5

Characterization and Properties of Chitosan

Elson Santiago de Alvarenga
Universidade Federal de Viçosa, Departamento de Química, Viçosa, MG, Brazil

1. Introduction

The biopolymer is characterized as either chitin or chitosan according to the degree of deacetylation (DD) which is determined by the proportion of D-glucosamine and N-acetyl-D-glucosamine. Structurally, chitosan is a straight-chain copolymer composed of D-glucosamine and N-acetyl-D-glucosamine being obtained by the partial deacetylation of chitin. Chitosan is the most abundant basic biopolymer and is structurally similar to cellulose, which is composed of only one monomer of glucose (Fig. 1). Chitosan solubility, biodegradability, reactivity, and adsorption of many substrates depend on the amount of protonated amino groups in the polymeric chain, therefore on the proportion of acetylated and non-acetylated D-glucosamine units. The amino groups (pKa from 6.2 to 7.0) are completely protonated in acids with pKa smaller than 6.2 making chitosan soluble. Chitosan is insoluble in water, organic solvents and aqueous bases and it is soluble after stirring in acids such as acetic, nitric, hydrochloric, perchloric and phosphoric (Guibal, 2004; Kluget al., 1998; Kubota et al., 2000; Kurita, 2006; Anthonsen & Smidsroed, 1995; Rinaudo, 2006; Sankararamakrishnan & Sanghi, 2006).

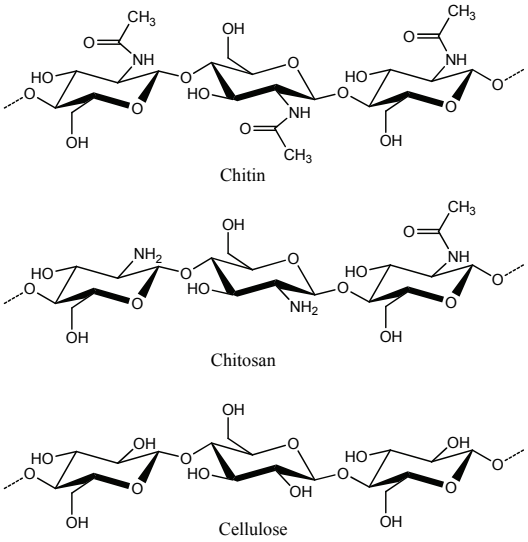

Fig. 1. Structures of chitin, chitosan, and cellulose

Food contaminations and the negative environmental impact of packaging materials currently in use have prompted the research of safer materials. Chitosan have shown great promise to be used in food industry as preservative because of its high antimicrobial activity against various microorganisms. The quality of a variety of food products are maintained by chitosan based films (Dutta et al., 2009).

Chitosan is considered one of the most valuable polymer for biomedical and pharmaceutical applications due to its biodegradability, biocompatibility, antimicrobial, non-toxicity, and anti-tumor properties. Nanoparticles, microspheres, hydrogels, films, and fibers are typical chitosan based forms for biomedical and pharmaceutical applications. Examples of such applications include nasal, ocular, oral, parenteral and transdermal drug delivery (Kumar, 2000).

The science for creating three dimensional body parts by seeding cellular material in vitro on a scaffold, to replace diseased body parts is defined as tissue engineering. The scaffold should be biodegradable and elastic for contractile tissues such as blood vessels and heart valves. It is therefore necessary to develop tissue like matrix with interconnected network, to act as templates to guide cell growth, transfer of nutrients, oxygen and waste products (Kathuria et al., 2009).

Cross-linked chitosan hydrogels as potential tissue engineering scaffold can be prepared by Schiff base reaction between the N-succinyl-chitosan and the aldehyde group from the oxidized hyaluronic acid. Encapsulation of bovine chondrocytes within the cross-linked hydrogel demonstrated that the composite hydrogel supported cell survival and the cells retained chondrocytic morphology. Hydrogels are attractive candidates for tissue regeneration due to its extracellular matrix mimic structure and the ability to form under mild conditions (Tan et al., 2009).

Chitosan microspheres as delivery drug carriers can be prepared by the water-in-oil emulsion solvent diffusion method, being ethyl acetate the oil phase. Figure 2 displays Scanning Electron Microscopy (SEM) image of the chitosan microspheres with drug entrapment (Phromsopha & Baimark, 2010).

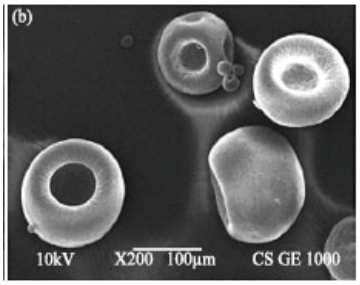

Fig. 2. Scanning Electron Microscopy (SEM) of drug-loaded chitosan microparticles (reproduced from Phromsopha & Baimark, 2010).

Chitosan has been largely employed in many areas, such as photography, biotechnology, cosmetics, food processing, biomedical products (artificial skin, wound dressing, contact lens, etc.), system of controlled liberation of medicines (capsules and microcapsules), treatment of industrial effluents for removal of metallic and coloring ions. The amino groups are responsible for the distinct characteristics attributed to this basic polymer (compared to an acidic biopolymer). Therefore, the characterization of the polymer in either chitin or

chitosan is extremely important according to the structure-properties relationship, defining a possible industrial application. Thus many techniques are available to determine the degree of deacetylation, such as:

- Elemental analysis (Davies & Hayes, 1988; dos Santos et al., 2009);
- Titration (Arcidiacono & Kaplan, 1992; Balázs & Sipos, 2007; de Alvarenga et al., 2010; Hattori et al., 1999; Park et al., 1983; Raymond et al., 1993; Tôei & Kohara, 1976; Zhanga et al., 2011);
- Hydrolytic methods (Davies & Hayes, 1988; Nanjo et al., 1991; Niola et al., 1993; Sato et al., 1998; Zamani et al., 2008);
- HPLC - Ultraviolet (Aiba, 1986; Muzzarelli & Rocchetti, 1985);
- Infrared (Baxter et al., 1992; Duarte et al., 2002; Kasaai, 2008; Miya et al., 1980; Moore & Roberts, 1980; Sannan et al., 1978);
- ^{1}H nuclear magnetic resonance (Brugnerotto et al., 2001; de Alvarenga et al., 2010; Fernandez-Megia et al., 2005; Lavertu et al., 2003; Varum et al., 1991);
- CP-MAS ^{13}C NMR (Heux et al., 2000; Manni et al., 2010; Raymond et al., 1993);
- CP-MAS ^{15}N NMR (Kasaai, 2009; Yu et al., 1999);
- Many other methods are described in the literature but with a somewhat smaller appeal. Some of these methods are: steric exclusion chromatography (Brugnerotto et al., 2001), nitrous acid deamination (Sashiwa et al., 1991), thermal analysis (Garcia Alonso et al., 1983), gas chromatography with columns packed with chitin and chitosan (Muzzarelli et al., 1980), etc.

2. Elemental analysis

A known amount of chitosan is heated for 1 h at 600ºC, and the residue is weighed to find the quantity of inorganic material. The percentages of nitrogen in fully deacetylated chitosan (8.695), in fully acetylated chitin (6.896), and in the organic fraction of the analyzed material (%N) are related to the DA by formula (1).

$$\%DA = \frac{(8.695 - \%N)}{8.695 - 6.896} \cdot 100 \quad (1)$$

Samples with varying DA (degree of acetylation) present relatively small variations in nitrogen content, thus results obtained by elemental analysis (EA) are not precise, especially if contaminants are present. This technique was used to define chitosan as having a nitrogen content of more than 7% and chitin with less than 7% nitrogen (Davies & Hayes, 1988; dos Santos et al., 2009).

3. Titration methods

3.1 Acid-base titration

A known amount of chitosan was solubilized in 0.1 N HCl with gentle shaking overnight. Titration with 0.1 N NaOH was performed on an automatic titration system. D-glucosamine and N-acetyl-D- glucosamine were used as 100% and 0% deacetylated controls, respectively. The DA is determined from first and second inflection points of the titration curve. Some samples presented large standard deviations possibly due to solution viscosity and precipitate formation (Arcidiacono & Kaplan, 1992; Park et al., 1983).

3.2 Potentiometric titration

Chitosan (ca. 100 mg) is dissolved in a known volume of aqueous HCl (0.010 mol L^{-1}) and the solution is then titrated with 0.1 mol L^{-1} NaOH, while the pH of the solution is measured at constant ionic strength (0.1 mol L^{-1} NaCl). The graph with the variation of pH versus the added volume of base has two inflexion points: the first corresponds to neutralization of HCl, and the second to neutralization of the ammonium ions from chitosan. The difference between the two inflexion points gives the amount of amino groups in chitosan (degree of deacetylation, DD). The degree of acetylation (DA) is obtained from formula (2):

$$\%DA = 100 - \%DD \tag{2}$$

This method presented some difficulties such as: The chitosan samples have to be purified and dried prior to measurements. The moisture and ash contents have to be determined and the weighed chitosan mass corrected. Low grade samples are not adequately measured by potentiometric titration (Balázs & Sipos, 2007; Zhanga et al., 2011).

3.3 Colloid titration

The principle of this method is based on a stoichiometric combination between positive and negative ions. In colloid titration, normality of the titer solution is defined by the number of equivalents of the dissociable groups of the polymer in 1 liter of solution.

A chitosan sample (1 a 10 x 10^{-5} mol) was dissolved in aqueous acetic acid (5 mL, 3 mol^{-1}) and diluted with deionized water (100 mL). The solution was titrated with potassium poly(vinyl sulfate) (PVS, 2.5 x 10^{-3} mol L^{-1}) using 3-amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride (toluidine blue, 1% m/v solution) as indicator. The positive ammonium groups of the protonated chitosan were directly bound to negatively charged sulfate groups of PVS. After the equivalent point is reached a minute excess of PVS binds to toluidine blue and the color change from blue to red.

This method presented some difficulties such as: The PVS had to be purified by dialysis before use and its solution had to be padronized by acid base titration. The chitosan samples suspended in aqueous acid had to be filtered to remove insoluble matter and the resulting solution had to be purified by dialysis. A clear change of color was observed for some chitosan samples and the end-point for other samples was determined only by precipitation. However neither change of color nor precipitation was observed for low DA chitosan samples. The low stability constant of the chitosan-PVS complex was attributed as the cause for the discrepant results (Hattori et al., 1999; Tôei & Kohara, 1976).

3.4 Conductometric titration

Conductimetry is the measurement of a solution conductance. During conductometric titration the solution electrical conductivity is constantly measured while the chemical reactions occur. $H^+$and OH^- contribute greatly for the solution conductance as these are the most conductive ions. The chitosan samples (0.2000 g) were dissolved in HCl (54.00 x 10^{-3} mol L^{-1}, 40.00 mL) and titrated with portions of 0.500 mL of NaOH (165.0 x 10^{-3}mol L^{-1}) in 20 sec. interval.

The values of conductance (mS cm^{-1}) with the corresponding titrant volumes were plotted in a graphic to find the linear variation before and after the equivalence point (Fig. 3). Three line segments are observed. The first rapid descending branch corresponds to neutralization of HCl in excess (A–B), the second segment refers to neutralization of the ammonium group (B–C) and the third to the excess of base (D). The two stoichiometry points are found by intersection of the three lines and the difference between the two points corresponds to the volume of base required to neutralize the ammonium groups (de Alvarenga et al., 2010).

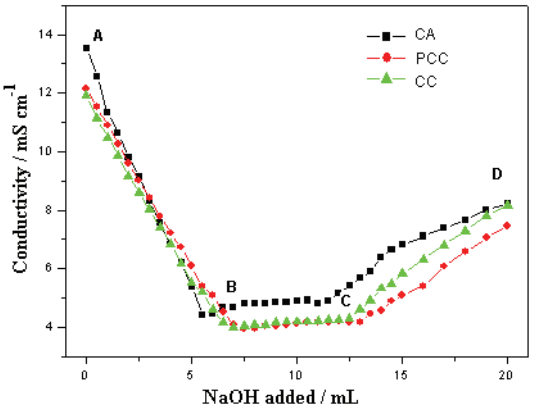

Fig. 3. Conductometric titration of three chitosan samples (reproduced from de Alvarenga et al., 2010).

The percentage of amino groups represented by the degree of deacetylation (DD) was calculated using formula (3):

$$\%DD = \frac{[base](V_2 - V_1)161}{m} \tag{3}$$

where [base] is the concentration of the NaOH solution (in mol L^{-1}), V_1 and V_2 are the volume of NaOH (in mL) used in the titration, 161 is the molar mass of the monomer ($C_6H_{11}O_4N$) and m is the mass of chitosan (in mg).
The content of D-glucosamine hydrochloride (DG-HCl) and N-acetyl-D-glucosamine (NADG) mixtures determined by conductimetry were coincident with their known concentration even for high NADG content. Thus high DA is not a barrier for conductimetry. However this method is not applicable to chitosan with high DA due to its low solubility. The difficulty presented by this method is that the sample has to be soluble and must be well dried prior to analysis.

4. Hydrolytic methods

4.1 Acid hydrolysis, distillation and titration

A known amount of chitosan was hydrolyzed with sodium hydroxide and acidified with phosphoric acid to convert the salt to acetic acid. The aqueous acetic acid is distilled, and when the distilling flask begins to go dry, 15 mL of hot distilled water was added to the flask. Aliquots of 25 mL were titrated with 0.01 N sodium hydroxide using phenolphthalein as indicator. The volume of base was multiplied by ten to give the total volume of the distillate (250 mL). The DA was determined from formula (4):

$$\%DA = \frac{V\ x\ 0.04305}{m} \tag{4}$$

where V is the volume of sodium hydroxide multiplied by ten and m the mass of chitosan (Davies & Hayes, 1988).

The drawback of this method is that it requires many tedious manipulations such as sample drying, hydrolysis with base, acidification, azeotropic distillation, and titration. However no expensive apparatus and reagents difficult to acquire are necessary for this method.

4.2 Acid hydrolysis - HPLC

A Varian Model 5000 equipped with 300 x 7.8 mm cation-exchange resin column and UV detector was employed for the HPLC measurements. The finely powdered polymer was hydrolyzed with aqueous sulfuric, oxalic and propionic acids at 155ºC for 1 hour. After cooling for 2 hours the mixture was filtered and 10 μL was injected in the HPLC. The detection of the carboxylic acids was carried out with a UV detector at 210 nm. The calibration curve was made with various dilutions of acetic acid and the DA was calculated according to formula (5).

$$\%DA = \frac{161M}{43 - 42M} \cdot 100 \tag{5}$$

Where M is the amount of acetyl groups liberated by acid hydrolysis divided by the mass of organic material (mass of analyzed material discounted the inorganic matter); 161 is the molar mass of 2-amino-2-deoxy-D-glucose; and 43 the molar mass of the acetyl group. M is calculated from the areas of the acetic acid (A_{Ac}) and the internal standard (A_{IS}) peaks and from the mass of propionic acid (M_{IS}) according to formula (6). K is the response factor.

$$M = K \cdot \frac{A_{Ac}}{A_{IS}} \cdot M_{IS} \tag{6}$$

The HPLC chromatogram of the chitosan reaction mixture is shown in Fig.4. Formic acid is formed by hydrolysis of oxalic acid (peak 1), acetic acid (2) is liberated from the hydrolysis of chitosan, and propionic acid (3) is the internal standard.

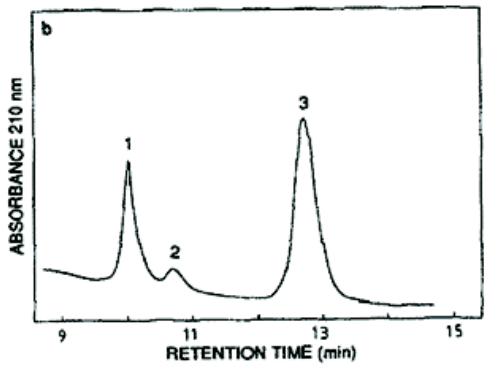

Fig. 4. HPLC of the reaction mixture of chitosan: (1) formic acid, (2) acetic acid, and (3) propionic acid (reproduced from Niola et al., 1993).

4.2.1 Drawbacks

- A calibration curve with various concentrations of acetic acid is required.
- The content of water and inorganic material in the sample must be determined prior to analysis.

- A long analysis time is required even after calibration and sample preparation.
- Sample degradation due to the high temperature employed for the hydrolysis.
- Separation of the acetic acid from the other carboxylic acids is not very trivial. A suitable HPLC column is required.
- The hydrolytic conditions employed degrade the HPLC column life time.

4.2.2 Advantages

- This method is valid over the complete range of DA.
- The HPLC equipment is quite affordable compared to solid state NMR.
- Insoluble chitin samples can be analyzed by this method (Niola et al.,1993; Zamani et al., 2008).

4.3 Enzymatic hydrolysis – colorimetry - HPLC

A Waters HPLC equipped with C-R3A integrator, column YMC-Pack PA-03(4.6 x 250 mm) and R-410 differential refractometer detector was employed in this technique.
Chitosan was hydrolyzed by cooperative action of exo-β-D-glucosaminidase, β-N-acetylhexosaminidase, and chitosanase and the amounts of D-glucosamine and N-acetyl-D-glucosamine were determined by colorimetry and HPLC. The analysis by HPLC was carried out on 0.2 mL solution of the aqueous hydrolysate. The peak area was converted into a molar concentration with standard curves obtained with authentic D-glucosamine and N-acetyl-D-glucosamine. Fig. 5 shows the HPLC chromatograms of the standards and of hydrolyzed chitosan.

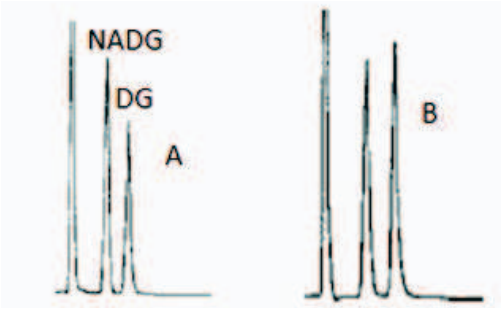

Fig. 5. HPLC chromatograms of: (A) Standard D-glucosamine (DG) and N-acetyl-D-glucosamine(NADG); B) Products of hydrolysis of chitosan (reproduced from Nanjo et al., 1991).

The degree of deacetylation (DD) was calculated according to formula 7:

$$\%DD = 100xDG / (DG + NADG) \tag{7}$$

Where DG and NADG are the concentration in μmol/mL of D-glucosamine and of N-acetyl-D-glucosamine, respectively. These concentrations are obtained from the HPLC peak areas.

4.3.1 Drawbacks

- Standard curves with authentic samples of D-glucosamine and N-acetyl-D-glucosamine are required.

- A longer analysis time is required for the colorimetric than the HPLC method.
- The HPLC results are affected by the presence of impurities in the enzyme extracts.
- The reaction conditions must be carefully controlled to ensure complete hydrolysis of chitosan (especially for distinct enzymes).
- These methods are valid only for samples soluble in 2% aqueous acetic acid.

4.3.2 Advantages

- The HPLC equipment is quite affordable compared to solid state NMR.
- No expensive apparatus and reagents are necessary for the colorimetric method.
- The HPLC column from the enzymatic method possess a longer life time than the HPLC column from the acid hydrolysis method (4.2) (Nanjo et al., 1991).

4.4 Pyrolysis – gas chromatography

A gas chromatograph equipped with a flame ionization detector (FID) and a vertical microfurnace pyrolyzer were used to characterize chitosan. GC / mass spectrometer was used to identify the fragments. The chitosan sample (ca. 50 μg) suspended in aqueous oxalic acid (3 μL, 1.0 mol^{-1}) in a platinum cup was heated at 450 ^{o}C in pyrolyzer under helium atmosphere. The flow rate of 50 mL/min of carrier gas at the pyrolyzer was reduced to 1.0 mL/min at the capillary column by means of a splitter. A metal capillary column coated with immobilized poly(ethylene glycol) (PEG) (0.25 μm thickness) was used. The column temperature was initially set at 35 °C for 5 min and then heated to 220 °C at a rate of 5 °C/min.

N-acetyl-D-glucosamine (NADG) was employed as standard to calculate the degree of acetylation (DA) of chitosan. This assumption is correct only if the characteristic products (acetonitrile, acetic acid, and acetamide) are quantitatively formed from both chitosan and NADG (Sato et al., 1998).

4.4.1 Drawbacks

- DA is overestimated by carbohydrate contamination.
- Excess of oxalic acid alters the DA.
- Life time of GC column is shortened by acid.
- The sample is destroyed by pyrolysis (destructive method).

4.4.2 Advantages

- This method can be used for entire range of DA.
- Very small amount of sample needed for analysis.
- Very short analysis time.
- Adequate for routine analysis.

5. Spectrometric methods

5.1 HPLC - ultraviolet spectroscopy

A Shimadzu LC-3A equipped with an integrator C-R1A, column Shimpack DIOL-300 and UV detector was employed for the gel permeation chromatography (GPC) measurements. Characterized chitosan samples (C-1, 2, 3, 4, 5, and 6) were employed as standards to determine the DA of the unknown polymers. The chitosan standards (C-2, 3, 4, 5, and 6)

were prepared by hydrolysis of sample C-1. The NADG was not used as standard because its concentration was not matched with the peak areas. The DA could be estimated because the UV absorption peak areas of the acetamide groups were proportional to the concentration of the chitosan samples (Fig. 6).

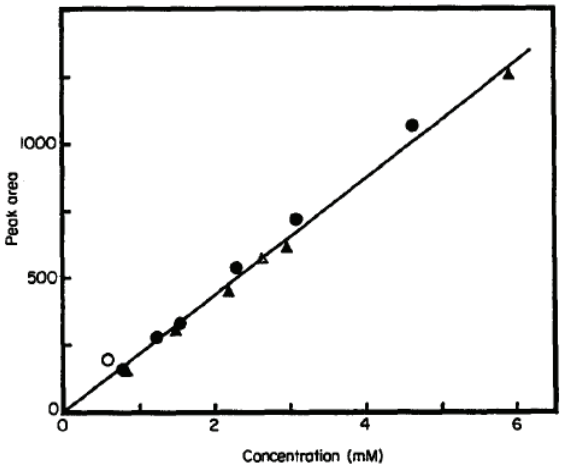

Fig. 6. Relationship between peak areas and concentration of acetamide groups of four different chitosan samples.o, C-1 chitosan; •, C-2 chitosan; Δ, C-4 chitosan; ▲, C-5 chitosan (reproduced from Aiba, 1986).

The GPC can be applied also for following chitosan reactions with low molecular weight reagents as the latter possess different retention time from chitosan and laborious purification of the chitosan derivative is avoided.
The results of the GPC method were reasonably coincident for 50% of the samples with the results obtained by colloid titration and IR.

5.1.1 Drawbacks

- Standard curves with characterized chitosan samples are required.
- Chitosan standards with varying DA have to be prepared and purified.
- This method is valid only for samples soluble in aqueous acetic acid.
- The DA obtained by GPC, colloid titration, and IR were coincident for only 50% of the samples evaluated.

5.1.2 Advantages

- The HPLC equipment is quite affordable compared to solid state NMR.
- Chitosan reactions with low molecular weight reagents can be accompanied by GPC.
- Chitosan molecular weight can be determined by GPC (Aiba, 1986).

5.2 Infrared spectroscopy

Infrared absorption spectroscopy can be employed in quantitative analyses and structure determination of compounds. The fact of certain groups of atoms presenting bands at or near the same frequency and the unique IR fingerprint of molecules allow the chemists, with

the aid of other technics, elucidate the structure of a compound. The DA is calculated by formula 8, where A_{1655} and A_{3450} are the absorbance of bands at 1655 and 3450 cm^{-1} respectively.

$$DA = (A_{1655} / A_{3450}) \times 115 \quad (8)$$

This relationship is valid for samples with DA up to 55%. The amount of sample in the beam must be small enough to ensure that the 3450 cm^{-1} band has a transmission of at least 10%. Samples prepared by N-acetylation of chitosan, must be kept in 0.5 mol^{-1} ethanolic KOH prior to recording the spectrum, to hydrolyze the esters.
The IR spectra can be obtained with KBr disk or as film. The disk is prepared by pressing the sample mixed with dry KBr. A film of aqueous acetic acid solution of chitosan is washed with methanolic ammonia, distilled water and methanol, and dried overnight in vacuum desiccator to afford the chitosan film. Fig. 7 shows the IR spectrum of chitosan and the baselines for calculating the absorbance ratio A_{1655}/A_{3450}.

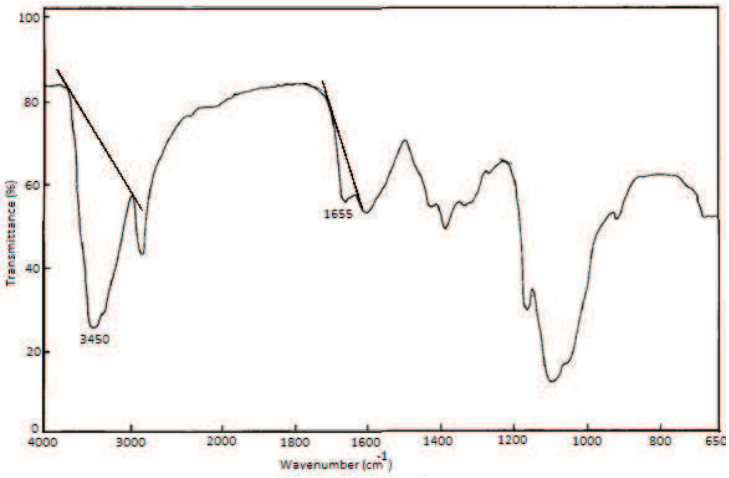

Fig. 7. IR spectrum of chitosan showing the baselines for calculating the absorbance ratio A_{1655}/A_{3450} (reproduced from Baxter et al., 1992).

5.2.1 Drawbacks

- The ester groups of synthetically N-acetylated chitosan have to be hydrolyzed in ethanolic KOH prior to analysis.
- This method is valid only for chitosan with DA in the range of 0-55%.
- Spectra with bad resolution for samples with low DA.
- Problems associated with OH absorption from water and polysaccharides. Thus samples have to be purified and well dried.
- DA results obtained by IR have quite a large margin of error.

5.2.2 Advantages

- Infrared spectrometers are widely available.
- This technique is the best choice considering only cost and time required for analysis.

- DA of insoluble samples could be determined by this technique (Baxter et al., 1992; Duarte et al., 2002; Kasaai, 2008; Miya et al., 1980; Moore & Roberts, 1980; Sannan et al., 1978).

5.3 Liquid state ^{1}H Nuclear Magnetic Resonance

Chitosan was suspended in DCl and the mixture was stirred or 24 h at room temperature. The ^{1}H NMR spectra were acquired with 16 transients, acquisition time of 3.642 s and delay of 1.500 s. The temperature was controlled at 70 ^{o}C to increase chitosan solubility. The ^{1}H NMR of a chitosan sample is shown in Fig. 8 (de Alvarenga et al., 2010).

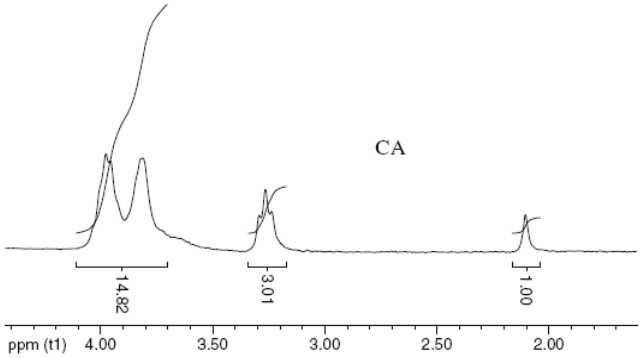

Fig. 8. ^{1}H NMR of chitosan in 1% DCl at 70 ^{o}C (reproduced from de Alvarenga et al., 2010).

The degrees of acetylation of chitosan was calculated from the areas of the signals in 2.1 ppm (methyl) and the sum of the areas from 3.2 to 4.2 ppm (H2, H3, H4, H5, H6, and H6') in the ^{1}H NMR according to formula (9).

$$\%DA = \left(\frac{2\ x\ A_{CH_3}}{A_{H2-H6}} \right) x100 \tag{9}$$

5.3.1 Drawbacks

- This method is valid only for soluble samples.
- High-cost technique.

5.3.2 Advantages

- NMR spectrometers are widely available nowadays.
- Analysis time is very short.
- Small amount of material necessary for analysis.
- The results obtained by this technique are very reproducible and reliable.
- Could be used for routine analysis.

5.4 Solid-state ^{13}C Nuclear Magnetic Resonance

The NMR spectra are mostly obtained from liquids, because a typical spectrum of an organic compound in solution provides detailed information on the structure, conformation and molecular motion. On contrary a conventional solid-state NMR spectrum is a broad

hump hiding most structural information, as the nuclei are static and cannot average out the anisotropic interactions. However an adequate dissolution of a sample for NMR analysis cannot always be obtained (Heux et al., 2000; Manni et al., 2010; Raymond et al., 1993).
The integral area of the CH_3 peak was compared to the area of the glucoside carbons in solid-state ^{13}C NMR (Raymond et al., 1993) to determine the DD (degree of deacetylation) of chitosan samples (Fig. 9). The DD could be calculated also by the integrated areas of the acetate carbonyl in relation to the C1 carbon of the glucoside ring (Pelletier et al., 1990).

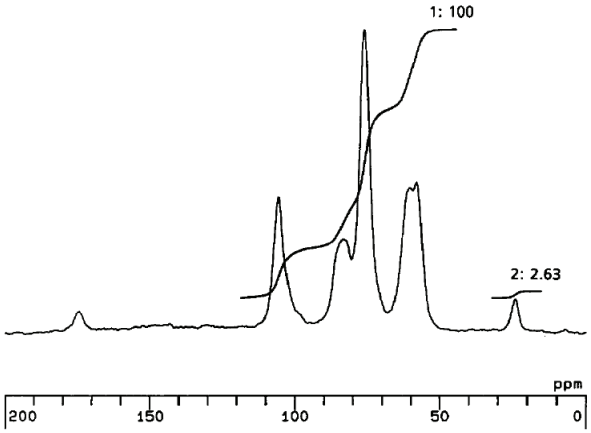

Fig. 9. Solid-state ^{13}C NMR spectrum of chitosan. Integration 1 corresponds to the glucosidic carbon atoms, whereas integration 2 corresponds to the methyl group, which is proportional to the acetyl content (reproduced from Raymond et al., 1993).

DD obtained by solid-state ^{13}C NMR for low acetyl chitosan was not coincident with the results from other technics. However, solid-state NMR is the method recommended for high DA samples, as these samples are insoluble in most common solvent.

5.4.1 Drawbacks

- This method requires high-cost accessories for the NMR machine.
- Very long analysis time.
- These accessories are not widely available yet.
- This technique could not be used for routine analysis, unless a dedicated NMR for solid state analysis is available in the laboratory.
- The results for low DA samples are not coincident with other techniques.
- DA is underestimated by carbohydrate contamination.

5.4.2 Advantages

- Small amount of material necessary for analysis.
- DA of insoluble samples could be determined by this technique, where most of the techniques are not applicable.
- Dry samples are not necessary.
- The sample is recovered intact after analysis (nondestructive method) (Heux et al., 2000; Manni et al., 2010; Pelletier et al., 1990; Raymond et al., 1993).

5.5 Solid-state ^{15}N Nuclear Magnetic Resonance

There is no unique technique that can measure the DA with high precision for all chitin/chitosan samples. For example, the lack of solubility of chitin points to solid-state ^{15}N NMR spectroscopy as one of the technics for its analysis. ^{13}C, ^{31}P, and ^{15}N are the nuclei most studied by solid-state NMR, because of the difficulties involved in obtaining high-resolution solid-state ^{1}H NMR spectra. The drawbacks of directly detecting ^{13}C and ^{15}N are low isotopic abundances, low spin polarization, and low signal intensity. Quadrupolar nuclei (e.g. ^{14}N, spin 1 nucleus) possess a nonspherical charge distribution that can couple to electric-field gradients present in most solid-state materials. Therefore solid-state ^{14}N NMR spectra are highly affected by this quadrupolar coupling.

A spectrometer operating at 30.35 MHz equipped with cross-polarization (CP) magic-angle spinning nitrogen NMR (CP-MAS ^{15}N NMR) has been used to record the spectra of chitin/chitosan samples. Samples were spun at 4000 Hz and the spectra were obtained with ca 80000 scans and recycle delay of 1 s. The CP technique is used to transfer the polarization from the abundant ^{1}H to the rare ^{15}N nuclei thus enhancing the signals. The MAS (magical angle spinning) technique removes the dipolar broadening of signals by spinning the sample at the magic angle (ca. 54.74°) with respect to the direction of the magnetic field, increasing the resolution for better analysis of the spectrum.

Figure 10 shows solid state ^{15}N NMR spectrum for chitosan. The amide, amine and ammonium nitrogen atoms are displayed at δ = ~ 101, 0, and 13 ppm respectively. The solid-state ^{15}N NMR spectra are simpler than the ^{13}C NMR spectra, because the latter have more distinct nuclei than the first. Besides determination of DA, solid-state ^{15}N NMR could be used also to evaluate the extent of grafting poly(3-hydroxybutyrate) to chitosan.

The DA is calculated by formula 10, where A_{101} and A_0 are the integral areas of peaks at δ = 101 and 0 ppm, respectively (Kasaai, 2009; Yu et al., 1999).

$$DA = A_{101} / \left(A_{101} + A_0 \right) \tag{10}$$

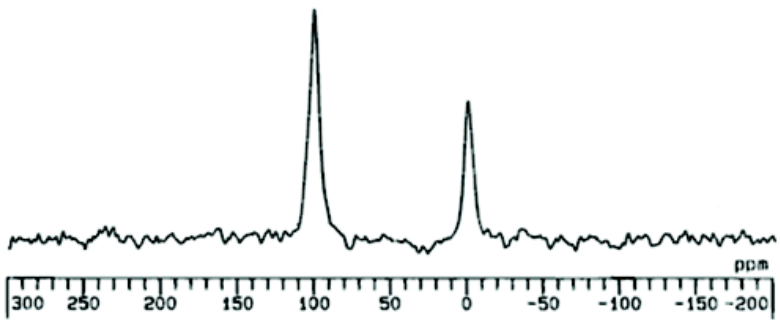

Fig. 10. Solid state ^{15}N NMR spectra of chitosan (reproduced from Yu et al., 1999).

5.5.1 Drawbacks

- This method requires high-cost accessories for the NMR machine.
- Very long analysis time.
- The accessories are not universally available.

- This technique could not be used for routine analysis, unless a dedicated NMR for solid state analysis is available in the laboratory.
- Impurities containing nitrogen (like proteins) alter the DA obtained by this technique.

5.5.2 Advantages

- Relatively small amount of material necessary for analysis.
- DA of insoluble samples could be determined by this technique, where most of the techniques are not applicable.
- Time demanding drying of samples is avoided.
- The chitosan samples are not destroyed by analysis.
- Could be employed to characterize synthetically modified chitosan, where solid state ^{13}C NMR could not be used.
- The spectra are very easy to interpret as only two signals are observed.
- DA is not affected by polysaccharide contamination.

6. Conclusion

The DA can be determined by elemental analysis, titration, conductometry, hydrolytic methods, HPLC – ultraviolet, infrared, NMR spectrometry, and other techniques. Many factors have to be considered before choosing the method of analysis, such as cost, analysis time, and accuracy. For example, some methods present the price of analysis at its favor, but are not very accurate. Some of these methods are not viable for chitosan samples with high DA, due to solubility problems. The samples have to be purified for some of these techniques to remove impurities such as insoluble highly acetylated chitosan, proteins, humidity and inorganic matter.

Considering the costs of analysis, titrations are the most indicated methods. One drawback is that these techniques demand tedious preparations and very long time of analysis.

Errors associated with the arbitrary definition of the baseline and the need of very well dried samples is a problem for the infrared spectroscopy. The advantage is that the sample can be blended with KBr for the infrared spectroscopy (no need to prepare a solution with the sample).

Solubility is one limiting factor for conductometry and liquid state ^{1}H NMR analysis. Therefore CP-MAS ^{15}N and ^{13}C NMR are the techniques most indicated for insoluble chitin where the other techniques could not be employed. The drawback in the solid state NMR analysis is the availability of the accessories (probe and console for solid-state analysis attached to the NMR machine), which are very expensive and not commonly used for routine analysis. The ^{1}H NMR is the technique most widely used for chitosan characterization, because of its accuracy, low variation of results, and analysis time. American Standart Test Method organization has adopted the ^{1}H NMR as a standard method to determine the DA for chitosan.

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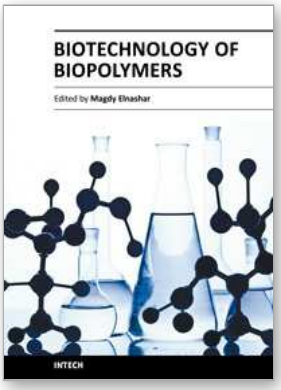

Biotechnology of Biopolymers

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The book "Biotechnology of Biopolymers" comprises 17 chapters covering occurrence, synthesis, isolation and production, properties and applications, biodegradation and modification, the relevant analysis methods to reveal the structures and properties of biopolymers and a special section on the theoretical, experimental and mathematical models of biopolymers. This book will hopefully be supportive to many scientists, physicians, pharmaceutics, engineers and other experts in a wide variety of different disciplines, in academia and in industry. It may not only support research and development but may be also suitable for teaching. Publishing of this book was achieved by choosing authors of the individual chapters for their recognized expertise and for their excellent contributions to the various fields of research.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821