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A perspective on 30 years research on chitin and chitosan

A. Domard *

Laboratoire des Matériaux Polymères et des Biomatériaux, UMR-CNRS 5223, Université Claude Bernard Lyon 1, Bâtiment ISTIL, 15 Bd. André Latarjet, 69622 Villeurbanne Cedex, France

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ABSTRACT

Chitosan is a complex linear amino co-polymer which, depending on a great variety of parameters, exhibits a more or less amphiphilic structure. This makes this polysaccharide one of the most versatile macromolecules for which it is sometimes difficult to control all the factors responsible of its properties. The purpose of this paper is to present a review of about 30 years of research in our team in the context of a precise scientific strategy. Thus, these years were devoted to improve the production of chitin and chitosan, produce series of co-polymers and co-oligomers, improve their characterizations, reveal a general law of behavior, generate nano-particles, physical gels and derived forms, show a continuum of structure from solutions to other physical states, propose the concept of materials decoys of biological media, etc.

1. Introduction

Starting in 1982, our works focused on the study of the physicochemical properties of chitosan in solution through the investigations on various interaction mechanisms and the elaboration of new materials with applications in numerous fields. Due to a non-accessible glass transition temperature before thermal decomposition, solutions were at the core of our activities related to the elaboration and the study of the properties of other physical forms like gels or solids (fibers, films, particles, capsules) processed from this physical state.

An accurate study of chitosan properties requires the use of well-characterized high quality products. For numerous applications it is also essential to produce a large spectrum of structures with perfect control of the chemical architectures, molecular weights and molecular weight distributions. In the first case, it is necessary to manage both the degree of acetylation (DA) and the distribution of the two kinds of residues along the polymer backbone. This is quite difficult for long co-polymer chains, and, up to now, the production of only statistic distributions is under control. The case of co-oligomers, more accessible in theory, remains complex. Series of fully acetylated/de-acetylated oligomers, or statistic co-oligomers of chosen DA were produced as well as pure co-oligomers of controlled architecture and DA.

All these considerations required that we could also control the production of both chitin and chitosan. They were necessarily founded on the fact that the control of both the chemical structures and the derived materials should allow us to find interesting applications, especially to illustrate new concepts in the field of life sciences.

2. Results and discussions

2.1. Production of chemical structures

The first challenge was to produce a fully de-acetylated chitosan with the highest possible molecular weight to access almost all the structures made from glucosamine and N-acetyl glucosamine residues. This required extracting chitin with a maximum preservation of its structure (molecular weight, DA). Two industrial sources were considered related to the two crystalline allomorphs, α and β, present in biomass, in shrimp shells and squid pens, respectively.

We first studied the extraction of chitin from shrimp shells (Percot, Viton, & Domard, 2003a; Percot, Viton, & Domard, 2003b). We noticed that after peeling of fresh shrimps, the best preservation of the cuticles is achieved by adding solid sodium chloride (10 kg NaCl/500 g shells). Then, the demineralization was performed under soft conditions: at room temperature, in the presence of a stoichiometric amount of 0.25 M HCl with regards to the calcium carbonate content, for only 15 min; all acid excess only contributing to the polymer degradation. The de-proteinization was later classically processed at 70 °C for 24 h in 1 M NaOH. These conditions allowed a good preservation of the chitin structure, with a high molecular weight and a DA remaining above 95%. A kinetic study of de-proteinization (Percot et al., 2003a,b) was then performed. Three different rate constants were observed and their

* Tel.: +33 472 448 587.
E-mail address: alain.domard@gmail.com.

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energies of activation and pre-exponential factors were deduced. The use of amino-acid analyses as a function of time revealed the degradation of 3 kinds of proteins, successively: acidic-, basic- and lipo-proteins; the latter being the most difficult to eliminate. The de-proteinization was also studied on squid pens (Chaussard & Domard, 2004). Optimal conditions consisted in a preliminary extraction of lipids and lipo-proteins before treatment in NaOH 1 M for 3 h at room temperature. A chitin of viscometric-average molecular weight over 1.3×10^6 g/mol and a DA beyond 93% was obtained.

The objective of the de-acetylation of chitin is to obtain chitosan, a more versatile polymer. The challenge is then to produce a fully de-acetylated sample with both the highest molecular weight and a low polydispersity index (I_p). First studies (Domard & Rinaudo, 1983) starting from a chitosan of moderate DA (<25%) allowed us to produce a fully de-acetylated sample with good preservation of the molecular weight, provided the reaction was repeated, instead of a continuous treatment, and using sodium thiophenolate as catalyst. A comparative study of a first heterogeneous de-acetylation of both chitin allomorphs was performed at different temperatures (Lamarque, Viton, & Domard, 2004a; Lamarque, Viton, & Domard, 2004b). The use of NMR and X-ray diffraction spectroscopies (synchrotron beam) exhibited two kinds of behaviors depending on a critical DA where the crystalline network vanishes. Before this DA, α and β forms are quite different. Thus, β chitin, more brittle, due to weak network of hydrogen bonding becomes rapidly amorphous and then, is easily and rapidly de-acetylated, inducing from the first stages of de-acetylation a statistical distribution of glucosamine and N-acetyl-glucosamine residues. In the case of α chitin, the network of hydrogen bonding is stronger and it is necessary to achieve a DA of about 45% to be fully amorphous. Before this value, we notice a de-acetylation in the amorphous parts, responsible for a block distribution of the two residues (the crystalline parts remaining acetylated). We also find a small fraction of low molecular weight polymers, produced from partial thermal and alkaline degradations, exhibiting a statistical distribution. A similar study was operated during the second and third steps of de-acetylation (Lamarque et al., 2004a,b). We showed that the de-acetylation of chitin was hindered by the non-accessibility of the crystalline domains to the catalyst (NaOH), thus justifying a faster de-acetylation of β chitin. The alkaline degradation was essential to destabilize these domains and was also easier with β chitin. Nevertheless, we demonstrated that operating under optimal conditions, it was better to start with β chitin to produce the highest molecular weight chitosan with a more homogeneous distribution of the residues. Thus, with a simple multi-step process, starting with a β chitin of weight-average molecular weight (M_w) close to 1.34×10^6 g/mol, after 3 steps of de-acetylation in NaOH at 50% (w/v) and within 100–110 °C, a chitosan of DA 11.48% and M_w of 318 000 g/mol was produced. A unique step of 10 h let to a DA of 15.6% and M_w of 163 000 g/mol. The high initial value of M_w and a relatively lower time for the alkaline degradation played a major role. These results let us to act on the possibility of avoiding the obstacle represented by the crystallinity. Thanks to a method of freeze-pump out-thaw cycles we could considerably improve the de-acetylation reaction (Lamarque, Lucas, 2005; Lamarque, Viton, 2005). This technique allowed the opening of the crystalline structure of both chitin allomorphs and then, a better permeability to alkaline solutions. Compared to the older method, it led to an earlier access to a statistic co-polymer, whatever the allomorph; then, to an easier dissolution of the polymer in the alkaline medium and, as a consequence, a better reactivity. It also allowed the absence of O_2, thus avoiding the oxydo-reductive process of degradation. We were in conditions very similar to that of a homogeneous reaction without difference of reactivity between the allomorphs, and limiting considerably the degradation. This allowed us to produce the highest M_w obtained for a fully de-acetylated chitosan (450 000 g/mol), with a low I_p close to 1.6, by means of a 4 steps process. The use of the best conventional method required 5 steps for a final M_w of only 260 000 gmol and I_p over 2.3. To complete this study, it was important to understand in detail the mechanism of the heterogeneous de-acetylation reaction (Lamarque, Chaussard, & Domard, 2007). We particularly investigated the role of the structure of the catalyst, the temperature and the formed sodium acetate. In the first part of the study, varying both the sodium hydroxide concentration and temperature, we evidenced that the activation energy of the reaction varied with the soda concentration. Then, the de-acetylation was the most efficient in presence of the less hydrated forms of the catalyst corresponding to the mono- or di-hydrate of sodium hydroxide. We concluded that a complex involving one of these hydrates was formed with the polymer. The non- or more hydrated forms were non efficient. This confirmed that depending on temperature (95–110 °C) the optimal range of NaOH concentration was within 50–55% (w/v). This also led us to point out the very important role of the consumption of water during the reaction, corresponding to: the formation of sodium hydrates, the alkaline hydrolysis of acetyl groups, the delivery of sodium acetate in a hydrate form, and possibly, the evaporation of water in the case of an open reactor. Then, the consumption of water (the reactive) was prejudicial to the alkaline hydrolysis reaction. It necessarily led to the transformation of sodium mono-and di-hydrates into non-hydrated soda and then, for both reasons, to the reaction level-off revealed by the well known plateau on the kinetics curves. We could finally propose a detailed scheme for the de-acetylation including five elemental reactions. We particularly pointed out the role of two reactions in the consumption of water. The first, the most water consuming, concerns the formation of a complex between the polymer chains and the tri-hydrate of the sodium acetate produced during the de-acetylation. The second, less water consuming is related to any kind of chain degradation inducing the rupture of the glycosidic bond.

The above studies allowed us to dispose of high molecular weight/high DA chitins, and fully de-acetylated chitosans of high M_w and low I_p. This was essential to: demonstrate particular properties only accessible with this kind of molecules and produce a large range of derived structures. The difficulty when studying the properties of chitosan is to consider separately the role of the molecular weight and the distribution of the two residues. This is certainly at the origin, in part, of a large discrepancy between published results in the previous century. We thus produced two kinds of homogeneous series of chitosans. A first corresponded to the re-acetylation of a highly de-acetylated polymer from squid pens (Sorlier, Denuzière, Viton, & Domard, 2001). Thanks to the mild conditions consisting to work in a hydro-alcoholic solution of chitosan using a stoichiometric amount of acetic anhydride as reactive, a series of chitosans of chosen DA, up to about 80%, could be prepared. NMR spectra illustrated a statistical distribution of the two kinds of residues, whatever the DA, and chromatographic analyses demonstrated that for all DAs, the size distribution was similar with I_p below 1.4. The quality of this kind of series was confirmed for a large range of DAs and molecular weights (Lamarque, Lucas, 2005; Lamarque, Viton, 2005; Schatz, Pichot, 2003; Schatz, Viton, 2003). A second series corresponded to chitosans with a statistical distribution of the residues, having the same DA but different DPs. In a first step, we worked with different batches of about the same DA but different DPs with a low I_p, below 1.4 (Lamarque, Lucas, 2005; Lamarque, Viton, 2005). More recently we preferred a new method consisting in an ultra-sound treatment of a high molecular weight chitosan from squid pens. This allowed us to produce series of same DA with an I_p close to 1.1 (Popa-Nita, Lucas, 2009; Popa-Nita, Rochas, 2009). It was the opportunity to elucidate the mechanism of the reaction, which is purely a-thermal and then of only mechanical

origin. Two mechanisms were evidenced. One first, corresponding to a specific rupture of weak bonds statistically distributed every 100 glycosidic bonds, responsible for a decrease of the polydispersity. A second, related to the cleavage of any bond, energetically equivalent when DP becomes below 100, was observed, with an increase of I_p and the production of oligomers up to the monomer. Knowing the values of the experimental parameters, a general law was elaborated giving a direct relation between a chosen DP and the reaction time.

Another important objective concerned the production of oligomers, very useful for the modeling, and then, the understanding of the physicochemical properties of chitosan, but also for some of their specific biological properties. In the first study, we obtained, in a pure state, glucosamine oligomers from the monomer up to DP 13, and fractions of narrow distribution up to a DP of about 90. For that, we determined optimal conditions of hydrolysis in presence of HCl as catalyst. Thus, a fully de-acetylated chitosan, in the hydrochloride form, was placed in presence of HCl ~ 12 M at 70 °C for 40′, if a distribution centered on DP 5–6 was expected. Then, a separation by steric exclusion chromatography, at the preparative scale, on Biogel P-4, with a 0.23 M acetic acid/0.05 M ammonium acetate buffer as eluent, was operated. 0.8 g of a mixture of oligomers could be injected at each run (Domard & Cartier, 1989). The hydrolysis was also produced by means of an ultrasound treatment. Indeed, for longer times than above, it was possible to obtain a chosen distribution of oligomers up to the monomer (Popa-Nita, Lucas, 2009; Popa-Nita, Rochas, 2009). The advantage in this case was to avoid any use of chemicals except water and any production of byproducts, then, no purification was necessary at the end, and the products were simply recovered after lyophilization. The distribution of oligomers, whatever the method used for their production can be modified by means of simple physicochemical treatments (Trombotto, Ladavière, 2008; Trombotto, Pernet-Poil-Chevrier, 2008). Thus, a hydrolysate was first subjected to an alkaline medium to eliminate oligomers over DP 12 by precipitation, then, treated with an alcohol to decrease the amount of soluble oligomers of DP below 4. The resulting fraction of narrow distribution, centered on DP 5–6 could be subsequently re-acetylated as above to produce a series of oligomers with a selected distribution of sizes and a chosen average DA. The re-acetylation could also be performed on a pure oligomer of a given DP to obtain series of oligomers of same DP with a chosen average DA. Some biological properties are so specific that among a series of co-oligomers produced as above, it is necessary to determine the exact architecture, with the precise location of the two kinds of residues, responsible for a particular biological response. Up to now only the full synthesis can lead to these oligomers. This is a very hard and long job since for a DP X, 2^X different structures have to be synthesized (Trombotto, Ladavière, 2008; Trombotto, Pernet-Poil-Chevrier, 2008).

2.2. Properties in solution

The very large range of accurately characterized structures prepared in the laboratory allowed us to approach comfortably the study of their solution properties. Moreover, our re-acetylated structures, due to the statistical distribution of the residues provided us with a large range of DAs soluble in water from 0 up to over 70%. Chitosan in aqueous solution corresponds to an amphiphilic cationic polyelectrolyte. Then, its properties depend on the balance between hydrophilic and hydrophobic interactions. They correspond to interactions between polymer chain segments of the same or different chains, and interactions between chain segments and molecules of solvent or solute. All this essentially depends on the electrostatic potential of the polymer chains. The latter is influenced by numerous parameters belonging to three different groups. One first concerns structural parameters including: the DA with the distribution of the two residues, the degree of polymerization (DP_w), I_p, and the contour length (L_c). A second set is related to environmental parameters such as: pH, the ionic strength, the dielectric constant of the solvent, the temperature and time. Finally, the polymer concentration constitutes a third group.

We first studied the role of the variation of the chemical structure (DA, DP, distribution of the residues) on the value of pK_0, the intrinsic pK of the polymer chains. Thanks to a homogeneous series of chitosans having the same DP, with a low I_p and covering a very large range of DAs, within 0 and 89%, we could study the variation of pK_0 as a function of DA. We obtained a curve showing 3 successive domains. A first, located below DA ~ 28%, a second for DA > 50%, and between these regions, a transition range (Sorlier et al., 2001). pK_0 is sensitive to the structure, especially the dielectric environment of the ionic site. Thus, it decreases when the structural charge density increases. This is what clearly happens in the first range. We are in the presence of a strong polyelectrolyte subjected to the ionic condensation as described in the theory of Manning (1969). In the third one, we may consider that charges are particularly less sensitive to other ionic sites becoming too far, the charge density is very low. Thus, the new increase of pK_0 with DA illustrates more the increase of the hydrophobicity of the charge surrounding. In the case of homo-oligomers of glucosamine, pK_0 decreases on increasing the number of ionic sites, and then with the increase of the electrostatic potential (E_p), from the value of the monomer, 7.55, to that of the polymer achieved for a DP ~ 10. In the case of co-oligomers, we already notice interesting results when considering the different dimers. Then, in the case of the two hetero-dimers, the position of each residue on the reducing- or non-reducing-end is quite important and induces significative differences in favor of a lower pK_0 for the glucosamine residue on the reducing end (Pernet-Poil-Chevrier, 2009).

These results allowed us to investigate the role of various parameters on the apparent charge density (λ_a), then, on E_p, and therefore, on the balance between hydrophilic and hydrophobic interactions (the H/H balance). The first was pH and in one old paper (Domard, 1987), we observed that the curves of the variation of pH as a function of the neutralization degree (α), for a fully de-acetylated chitosan exhibited two kinetics phenomena. Thus, depending on time, a mechanism of aggregation, at α close to 0, and a process of precipitation, at α over 0.4, were identified. A more recent study was extended to the homogeneous series mentioned above (Sorlier, Viton, & Domard, 2002). At very low α values (<0.15) and high DAs, ageing of the solutions, corresponding to the formation of aggregates, resulted in a slight increase of pH. This was attributed to an increase of the pK_a provoked by a slight decrease of the apparent charge density due to the hydrogen bonding formation. For α values over 0.4, ageing responsible for precipitation was illustrated by a decrease of pH with time. This decrease was faster at low DA to achieve the value of water used to prepare the solution at day 1 illustrating a full precipitation of the polymer by the formation of a strong network of intra- and inter-molecular hydrogen bonding and hydrophobic interactions. In the case of high DAs, the kinetics was slower and the full precipitation was not observed even after 3 days. This behavior revealed the important role played by N-acetylated residues inducing a better stability of the solutions in a larger range of pH by increasing both the pK_a and the stiffness of the polymer chains. In the same study, the representations of the value of pK_0, the critical value of α where ageing appeared, and the pH on this point, with DA had the same shape, confirming a generalization of the law of behavior described above when representing a property as a function of DA with the 3 domains: for DA < 28%, between 28 and 50% and > 50%.

A third aspect of the electrostatic properties concerns the interactions between chitosan and small- or polymeric-molecules.

These interactions depend on the pK_a and the charge density of both the counter-ion and chitosan. They also depend on other possible interactions between glucosamine residues and the counter-ion. When comparing chloride and ethanoate anions, molecular dynamic, using the Glycam Forcefield method allowed us to modelize the interaction and show some interesting differences between the two counter-ions (Terreux, Domard, & Domard, 2006). Ethanoate ions are more strongly interacting with chitosan than chlorides by the fact that the first form a complex involving 2 H-bonds with one glucosamine residue although the second form strong ion-pairs. Then, the ethanoate form is more hydrophilic than the chloride, justifying that chitosan is more soluble in water in the ethanoate form. It is also apparent that the number of ions interacting with the polymer is more important with Cl^-, in relation with a higher pK_a of the ethanoate ion (the salt form is never fully produced). The interaction with weak acids is also interesting when we consider the salt forms of chitosan in the solid state. Indeed, these salts are easily hydrolyzed, and, in wet atmosphere, an equilibrium between the salt and the free acidic form of the counter-ion always exists. Then, provided the acid is volatile as acetic acid, the film can be fully converted into the free amine form by simple evaporation of the acid liberated by the chitosan salt hydrolysis, in presence of water (Demarger-André & Domard, 1994). Moreover in particular conditions of drying the ahydrous allomorph of chitosan can be formed.

A fourth aspect of the electrostatic properties of chitosan is that as unique natural polymer to be only cationic it finds interesting applications in the formation of polyelectrolyte complexes (PEC) with the numerous anionic polyelectrolytes present in biomass. The formation of a PEC is due to an ionic condensation between charges of opposite sign born by two different polymer chains. Thus, at least one of the two polymers must induce this mechanism, which signifies that its electrostatic potential has to be over a critical value described by Manning. As a consequence, in numerous cases, a PEC is of 1/1 stoichiometry. Nevertheless, due to various parameters (chemical structure, involvement of other interactions) this stoichiometry is sometimes not verified. This was the case when we studied the interaction between a fully de-acetylated chitosan and collagen, the most widespread protein in mammal tissues (Taravel & Domard, 1993). Globally, the electrostatic interaction was weak and as shown by IR and CD spectroscopies, only a small part of collagen was in strong interaction with chitosan, the other part remaining completely free. In fact, chitosan was encapsulating microgels of collagen thus preventing the diffusion of other chitosan chains to the core of the collagen particles. It was possible to improve the complexation when collagen was denaturated but there also the 1/1 stoichiometry could not be reached (Taravel & Domard, 1995). Another way of forcing the interaction is by increasing the charge molar ratio chitosan/collagen well above 1. This did not really contribute to favor the PEC formation but induced a mechanism of strong interactions by hydrogen bonding and hydrophobic interactions between the two polymers inducing a denaturation of the protein. In the latter case, the proportion of collagen in the H-bond complex was much higher than in the first (<10% in weight). This contributed to strongly protect collagen toward its hydrolysis by specific enzymes (Taravel & Domard, 1996). The case of PECs between chitosan (fully de-acetylated) and hyaluronic acid (HA) or chondroitin sulfates (CS) was also studied (Denuzière, Ferrier, & Domard, 1996). When the complex is formed using polymers in the salt form, their charge density is maximum, and, whatever the case, we are in good conditions to form a perfect 1/1 complex. CS is an alternated co-polymer constituted of glucuronic acid and N-acetylgalactosamine-4- or -6-sulfate residues. The sulfate sites are always fully ionized although the carboxyl groups of glucuronic residues have a pK_0 of 3.83, corresponding to a weak acid. Thus, if a solution of CS with a full protonation of its carboxyl groups is used, we observe a continuous decrease of pH when adding a chitosan hydrochloride solution, to obtain a PEC in which all sulfate ions are complexed and only half the carboxyl groups are used. Then, we have an unstable PEC, which can become anionic on increasing pH. PECs find various applications as materials, especially membranes or particles. In the latter case, we prepared sub-micrometric particles by mixing in one-shot solutions of dextrane sulfate and chitosan. It was interesting to notice that depending on an excess of one of the polyelectrolytes, we obtained sorts of core–shell particles with a core constituted of the hydrophobic 1/1 complex and a hydrophilic shell positively or negatively charged according to the polymer in excess. Then, these particles could be used to vectorize various kinds of substrates by means of electrostatic and/or hydrophobic interactions (Schatz, Domard, Viton, Pichot, & Delair, 2004).

In solution, the H/H balance plays a major role on the conformations, and all the parameters mentioned above must be considered. Light-scattering experiments gave an interesting access to a large range of dimensions. Before starting such experiments it was necessary to determine the refractive index increment dn/dC. This parameter is related to the dielectric properties of the polymer chains and then, to the H/H balance. If we represent the variation of dn/dC as a function of DA for different neutralization degrees (α), using a homogeneous series of chitosan (Sorlier, Hartman, 2003; Sorlier, Rochas, 2003), we notice that for a given α, the values are located on a curve illustrating the 3 domains of DA mentioned above. The first range, for DA < 28% is highly sensitive to α, and when DA or α increase, the electrostatic potential and then, dn/dC decrease. We are in the range of strong polyelectrolyte properties where we observe the ionic condensation. In a third range, for DA > 50%, the polymer is a weak polyelectrolyte with more or less isolated charges in an hydrophobic environment becoming more important with DA. On increasing DA, whatever α, dn/dC becomes progressively independent of DA and tends toward 0.145, a value classically observed for neutral polysaccharides. In between, we have a transition range of the H/H balance from a hydrophilic to a hydrophobic behavior. This law of behavior became definitely a general law for all properties of chitosan studied as a function of DA with diverse homogeneous series. This was the case of: the second virial coefficient, the gyration radius, the intrinsic viscosity, the constant and exponent of the Mark–Houwink–Sakurada law, the persistence length (L_p), etc. (Lamarque, Lucas, 2005; Lamarque, Viton, 2005; Schatz, Pichot, 2003; Schatz, Viton, 2003). As already mentioned, other parameters influence the H/H balance. When studying homogeneous series of chitosan with low I_p, it was possible to also observe the variation of L_p as a function of DP_w, and thus, a continuous decrease of L_p on increasing DP_w occurred for DAs in the first range of the general law and the contrary in the third one. In the latter case, the increase of L_p especially for DP_w over 1500 could be attributed to the important increase of the steric contribution to the excluded volume counterbalancing the loss of the electrostatic parameter (Lamarque, Lucas, 2005; Lamarque, Viton, 2005).

The displacement of the H/H balance in favor of the chain hydrophobisation is also at the origin of important changes of molecular organizations. We observed solutions of a homogeneous series of different DAs by means of quasi-elastic light scattering (QELS) in the dilute regime, at $C_p < C^*$, with C^*, the critical concentration of chain entanglement. At full ionization ($\alpha = 0$), without added salt, whatever the DA, up to about 70%, a unique distribution of hydrodynamic radii (R_h) was observed centered at near 25 nm. In this case, the chains are at their maximum of both their hydration and expansion (maximum L_p) and are isolated in solution. On increasing α, a second distribution appears at higher R_h values, centered on 250 nm (Sorlier, Hartman, 2003; Sorlier, Rochas, 2003). This distribution is attributed to the formation of hydrophobic nano-aggregates that play a major role in all the morphologies

of other physical forms we may study like gels and solids based on chitosan. These nano-aggregates are also identified by electron microscopy when we achieve a critical pH just before the polymer precipitation. In the latter case, they have a size within 250–350 nm (Schatz, Pichot, 2003; Schatz, Viton, 2003). Their formation is also favored by an increase of concentration, which contributes to the hydrophobisation, especially over C^*. Thus, for a DA over 70%, at $C_p > C^*$, they are observed at any pH by QELS or electron microscopy (Popa-Nita, Alcouffe, Rochas, David, & Domard, 2010), by means of the interesting technique of wet STEM. Moreover, they look like core–shell structures. Over C^*, recent studies by means of SAXS (synchrotron beam), showed the presence of a scattering peak (the so-called polyelectrolyte peak). The value of the scattering vector q_{max} at the maximum intensity was studied as a function of DA, C_p and M_w. Its variation with C_p, for different DAs, illustrated a power law which exponent (a) showed a transition between 1/2 and 1/3, in relation with the model of the "necklace", proposed to illustrate the conformation of polyelectrolytes (Dobrynin & Rubinstein, 1999). When a is 1/2, the polymer is highly hydrophilic and the conformation is controlled by the strings. On the contrary, for 1/3, it becomes highly hydrophobic and is controlled by the pearls. The critical concentration C_b between both values revealed a second critical phenomenon to be related to C^{**}, the second critical concentration, we already identified in previous studies (Boucard, Viton, & Domard, 2005; Montembault, Viton, & Domard, 2005a; Montembault, Viton, & Domard, 2005b; Montembault, Viton, & Domard, 2005c) where a nano-phase separation occurs with one phase constituted of more or less isolated and hydrophilic chains and a second of hydrophobic nano-aggregates. The variation of C_b with DA followed again the general law of behavior (Popa-Nita, Rochas, 2009).

2.3. From solutions to other physical forms

The knowledge of the properties in solution including changes of chain conformation and molecular organization is quite useful to understand the formation of derived forms. It is thus relatively easy to form a physical hydrogel of chitosan with no use of an external cross-linking agent provided we consider three essential conditions. The first is to be initially at a concentration over C^* to favor polymer chain junctions. The second is to displace the H/H balance to overcome C^{**}, and the third is to generate a bi-dimensional sol–gel transition. We identified two different ways.

In the first case, the second criterion was fulfilled using a hydro-alcoholic solvent inducing a lower dielectric constant of the medium, thus becoming more hydrophobic than pure water. In this medium the acid used to dissolve chitosan was less dissociated, even in the case of HCl. Then, the third criterion could be achieved by means of a simple evaporation of water and the non-dissociated acid (acetic acid, hydrochloric acid), both allowing to also overcome C^{**}, provided the alcohol used had a higher boiling temperature than water. A kinetics study confirmed the hydrophobic role of an increase of temperature favoring a faster gelation with two different energies of activation and a transition at near 40 °C. We also noticed a decrease of the neutralization degree at which gelation occurred, as a function of DA, illustrating again the general law of behavior. This law was also observed when studying the variation of the storage modulus of the gels increasing with DA, thus showing the important role played by acetyl groups in the formation of hydrophobic junctions (Montembault et al., 2005a,b,c). The alcohol used was shown to participate in the gelation and only a few alcohols allowed gelation to undergo (propandiol, glycerol ...). SAXS showed that during the evaporation of water and even over the gel point, the polyelectrolyte peak was always present, confirming the formation of a polyelectrolyte alcohol gel (Boucard et al., 2005). Therefore these gels could be neutralized in an alkaline medium and after thorough washings in water, they only contained water and chitosan in the $-NH_2$ form. The neutralization is a very interesting step. Indeed before neutralization, no order was observed in the gels. When NaOH 2 M was used to neutralize the gel, after washing, law-angle laser-light scattering and scanning electron microscopy revealed an organization at two levels. The gel was then constituted of an assembly of raspberry-like structures which diameter is between 1 and 3 μm and these objects are themselves formed by the assembly of nano-particles of average size corresponding to those described above in solution (Popa-Nita et al., 2010).

In the second, the gel was directly produced from an aqueous solution initially over C^{**}. The second and third criteria were fulfilled by means of the hydrophobisation of the polymer chains using either an alkaline gaz, like ammonia (Montembault et al., 2005a,b,c), or a concentrated solution of sodium hydroxide (unpublished results). The variation of the storage modulus as a function of DA also illustrated the general law of behavior. During the neutralization/gelation, hydrophobic nano-aggregates were collapsed together (gel point) by condensation on their surface of isolated chains turned hydrophobic. In a similar manner as above, but somewhat different, as shown by low-angle laser-light scattering (to be published), these particles constitute the walls of a parallel assembly of micrometric tubes, in the direction of the base flux.

The neutralization of an alcohol gel in presence of 1–2 M NaOH induces an hydrophobisation of the polymer chains, which, becoming highly hydrophobic, condense together to form a denser gel from the surface of the material. If we stop the neutralization for a few tenth seconds, we contribute to generate an inter-phase without polymer chains between the dense gel and the unmodified alcohol gel. The repetition of this sequence generates a closed multi-membrane material constituted of fully independent membranes. During the neutralization, the inside of the nano-aggregates is re-organized into a parallel assembly of smaller ellipsoidal aggregates of length close to 15 nm, oriented perpendicular to the base flux (Ladet, David, & Domard, 2008). The gelation in water was also used to generate multi-membrane hollow fibers. Thus, the spinning of an aqueous solution of chitosan at $C_p > C^{**}$ was subjected to a succession of coagulation (aqueous NaOH)/washing (water) steps; each sequence generating a membrane and an inter-membrane space, the core of the fiber, remaining a solution, could be easily eliminated to constitute a hollow fiber (Domard et al., 2009). The presence of nano-aggregates was also observed in the structure of chitosan fibers. Indeed, when a gel spinning of a chitosan solution over C^{**} is operated, followed with a coagulation by ammonia gaz, the observation of the fibers by scanning electron microscopy, after washing and drying, revealed that they were constituted of a parallel assembly of sort of necklaces oriented according to the extrusion axis. The size of the pearls was then equivalent to that of the above nano-aggregates (250–350 μm) (Notin et al., 2006).

Our results allow us to illustrate a continuum of structure from solutions to other physical forms where the H/H balance plays the major role, schematized in a recent paper (Popa-Nita et al., 2010). In a reference state where $C_p < C^*$, at maximum ionization, without salt, and DA < 28%, chitosan is at its maximum hydrophily and behaves like a semi-rigid chain with a persistence length close to 10 nm. Then, any contribution to the hydrophobisation induces the formation of hydrophobic nano-aggregates, which size is almost constant. Only their number increases on increasing the hydrophobicity up to overcome C^{**} where we observe a nano-phase separation. The latter behavior is attributed to a dynamic equilibrium between isolated chains and nano-aggregates, as in the formation of micelles, characterized by a sort of critical micellar concentration and a relatively weakly polydispersed system. One phase is constituted of numerous hydrophobic nano-particles, and a second of more or less isolated chains remaining hydrophilic. This situation is essential to produce physical hydrogels or fibers by

the condensation of isolated chains, became hydrophobic, on the surface of the particles, up to their collapse corresponding to the gel point. Depending on the context, these particles constitute the walls of raspberry- or micrometric tube-like structures in gels or the backbone of necklaces in fibers.

2.4. Biological properties

The knowledge of the structures (chemical and physical) and physicochemical properties is essential for the understanding of the biological properties of chitosan-based biomaterials. It is particularly important to establish the relationship between the structures and the biological responses. Our studies were all founded on the new concept of "materials decoys of biological media" proposed a few years ago (Montembault et al., 2006). This concept is based on two criteria. The first concerns the chemical structure of the considered polymer. The latter must be a co-polymer fully absent in the chosen living tissue for its application, but any of its elemental stones constituting its backbone must be all present in biomass to limit problems of biocompatibility. It is then interesting to consider a few polysaccharides, especially those of the family of glycosaminoglycans. In this case, some parts of the structure must be fully absent of the considered tissue, like a glucosamine residue, but some others, like a N-acetylglusamine unit or a $\beta-(1->4)$ glycosidic bond, fully present. The second criterion is related to the physical structure. As for living tissues, it is important to use a highly hydrated material (>70% of water) for which the minimum porosity corresponds to a mesh of the polymer network below 500 nm, thus precluding any possibility of physical transfer of microorganisms like bacteria or cells. This is the case of a physical hydrogel, a film, or a fiber of chitosan. Nevertheless, the morphology and the polymer chain organization of the material must be different from what we can observe in a living tissue. A material responding to both criteria is a bio-inspired and not a bio-mimetic system, and this is the case of the chitosan-based materials mentioned above with an optimum represented by physical hydrogels. The latter will induce interesting positive, but necessarily erroneous biological responses.

One first aspect of the biological properties of chitosan is that it is an elicitor of numerous activities when it is in contact whether with animal or plant cells. This means that a very low amount induces an amplification of cell signals. In the case of plant cells, the result depends on DA and the molecular weight but also on the cell line. Thus, when a chitosan solution was subjected to a suspension of *Chatarantus roseus* cells, a production of callose was noticed. The response was dose dependent and increased with DP up to a plateau. It also increased on decreasing DA. These results showed an exclusion mechanism toward very high molecular weights due to a barrier effect of the cell wall. Indeed, in the case of protoplasts, no limit DP was observed. It also revealed that interactions between chitosan and cells were reinforced by the presence of the amine groups of glucosamine residues, in relation with both hydrogen bondings and weak electrostatic interactions (at the pH of the experiments). It was thus proposed that this kind of interaction could take place with some negatively charged phospholipids of the membrane, with an alteration of the membrane fluidity, especially the ion transport known to be associated to the production of callose (Kauss, Jeblick, & Domard, 1989). This result was confirmed when studying the elicitation of the laminarinase activity by a series of fully de-acetylated chitosans, including low DP oligomers, in *Rubus* cells or protoplasts. Thus, an increase of the elicitor interaction with DP was observed. In the same study we noticed a specific recognition of low DP oligomers with a maximum activity for a DP 4. This let us to suppose a specific recognition by membrane-receptor sites (Lienart, Gautier, & Domard, 1993). A lectin specific for oligomers could be identified and purified by chitosan affinity chromatography. This lectin was eluted by the oligomer of DP 4 in presence of L-α-phosphatidylserine dipalmitoyl and its molecular weight evaluated at 67 kg/mol. The best affinity was observed with a DP 4, but another weaker binding site was identified for a DP 6. Beyond, or below these DPs, the affinity decreased drastically (Lienart, Gautier, & Domard, 1991). It was important to test the eliciting properties directly on leaves. This was done on wheat leaves infested with *Titricum aestivum* L. The elicitation of both phenylanaline ammonia-lyase (PAL) and peroxydase (POD) were investigated. Fully de-acetylated oligomers had no influence on the POD activity, contrary to those of N-acetylglucosamine of DP > 6. Partially N-acetylated polymers elicited both PAL and POD activities with a maximum for intermediate DAs (30–40%) (Vander, Vårum, Domard, El Gueddari, & Moerschbacher, 1998).

As mentioned above, chitosan is also an elicitor of animal cells. We may first mention some interesting immunological results. Polyclonal anti-chitosan antibodies were prepared. A carrier protein linked to chitosan by electrostatic or covalent interaction was necessary to enhance the immune response and obtain reproducible and stable antibodies. There was no influence of DP between 22 and 2260, but the increase of DA decreased the affinity in relation with the important role played by the $-NH_2$ functions. No cross-reactivity with other GAGs was observed whatever the DA (Sorlier et al., 2003a,b).

As above we tested the eliciting properties of chitosan as a medium of cultivation of various human and/or animal cells. One first example concerned the cultivation of human fibroblasts and keratinocytes on chitosan films of various DAs. We first confirmed the very good cytocompatiblity of chitosan films, whatever their DA. We also noticed a decrease of cell adhesion on increasing DA, here also in relation with the important role played by the amino groups. Adhesion of fibroblasts was about twice more important than that of keratinocytes. The proliferation of keratinocytes decreased on increasing DA, in agreement with the cell adhesion. Although remaining alive, fibroblasts did not proliferate certainly due to a too strong adhesion on chitosan films, thus inhibiting the cell growth. In parallel with the role of the cell adhesion, we showed that smooth surfaces favored the cell proliferation much better than rough surfaces (Chatelet, Damour, & Domard, 2001).

We also tested the cultivation of chondrocytes, and in order to be in agreement with the concept of materials decoys described above, we used particles of physical hydrogel as culture medium. The experimentation was performed with rabbit cells, then, confirmed with human chondrocytes. We noticed that whatever the experiment time, the material was never penetrated by cells tightly bound to its surface, generating a spontaneous aggregation of both cells and gel particles. A neo-construct was built by means of the production of an important amount of extra-cellular matrix (ECM) distributed in the intercellular space and around the particles. This corresponded to another new concept: the concept of "reverse encapsulation" in which both cells and their ECM encapsulate the culture medium. This is what usually happens during the natural tissue regeneration. We showed that there was an optimal DA within 30–40%, *i.e.* in the intermediate range of the general law of behavior where both electrostatic and hydrophobic interactions coexist. The experimentation followed with human chondrocytes showed that chondrocytes who dedifferentiated in the primary culture, rapidly lost the expression of type I and immature type II collagens to only express type II mature collagen and aggrecans. Thus, chondrocytes maintained their phenotype for as long as 45 days, forming cartilage-like nodules. We could also consider that chitosan gel did not work as a scaffold (Montembault et al., 2006). The cultivation of chondrocytes was also performed by means of multi-membrane physical hydrogels of chitosan. Thus, 4 successive inter-membrane spaces were seeded with chondrocytes. After 21 days, but also up to 8 months, cells were alive, they had pro-

liferated without any dedifferentiation, producing a large amount of ECM with the same characteristics as described above. Moreover, we could notice that whatever the inter-membrane space, no invasion of the membranes could be seen neither by cells nor by the ECM, thus justifying again our two new concepts. A biochemical study revealed the only presence of mature type II collagen and an interesting involvement in the NO cascade with the inhibition of some matricial metalo-porphirines (MMPs), in relation with the limitation of the production of nitrites. This was explained by the possible action of chitosan as complexing agent to extract zinc ions from MMPs and also by the chemical consumption of nitrites to produce oligomers of chitosan acting both as reductive agents and pseudo growth factors (Domard et al., 2006; Ladet et al., 2008). Depending on their nature, MMPs play an important role in relation with the immune system and can either favor or disfavor the tissue regeneration as, for the latter case, the necrotic activity of NO.

We also tested our material directly on living tissues from several veterinary experiments. One of them concerned the use of a three-layer physical hydrogel for the treatment of third degree burns with the pig as animal model. Thus, gluing a soft gel, made directly from a water solution with a hard gel produced from a hydro-alcoholic solution, processed the biomaterial. The glue joint was a simple aqueous solution subjected to gelation. A thermal burn was made on the backs of animals to fully destroy the skin. After 2 days, to favor adverse responses toward healing, the necrotic tissue was surgically fully eliminated up to the aponeurosis. Then, the defect was perfectly filled by the material with the soft gel in contact with the basal membrane to ensure a good contact with the living tissue, and the hard one, constituting the external side, for its good mechanical properties. This coverage was a sort of skin substitute bringing: similar mechanical properties, exchanges of CO_2 and water with the outside, a good barrier toward the contamination by microorganisms of fungal or bacterial type. It also acted as a material decoy inducing a very good tissue regeneration (Boucard et al., 2007). It is important to mention that the coverage was never replaced all along the experimentation whatever the time. After three weeks, we noticed a complete regeneration of the dermis and the migration of keratinocytes from the edges of the wound induced a partial epidermalisation. The aesthetic aspect and the mechanical properties were very similar to those of the native skin of the surrounding. We also observed a very limited retraction of the scar probably physically prevented by the presence of the material. The rebuilt skin was analyzed by histology and immunohistochemistry. We could thus confirm that the new skin was highly vascular with numerous blood vessels oriented perpendicular to the surface of the skin. A well organized network of type I collagen was also present in the new dermis. The histology completed by the detection of type IV collagen demonstrated the formation of a dermo-epidermal junction. After 1 month, all the wounds were completely healed with a new skin very similar to the native skin of the surrounding. This experimentation was recently renewed giving the same results (unpublished results). The mechanical study of the new skin was tested immediately after collection of samples and the mechanical characteristics demonstrated to be very close to those of a native skin, much more than for the skin regenerated by classical coverages like "Tulle gras®". All along the time of the dermis regeneration, the material was fully absent of both cells and new ECM. Nevertheless, after 1 month, the analysis of the coverage revealed the presence of numerous cells with a new matrix in the part immediately in contact with the living tissue and a surface highly keratinized. The material resembled a natural skin. This behavior can be explained (Fig. 1) considering what happens when a physical hydrogel of chitosan is contacted with blood (personal unpublished results). The minimal porosity of the gel is below 500 nm and there is no possible physical transfer of cells inside the material. After the beginning of the gel dehydration at the end of the dermis reconstruction, the gel becomes multi-lamellar, the access is then possible to cells finding nutriments as well as growth factors brought initially by blood. The cells beginning to penetrate the material can also produce nitrites favoring the local degradation of the gel with the production of oligomers, and so on. Then, the material becomes a sort of artificial skin model.

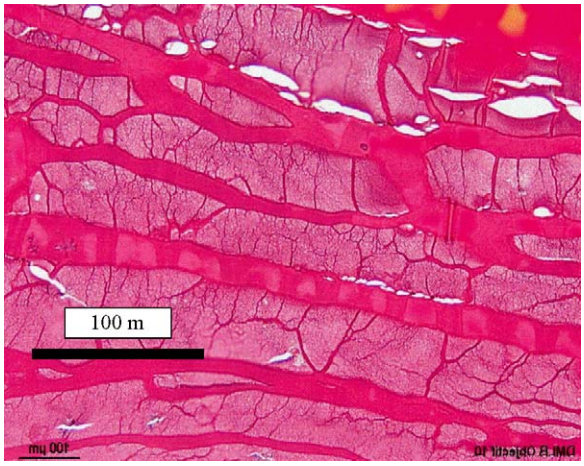

Fig. 1. Histology of a cut of chitosan physical hydrogel immersed in heparinized blood for 1 h. Breaks in white are artifacts of cut.

3. Conclusion

Progress over these 30 years can be considered in various steps.

One first concerns the increase of the molecular weight of the extracted chitin from either shrimp shells or squid pens. The objective would be to avoid any degradation before treatment. Thus, it would be important to treat the raw material immediately after its collection from living animals. We could thus approach a M_w of 3×10^6 g/mol close to that supposed in the native chitin.

The second is related to the control of the hydration of the reaction medium during the de-acetylation of chitin, to preclude the level-off due to the complete consumption of the reactive. Then, thanks to the recent studies and starting with a chitin of M_w near 3 Mg/mol we could overcome the million g/mol for a fully deacetylated chitosan, thus accessing to new physical properties for all the physical forms.

The production of co-oligomers of perfectly controlled architecture and size remains a challenge that must be regarded as particularly promising for their specific biological properties, but also their physicochemical properties: for example, the possibility to form self-assembling materials (nano-particles, organo-gels, etc.). They are also interesting to produce a new series of copolymers, especially that concerning the block co-polymers useful for their typical amphiphilic properties.

Solution properties of chitosan reveal the important role played by all parameters influencing the balance between hydrophilic and hydrophobic interactions, at the origin of their nano-structuration associated to the existence of a general law of behavior. This opens the understanding of all the interactions involving chitosan and allows us to show a continuum of structure from the solutions to any physical states. These advances are at the origin of the understanding of some biological properties also allowing us to propose new concepts; especially that of materials decoys of biological media. All these results should open a large and interesting access to a wide field of new properties and interpretations.

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