al*. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. Gene Ther 2004; 11: 1441-52.

[149] Thanou M, Florea BI, Geldof M, Junginger HE, Borchard G. Quaternized chitosan oligomers as novel gene delivery vectors in epithelial cell lines. Biomaterials 2002; 23(1): 153-9.

[150] Lavertu M, Methot S, Tran-khanh N, Buschmann MD. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation Biomaterials 2006; 27: 4815-24.

[151] Kiang T, Wen J, Lim HW, Leong KW, Kam KW. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. Biomaterials 2004; 25(22): 5293-301.

[152] Ho MH, Wang DM, Hsieh HJ, *et al*. Preparation and characterization of RGD-immobilized chitosan scaffolds. Biomaterials 2005; 26(16): 3197-206.

[153] Ma L, Gao C, Mao Z, *et al*. Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Biomaterials 2003; 24(26): 4833-41.

[154] Madihally SV, Howard WT. Porous chitosan scaffolds for tissue engineering. Biomaterials 1999; 20(12): 1133-42.

[155] Tığlı R, Karakeçili A, Gümüşderelioğlu M. In vitro characterization of chitosan scaffolds: influence of composition and deacetylation degree. J Mater Sci 2007; 18 : 1665-74.

[156] Amaral IF, Lamghari M, Sousa SR, Sampaio P, Barbosa MA. Rat bone marrow stromal cell osteogenic differentiation and fibronectin adsorption on chitosan membranes: The effect of the degree of acetylation. J Biomed Mater Res 2005; 75A: 387-97.

[157] Freier T, Montenegro R, Koh HS, Shoichet MS. Chitin-based tubes for tissue engineering in the nervous system. Biomaterials 2005; 26(22): 4624-32.

[158] Freier T, Koh HS, Kazazian K, Shoichet MS. Controlling cell adhesion and degradation of chitosan films by N-acetylation. Biomaterials 2005; 26(29): 5872-8.

[159] Chenite A, Chaput C, Wang D, *et al*. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. Biomaterials 2000; 21(21): 2155-61.

[160] Schuetz YB, Gurny R, Jordan O. A novel thermoresponsive hydrogel based on chitosan. Eur J Pharm Biopharm 2008; 68(1): 19-25.

[161] Tangsadthakun C, Kanokpanont S, Sanchavanakit N, *et al*. The influence of molecular weight of chitosan on the physical and biological properties of collagen/chitosan scaffolds. J Biomater Sci Polym E 2007; 18(2): 147-63.

[162] Sandford P. Chitosan Commercial uses and potential applications. In: Skjak-Braek TAaPS, Ed. Chitin and chitosan: Sources chemistry, Biochemistry, Physical properties and Applications. London: Elsevier Applied Science 1984; 51-69.

[163] Struszczyk MC. Global requeriments for medical applications of chitin and its derivatives. Polish Chitin Society Monography XI. 2006.

[164] Liu J, Zhang J, Xia W. Hypocholesterolaemic effects of different chitosan samples in vitro and in vivo. Food Chem 2008; 107(1): 419-25.

[165] Zeng L, Qin C, Wang W, Chi W, Li W. Absorption and distribution of chitosan in mice after oral administration. Carbohydr Polym 2008; 71(3): 435-40.

[166] Sumiyoshi M, Kimura Y. Low molecular weight chitosan inhibits obesity induced by feeding a high-fat diet long-term in mice. J Pharm Pharmacol 2006; 58: 201-7.

[167] Han L, Kimura Y, Okuda H. Reduction in fat storage during chitin-chitosan treatment in mice fed a high-fat diet. Int J Obesity 1999; 23: 174-9. .

[168] Chiang MT, Yao HT, Chen HC. Effect of Dietary Chitosans with Different Viscosity on Plasma Lipids and Lipid Peroxidation in Rats Fed on A Diet Enriched with Cholesterol. Biosci Biotechnol Biochem 2000; 64(5): 965-71.

[169] Aberg C, Chen T, Olumide A, Raghavan S, Payne G. Enzymatic Grafting of Peptides from Casein Hydrolysate to Chitosan. Potential for Value-Added Byproducts from Food-Processing Wastes. J Agric Food Chem 2004; 52: 788-93.

[170] Qin C, Li H, Xiao Q, Liu Y, Zhu J, Du Y. Water-solubility of chitosan and its antimicrobial activity. Carbohydr Polym 2006; 63(3): 367-74.

[171] Jing Y, Hao Y, Qu H, Shan Y, Li D, Du R. Studies on the antibacterial activities and mechanisms of chitosan obtained from cuticles of housefly larvae. Acta Biol Hung 2007; 58(1): 75-86.

[172] Liu N, Chen XG, Park HJ, *et al*. Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*. Carbohydr Polym 2006; 64(1): 60-5.

[173] Zivanovic S, Basurto CC, Ch S, Davidson PM, Weiss J. Molecular Weight of Chitosan Influences Antimicrobial Activity in Oil-in Water Emulsions. Food Protec 2004; 67(5): 952-9.

[174] Chung YC, Li YH, Chen CC. Pollutant removal from aquaculture wastewater using the biopolymer chitosan at different molecular weights. Environ Sci Healt A 2005; 40: 1775-90.

[175] Pan Y, Li YJ, Zhao HY, *et al*. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. Int J Pharm 2002; 249(1-2): 139-47.

[176] Babiker E. Effect of chitosan conjugation on the functional properties and bactericidal activity of gluten peptides. Food Chem 2002; 79: 367-72.

[177] Miralles B, Martínez-Rodríguez A, Santiago A, van de Lagemaat J, Heras A. The occurrence of a Maillard-type protein-polysaccharide reaction between [beta]-lactoglobulin and chitosan. Food Chem 2007; 100(3): 1071-5.

[178] Chien PJ, Sheu F, Huang WT, Su MS. Effect of molecular weight of chitosans on their antioxidative activities in apple juice. Food Chem. 2007; 102(4): 1192-8.

[179] Kim K, Thomas R. Antioxidative activity of chitosans with varying molecular weights. Food Chem 2007; 101: 308-13.

[180] Kamil JYVA, Jeon YJ, Shahidi F. Antioxidative activity of chitosans of different viscosity in cooked comminuted flesh of herring (Clupea harengus). Food Chem 2002; 79(1): 69-77.

[181] Shahidi F, Kamil JYVA, Jeon YJ, Kim SK. Antioxidant role of chitosan in a cooked cod (cadus morhua) model system. J Food Lipids 2002; 9(1): 57-64.

[182] Galed G, Fernandez-Valle ME, Martinez A, Heras A. Application of MRI to monitor the process of ripening and decay in citrus treated with chitosan solutions. J Magn Reson Imaging 2004; 22(1): 127-37.

[183] Santos C, Seabra P, Veleirinho B, Delgadillo I, Lopes da Silva JA. Acetylation and molecular mass effects on barrier and mechanical properties of shortfin squid chitosan membranes. Eur Poly J 2006; 42(12): 3277-85.

[184] El Ghaouth A, Arul J, Wilson C, Benhamou N. Biochemical and cytochemical aspects of the interactions of chitosan and Botrytis cinerea in bell pepper fruit. Postharvest Biol Tec 1997; 12: 183-94.

[185] Hernández-Muñoz P, Almenar E, Ocio M, Gavara R. Effect of calcium dips and chitosan coatings on postharvest life of strawberries (Fragaria x ananassa). Postharvest Biol Tec 2006; 39(3): 247-53.

[186] Ribeiro C, Vicente A, Teixeira J, Miranda C. Optimization of edible coating composition to retard strawberry fruit senescence. Postharvest Biol Tec 2007; 44(1): 63-70.

[187] Chien PJ, Sheu F, Lin HR. Quality assessment of low molecular weight chitosan coating on sliced red pitayas. J Food Eng 2007; 79(2): 736-40.

[188] Jeon Y, Kamil J, Shahidi F. Chitosan as an edible invisible film for quality preservation of herring and Atlantic cod. J Agric Food Chem 2002; 50(18): 5167-78.

[189] Kim S, No H, Prinyawiwatkul W. Effect of molecular weight, type of chitosan, and chitosan solution pH on the shelf-life and quality of coated eggs. J Food Sci 2007; 72 (1): S044-8.

[190] Schulz P, Rodríguez M, Del Blanco L, Pistonesi M, Agulló E. Emulsification properties of chitosan. Coll Polym Sci 1998; 276(12): 1159-65.

[191] Rodriguez MS, Albertengo LA, Agullo E. Emulsification capacity of chitosan. Carbohydr Polym 2002; 48(3): 271-6.

[192] Laplante S, Turgeon SL, Paquin P. Emulsion stabilizing properties of various chitosans in the presence of whey protein isolate. Carbohydr Polym 2005; 59(4): 425-34.

[193] Aoki T, Decker E, McClements D. Influence of environmental stresses on stability of O/W emulsions containing droplets stabilized by multilayered membranes produced by a layer-by-layer electrostatic deposition technique. Food Hydrocolloid 2005; 19(2): 209-20.

[194] Mun S, Decker EA, McClements DJ. Effect of molecular weight and degree of deacetylation of chitosan on the formation of oil-in-water emulsions stabilized by surfactant-chitosan membranes. J Colloid Interface Sci 2006; 296(2): 581-90.

[195] Price NC, Stevens L. The preparation and kinetics of immobilised enzymes. Fundamentals of enzymology, 2nd Edition: Oxford University Press 1990: 81-137.

[196] Bianchi G, Setti L, Spagna G, Pifferi PG. In: Wijffelds R, Buitelaar RM, Tramper J, Eds, Rhodococcus sp. immobilized by adsorption on chitin. Immobilized cells: Basics and Applications: Elseviere Science. 1996: 126-31.

[197] Strand SP, Varum KM, Ostgaard K. Interactions between chitosans and bacterial suspensions: Adsorption and flocculation Colloid Surface B 2003; 27(1): 71-81.

[198] Ohashi E. Microorganism immobilization in a beta-chitin carrier. Biotechnol Adv 1995; 13: 287-8.

[199] Martin A-B, Picciolato M, Heras A. In: Ballesteros A, Plou F-J, Iborra J, Halling P-J, Eds. Coimmobilization of enzymes and cells on chitin and derivatives. Progress in Biotechnology: Stability and Stabilization of Biocatalysts. Amsterdam, Elsevier 1998: 679-84.

[200] Setti L, Lanzarini G, Pifferi PG. In: Wijffelds R, Buitelaar RM, Tramper J, Bucke C, Eds. Immobilized cells for applications in non-conventional systems. Immobilized cells: Basics and Applications: Elseviere Science 1996.

[201] Feng-Ming H, Huang C, Lin TF, Chen YM, Lin JC. Study of sodium tripolyphosphate-crosslinked chitosan beads entrapped with Pseudomonas putida for phenol degradation. Proc Biochem 2008; 43: 83-92.

[202] Fierro S, Sánchez-Saavedra MP, Copalcua C. Nitrate and phosphate removal by chitosan immobilized Scenedesmus. Bioresource Technol 2008; 99: 1274–9.

[203] Long DD, Luyen DV. Chitosan-carboxymethylcellulose hydrogels as supports for cell immobilization. Pure Appl Chem 1996; 12: 1875-84.

[204] Lia GY, Huang KL, Jiang YR, Ding P. Production of (R)-mandelic acid by immobilized cells of Saccharomyces cerevisiae on chitosan carrier. Proc Biochem 2007; 42(10): 1465-0.

[205] Yu SH, Buchholz R, Kim SK. Encapsulation of rat hepatocyte spheroids for the development of artificial lliver. Biotechnol Tech 1999; 13: 609-14.

[206] Lee SL, Cheng HY, Chen CW, Chou CC. Production of Y-decalactone from ricinoleic acid by immobilized cells of Sporidiobolus salmonicolor. Proc Biochem 1998; 33: 453-9.

[207] Krajewska B. Chitin and its Derivatives as Supports for Immobilization of Enzymes. Acta Biotechnol 1991; 11 (3): 269-77.

[208] Krajewska B. Application of chitin and chitosan based materials for enzyme immobilizations : a review. Enzyme Microb Technol 2004; 35: 126-39.

[209] Muzzarelli R. Immobilization of enzymes on chitin and chitosan. Enzyme Microb Technol 1980; 2: 177-84.

[210] Hackmam R. Chitin III. Adsorption of proteins to Chitin. Aus J Biol Sci 1955; 8: 530-6.

[211] Aranaz I. Preparation and characterization of immoblized derivatives of Agrobacterium radiobacter extract on chitin and chitosan. Thesis dissertation. Madrid: Univeridad Complutense; 2005.

[212] Aranaz I, acosta N, Heras A. Encapsulation of an Agrobacterium radiobacter extract containing D-hydantoinase and D-carboamylase activities into alginate-chitosan polyelectrolyte complexes. Preparation of the biocatalyst. J Mol Catal B: Enzym. DOI: 10.1016/j.molcabt.2008.11.006. In press.

[213] Aranaz I, Ramos V, De la Escalera S, Heras A. Coimmobilization of D-Hydantoinase and D-Carboamylase on chitin: application to the synthesis of p-hydroxyphenylglycine. Biocatal Biotrans 2003; 21: 349-56.

[214] Durham D-R, Weber J-E. Properties of D-hydantoinase from Agrobacterium tumefaciens and its use for the preparation of N-carbamyl D-amino acids. Biochem Biophys Res Commun 1995; 216(3): 1095-100.

[215] Louwrier A, Knowles CJ. The purification and characterization of a novel D(-)specific carbamoylase enzyme from an Agrobacterium sp. Enzyme Microb Technol 1996; 19: 562-71.

[216] Fernandesa S, Oliveira I, Vieira I. A green bean homogenate immobilized on chemically crosslinked chitin for determination of caffeic acid in white wine. Enzyme Microb Technol 2007; 40 (4): 661-8.

[217] Synowiecki J, Sikorski Z, Naczk M. The activiy of immobilized enzymes on different krill chitin preparations. Biotechnol Bioeng 1981; 23(10): 2211-5.

[218] Heras A, Acosta N. Proteases immobilized on chitin: Aplications to peptide synthesis in aqueous organic media. In: Muzzarelli R, Ed. Chitin enzymology: Eur Chitin Soc 1993: 453-60.

[219] Heras A, Acosta N. alpha-chymotrysin immobilized on chitin. Relatioships between the enzyme kinetic constant and support structure. Biocatalysis 1994; 11: 305-13.

[220] Rhazi MJ, Desbrières A, Tolaimate AA, Vottero P. Investigation of different natural sources of chitin: influence of the source and deacetylation process on the physicochemical characteristics of chitosan. Polym Int 2000; 49: 337-44.

[221] Synowiecki J, Sikorska-siondalska A, El-Bedawey A. Adsorption of enzymes on krill chitin modified with carbon disulfide. Biotechnol Bioeng 1989; 29: 352-4.

[222] Ohashi E, Koriyama T. Simple and mild preparation of an enzyme-immobilized membrane for a biosensor using β -type crystalline chitin. Anal Chim Acta 1992; 262: 19-25.

[223] Gary Delanoya QLaJY. Activity and stability of laccase in conjugation with chitosan. I J Biol Macromol 2007; 35(1-2): 89-95.

[224] Amorim RVS, Melo ES, Carneiro-da Cunha MG, Ledingham WM, Campos-Takaki GM. Chitosan from Syncephalastrum racemosum used as a film support for lipase immobilization. Bioresource Technol 2003; 89: 35-9.

[225] Alsarra IA, Betigeri SS, Zhang H, Evans Ba, Neau SH. Molecular weight and degree of deacetylation effects on lipase-loaded chitosan bead characteristics. Biomaterials 2002; 23: 3637-44.

[226] Munjal N, Sawhney SK. Stability and properties of mushroom tyrosinase entrapped in alginate, polyacrylamide and gelatin gels. Enzyme Microb Technol 2002; 30(5): 613-9.

[227] Taqieddin E, Amiji M. Enzyme immobilization in novel alginate-chitosan core-shell microcapsules. Biomaterials 2004; 25: 1937-45.

[228] DeGroot AR, Neufeld RJ. Encapsulation of urease in alginate beads and protection from alpha-chymotrypsin with chitosan membranes. Enzyme Microb Technol 2001; 29: 321-7.

[229] Simsek-Ege FA, Bond GM, Stringer J. Matrix molecular weigth cut-off for encapsulation of carbonic anhydrase in polyelectrolyte beads. J Biomat Sci Polym E 2002; 13: 1175-87.

[230] Boadi DK, Neufeld RJ. Encapsulation of tannase for the hydrolysis of tea tannins. Enzyme Microb Technol 2001; 28: 590-5.

[231] Crini G. Recent developments in polysaccharide-based materials used as adsorbents in wastewater treatment. Prog Polym Sci 2005; 30(1): 38-70.

[232] No HK, Meyers SP. Application of chitosan for treatment of wastewaters. Rev Environ Contam Toxicol 2000; 163: 1-28.

[233] Guibal E. Interactions of metal ions with chitosan-based sorbents: A review. Sep Purif Technol 2004; 38(1): 43-74.

[234] Guibal E, Van Vooren M, Dempsey BA, Roussy J. A review of the use of chitosan for the removal of particulate and dissolved contaminants. Sep Sci Technol 2006; 41(11): 2487-514.

[235] Roussy J, Van Vooren M, Dempsey BA, Guibal E. Influence of chitosan characteristics on the coagulation and the flocculation of bentonite suspensions. Water Res 2005; 39(14): 3247-58.

[236] Crini G, Badot P. Application of chitosan, a natural aminopolysaccharide, for dye removal from aqueous solutions by adsorption processes using batch studies: A review of recent literature. Prog Polym Sci 2008; 33: 399-447.

[237] Muzzarelli R, Tuberni O. Chitin and chitosan as chromatographic supports and adsrobents for collection of metal ions from organic and aqueous solutions and sea-water. Talanta 1969; 71: 1571-7.

[238] Thomé JP, Van Daele Y. Adsorption of polychlorinated biphenyls (PCB) on chitosan and application to decontaminated of polluted stream waters. In: Muzzarelli RAA, Jeuniaux C, Gooday GW, Eds. Proceedings of the third International Conference on chitin and chitosan. Senigallia 1986: 551-4.

[239] Crisafully R, Milhome MAL, Cavalcante RM, Silveira ER, De Keufeleire D, Nascimiento RF. Removal of some polycyclic aromatic hydrocarbons frompetrochemical wastewater using low-cost adsorbents of natural origin. Biores Technol 2008; 99: 4515-9.

[240] Saha TK, Karmaker S, Ichikawa H, Fukumori Y. Mechanisms and kinetics of trisodium 2-hydroxy-1,1-azonaphthalene-3,4,6-trisulfonate adsorption onto chitosan. J Colloid Interf Sci 2005; 286: 433-9.

[241] Zang H, Schiewew S. Arsenic (V) sorption on crab shell based chitosan. Proceedings of the 2005 World Water and Environmental Resources Congress; 2005; 2005. p. 296.

[242] Wong YC, Szeto YS, Cheung WH, McKay G. Effect of temperature, particle size and percentage deacetylation on the adsorption of acid dyes on chitosan. Adsorption 2008; 14(1): 11-20.

[243] kurita K, Sanna T, Iwakura Y. Studies on chitin VI. Binding of metal cations. J Appl Polym Sci 1979; 23: 511-5.

[244] Huang C, Chen S, Ruhsing Pan J. Optimal condition for modification of chitosan: A biopolymer for coagulation of colloidal particles. Water Res 2000; 34(3): 1057-62.

[245] Bough WA, Landes DR. Treatment of food processing wastes with chitosan and nutritional evaluation of coagulated by-products. In: Muzzarelli RAA, Pariser ER, Eds. Proceedings of the First International Conference on Chitin and Chitoswan. Cambridge: MIT SEA Grant Program 1978: 218-30.

[246] Chung YC. Improvement of aquaculture wastewater using chitosan of different degrees of deacetylation Environ Technol 2006; 27: 1199-208.

[247] Wibowo S, Velazquez G, Savant V, Torres JA. Effect of chitosan type on protein and water recovery efficiency from surimi wash water treated with chitosan-alginate complexes. Biores Technol 2007; 98: 539-45.

[248] Aburto J, Mendez-Orozco A, Borgne SL. Hydrogels as adsorbents of organosulphur compounds currently found in diesel. Chem Eng Process 2004; 43(12): 1587-95.

[249] Espinosa-García BM, Argüelles-Monal WM, Hernández J, Félix-Valenzuela L, Acosta N, Goycoolea FM. Molecularly imprinted Chitosan - Genipin hydrogels with recognition capacity toward o-Xylene. Biomacromolecules 2007; 8(11): 3355-64.

[250] Yu Q, Deng S, Yu G. Selective removal of perfluorooctane sulfonate from aqueous solution using chitosan-based molecularly imprinted polymer adsorbents. Water Res 2008; 42 (12): 3089-97.

[251] Guo TY, Xia YQ, Hao GJ, Song MD, Zhang BH. Adsorptive separation of hemoglobin by molecularly imprinted chitosan beads. Biomaterials 2004; 25(27): 5905-12.

[252] Guo T-Y, Xia Y-Q, Wang J, Song M-D, Zhang B-H. Chitosan beads as molecularly imprinted polymer matrix for selective separation of proteins. Biomaterials 2005; 26(28): 5737-45.

[253] Li F, Li J, Zhang S. Molecularly imprinted polymer grafted on polysaccharide microsphere surface by the sol-gel process for protein recognition. Talanta 2008; 74(5): 1247-55.

[254] Xi F, Wu J, Lin X. Novel nylon-supported organic-inorganic hybrid membrane with hierarchical pores as a potential immobilized metal affinity adsorbent. J Chromatogr A 2006; 1125(1): 38-51.

[255] Fu G, Zhao J, Yu H, Liu L, He B. Bovine serum albumin-imprinted polymer gels prepared by graft copolymerization of acrylamide on chitosan. React Funct Polym 2007; 67(5): 442-50.

[256] Xia YQ, Guo TY, Song MD, Zhang BH, Zhang BL. Hemoglobin recognition by imprinting in semi-interpenetrating polymer network hydrogel based on polyacrylamide and chitosan. Biomacromolecules 2005; 6(5): 2601-6.

[257] Li Q, Su H, Tan T. Synthesis of ion-imprinted chitosan-TiO2 adsorbent and its multi-functional performances. Biochem Eng J 2008; 38(2): 212-8.

[258] Su H, Wang Z, Tan T. Preparation of a surface molecular-imprinted adsorbent for Ni2+ based on Penicillium chrysogenum. J Chem Technol Biot 2005; 80(4): 439-44.

[259] Su H, Li Q, Tan T. Double-functional characteristics of a surface molecular imprinted adsorbent with immobilization of nano-TiO2. J Chem Technol Biot 2006; 81(11): 1797-802.

[260] Tianwei T, Xiaojing H, Weixia D. Adsorption behaviour of metal ions on imprinted chitosan resin. J Chem Technol Biot 2001; 76(2): 191-5.

[261] Li F, Du P, Chen W, Zhang S. Preparation of silica-supported porous sorbent for heavy metal ions removal in wastewater treatment by organic-inorganic hybridization combined with sucrose and polyethylene glycol imprinting. Anal Chim Acta 2007; 585(2): 211-8.

[262] Li Q, Su H, Li J, Tan T. Studies of adsorption for heavy metal ions and degradation of methyl orange based on the surface of ion-imprinted adsorbent. Process Biochem 2007; 42(3): 379-83.

[263] Li F, Jiang H, Zhang S. An ion-imprinted silica-supported organic-inorganic hybrid sorbent prepared by a surface imprinting technique combined with a polysaccharide incorporated sol-gel process for selective separation of cadmium(II) from aqueous solution. Talanta 2007; 71(4): 1487-93.

[264] Xia YQ, Guo TY, Song MD, Zhang BH, Zhang BL. Selective separation of quercetin by molecular imprinting using chitosan beads as functional matrix. React Funct Polym 2006; 66(12): 1734-40.

[265] Jiang Z, Yu Y, Wu H. Preparation of CS/GPTMS hybrid molecularly imprinted membrane for efficient chiral resolution of phenylalanine isomers. J Membr Sci 2006; 280(1-2): 876-82.

[266] Huang H, Yuan Q, Yang X. Preparation and characterization of metal-chitosan nanocomposites. Colloid Surface B 2004; 39(1-2): 31-7.

[267] Huang H, Yang X. Synthesis of polysaccharide-stabilized gold and silver nanoparticles: a green method. Carbohydr Res 2004; 339: 2627-31.

[268] Twu YK, Chen YW, Shih CM. Preparation of silver nanoparticles using chitosan suspensions. Powder Technol 2008; 185(2): 251-57.

[269] Murugadoss A, Chattopadhyay A. A 'green' chitosan-silver nanoparticle composite as a heterogeneous as well as micro-heterogeneous catalyst. Nanotechnology 2008; 19: 1-9.

[270] Wei D, Qian W. Facile synthesis of Ag and Au nanoparticles utilizing chitosan as a mediator agent. Colloid Surface B 2008; 62(1): 136-42.

[271] Chen P, Song L, Liu Y, Fang Y-e. Synthesis of silver nanoparticles by [gamma]-ray irradiation in acetic water solution containing chitosan. Radiat Phys Chem 2007; 76(7): 1165-8.

[272] Wu L, Shi C, Tian L, Zhu J. A One-Pot Method to Prepare Gold Nanoparticle Chains with Chitosan. J Phys Chem C 2008; 112: 319-23.

[273] Yang K, Wang X, Zhou Z, Xu J, Weng J, Zhang Q. One-step synthesis and characterisation of chitosan-mediated micro-sized gold nanoplates through a thermal process. IET Nanobiotechnology 2007; 1(6): 107-11.

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