Atypical Polysaccharide Physical Gels: Structure/Property Relationships

*Alexandra Clayer, Laurent Vachoud, Christophe Viton, Alain Domard**

Laboratoire des Matériaux Polymères et des Biomatériaux – UMR-CNRS 5627, Domaine Scientifique de la Doua, ISTIL, 15 Bd. Latarjet, 69622 Villeurbanne Cedex, France
Email: alain.domard@univ-lyon1.fr

Summary: Chitin and chitosan are polysaccharides produced by the biomass. They have the same general chemical structure and constitute the series of linear copolymers of linked β, (1–>4) glucosamine and N-actylglucosamine. We studied the possibility of forming physical gels with all the terms of this series, whatever the proportion of the two kinds of residues included in the polymer chains. We show that physical gelation is still possible through a percolating process when certain important conditions are met. Initially the concentration in polymer must be above C*; a critical value of the balance between hydrophobic and hydrophilic interactions must be achieved and gelation must occur simultaneously everywhere in the medium. These conditions were observed in several situations allowing the formation of different kinds of gels at all values of DA. In view of the rare bio-active properties of chitin and chitosan, these gels were tested for living tissue regeneration and constitute very interesting examples in illustration of our concept of decoys for biological media.

Keywords: chitin; chitosan; physical gels; bioactivity; tissue engineering

Introduction

Usually, the junctions responsible for the formation of a network of polymer chains may involve various kinds of interactions. On the one hand, they can be of relatively high energy, such as ionic bonds or chelation with metals. On the other hand, they can correspond to low energy interactions such as hydrogen bonding or Van der Waals, hydrophobic and charge transfer interactions. Moreover, almost all physical gels contain both amorphous and ordered domains, leading to a pseudo-solid behaviour. As a consequence, these gels should be both solvo- and thermo-reversible. If we consider the case of biopolymers, their physical gels are due to molecular aggregation involving a typical amphiphilic structure and, in addition, a particular thermodynamic situation. This particular situation is widely observed in living media and we may consider that all tissues constituting living matter, especially extra-cellular

 DOI: 10.1002/masy.200351001

matrices, must be regarded as more or less complex physical hydrogels. Among the molecules present in these tissues constituting what is called the extra-cellular matrix, glycosaminoglycans (GAGs) and glycoconjugates play a special role in favouring cell development. These saccharidic structures are composed of a limited number of sugars including N-acetylglucosamine, whether sulphated or not. We consider that physical gels made with polysaccharides containing this kind of sugar must be regarded as very interesting tools for tissue engineering.

Inside the family of GAGs, the series of linear copolymers of linked β, (1→4) glucosamine and N-acetylglucosamine exhibits particularly interesting properties of physical, physicochemical but also of biological nature. The chemical structure of these copolymers is displayed in Figure 1.

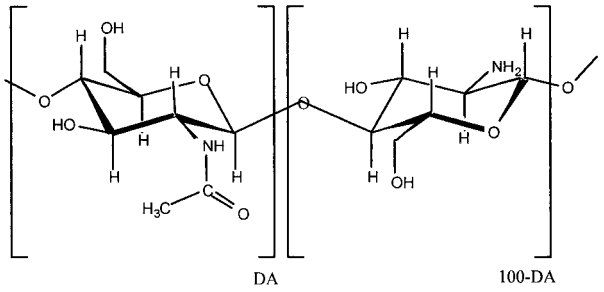

Fig. 1. Chemical structure of the linear copolymers of linked β, (1→4) glucosamine and N-acetylglucosamine.

When the degree of acetylation (DA), or the number fraction (%) of N-acetylglucosamine residues in the polymer chains is less than 60%, the polymer is soluble in water or in dilute acidic media and is termed chitosan. Above 60%, however, this solubility is completely lost and the name is then chitin. Chitin, with cellulose, is the most abundant biopolymer produced by the biomass. Nevertheless, like chitosan, it is completely absent in mammals.

The objectives of our work are to show that it is still possible to prepare typical forms of physical gels from this series of copolymers and to show that they constitute decoys for biological media responsible for particularly interesting biological properties.

Typical Gelation of Chitin and Chitosan

a- Basic Considerations

Usually, the gelation of polysaccharides involves the formation of junctions made from cross-links between chain segments in ordered conformation, by means of cross-linking agents generally corresponding to specific ions such as Ca^{2+} or K^{+}. In our series of copolymers, no ordered conformation or conformational transition has ever been observed in solution. Thus, the first important condition to be achieved to form a gel with these copolymers is to start from a situation where chain entanglements already exist and thus to be above C*.

The second condition to be observed concerns the critical value of the balance between hydrophilic and hydrophobic interactions where gelation can occur. Due to the high functionality of the chemical structure displayed in Figure 1, this value can be achieved by the involvement of numerous parameters and, according to the major parameter, thermo-reversible or non thermo-reversible gels can be formed.[1,2] Thus, when the junctions are due both to hydrophobic interactions and hydrogen bonding, the gel is necessarily non thermo-reversible. When only hydrogen bonding is involved in association with thermo-reversible interactions, such as ionic interactions, the gels are necessarily thermo-reversible. Nevertheless, due to the toxicity of the reagent used in these cases, possible applications of these gels for inducing biological responses are practically excluded. Thus, the first case appears to be the most interesting.

In the latter case, there are several possibilities for reaching the critical value of the balance between hydrophobic and hydrophilic interactions. We may consider three main situations. The first consists in chemically modifying chitosan by acylation of the free amino groups with a preference for acetylation. The second possibility consists in changing the dielectric constant of the solvents by progressively introducing a less polar solvent. The third case regards the decrease of the apparent charge density of the polymer chains up to a critical value, which depends on the hydrophobic environment of the glucosamine residues, especially on the DA.

The third condition to be observed must take into account the fact that several events are successively involved in the formation of these gels and, therefore, the initial perturbation of the medium must arise simultaneously everywhere in the solution. This is why we must effectively consider a process of percolating gelation. Indeed, a local perturbation without fast propagation leads essentially to precipitation or the formation of a membrane and then, to local

coacervation. It is also important that this first event should occur very rapidly compared to the rate of molecular organization responsible for gelation. We may consider that the gelation process occurs through the formation of aggregates of polymer chains whose size increases with time until a macro-gel is obtained.

b- Chitin Gels

The formation of chitin gels was observed for the first time by S. Hirano and then by G. Roberts.[1,3] Thus, when chitosan is acetylated in a hydro-alcoholic medium, whatever the alcohol chosen, gelation arises for a critical DA close to 77 %. The sole role of the alcohol is to avoid the reactions of O-acylation and to decrease the dielectric constant of the medium, thus favouring the presence of free amino-groups that are necessary for the N-acetylation reaction. The amphiphilic structure of the alcohol also contributes to maintain the critical balance between hydrophobic and hydrophilic interactions involving some molecules of alcohol in the formation of hydrophobic cross-links. Then, when we completely exchange the alcohol with water, we observe a strong depletion and a loss of transparency of the gels. The basic considerations discussed above are fully observed in this case since the reaction of acetylation occurs everywhere in the reaction vessel and the critical value of DA necessary to induce the gelation is always achieved for a time that is at least two times shorter than the time corresponding to the gel point determined by light scattering.[4]

It is also possible to form chitin gels directly from a solution of chitin (of DA>77%) in a solvent such as dimethylacetamide + 5% LiCl. If we directly dialyse the solution against water, the basic conditions are only partly respected and only a membrane or a precipitate is formed since the kinetics of aggregation is too fast. We can remedy this problem simply by using a hydro-alcoholic medium instead of pure water, which has the advantage of slowing the process of aggregation and also of decreasing the part of hydrogen bonding.

c- Chitosan Gels

It is still possible to form a chitosan gel directly whatever the DA if, here also, the basic conditions are scrupulously respected. In that case, there is no need for chemistry and only physicochemical parameters are needed for this conversion. For a given DA, the simplest way to achieve the critical value of the balance between hydrophobic and hydrophilic interactions is to decrease the apparent charge density of the polymer chains up to this critical point.

Nevertheless, the basic conditions are more difficult to achieve. Indeed, there is no possibility of reaching the critical point by simple addition of an alkaline solution, since this immediately leads to local precipitation. The charge density must be decreased very fast, simultaneously throughout the solution. It is also necessary that this event be completed before the molecular organization has really begun.

The first way we tested consisted in the use of a hydro-alcoholic solution of chitosan where the associated acid was acetic acid. These conditions were particularly important for several reasons. Acetic acid is a weak acid whose water solubility is limited. Then, if we evaporate a solution of chitosan prepared from this acid, we contribute to creating the conditions for percolating gelation. A gel is formed after a time of evaporation depending essentially on the initial polymer concentration, the DA, the temperature and the water/alcohol ratio in the medium. Thus, for an initial concentration in polymer close to 0.5%, the time necessary to reach the gel point, measured at ambient temperature by light scattering, decreases considerably with increasing DA. The curve representing this variation agrees quite well with the general law of behaviour of chitosan solutions when we consider the changes between a solution of a polycationic polyelectrolyte and that of a polymer bearing a few charges whose hydrophobic environment increases with DA.[5] It is also particularly interesting to note that the extrapolation to zero gelation time is close to DA 77%. Another interesting study is the measurement of the fraction of free amine groups at the gel point. Thus, we show that for a given water/alcohol ratio, this value appears to be independent of the degree of acetylation. This result reflects the fact that the apparent charge density plays a role, but is certainly not the major parameter. This is confirmed by the fact that if we work with two chitosans having different DAs, and a ratio of concentrations inversely proportional to the ratios of the DA values, the time necessary to reach the gel point is almost the same. Another parameter we studied is the temperature. Then, the curves showing the variation of the time of gelation as a function of temperature for two very different DAs (5.2 and 65.5%) show that in both cases, the rate of reaction increases with temperature. This behaviour agrees quite well with the very important role played by hydrophobic interactions in the gelation mechanism. This point is confirmed by a stronger influence of the temperature for the lower DA value. Above 50°C, the role of temperature becomes negligible compared to a gelation process where hydrogen bonding also contributes to the formation of cross-links, and thus becomes disfavoured above this temperature.

A second way to induce percolating gelation in chitosans is to use the fact that the diffusion of gas as well as the mobility of protons in water are both very high. In that case it is possible to form a gel very simply, without alcohol. In these conditions, we may follow the formation of the gel at various scale levels by means of techniques such as elastic light scattering and X-ray scattering. Then we may observe the changes at dimensions ranging from a few nm to over a thousand nm. It is particularly interesting to note the presence of a butterfly type spectrum in the case of low angle light scattering.

Typical Properties of Chitin and Chitosan Gels

a- Non Biological Properties

Physical gels of the series of copolymers of glucosamine and N-acetylglucosamine have obviously all the properties of this series, but in the gel form. Thus, as the value of the intrinsic pK, pK_0 of the free amino groups increases with increasing DA, in the cationic form these gels have the particularity of being charged even at values of pH above 7.5 when the DA is above 80%.[4-7]

These gels constitute a sort of three-dimensional membrane allowing certain osmotic phenomena to be observed. When this kind of gel is subjected to a solution of very high molecular weight substrates, the impossibility for the substrate to penetrate the gel induces very strong depletion of the gels up to concentrations in polymer inside the gel of 30%, a value which cannot be achieved directly in solution.

Another typical behaviour is the interactions of chitin gels with certain dyes such as congo-red. This dye has the particularity of having a chemical structure in which two ionic ends are separated by a long hydrophobic sequence. Thus, when the gels are exposed to a solution of this dye, we observe the formation of a boundary layer of dye at the extreme surface of the gel. This phenomenon is due to the formation of hydrophobic interactions between the gel surface and the dye. The interaction lets free the ionic ends of the dye thus creating a mechanism of electrostatic exclusion against the molecules of dye remaining in solution. At equilibrium, the presence of a red skin at the surface of the gel can be seen with no other molecules of the substrate inside the bulk gel.[6]

b- Biological Properties

Our series of copolymers is well known for its interesting biological properties.[8] Chitosans are completely absent in mammals. Nevertheless, the N-acetyl-D-glucosamine residue and the β, (1→4) glycosidic linkages are largely present in glycosaminoglycan and glycoconjugate structures. In contrast, the D-glucosamine residue is never present. Chitosan is then partially recognized by living media and the unidentified part (glucosamine residues) induces erroneous biological responses when it is exposed to living media. We attribute that to decoy behaviour. This observation is the consequence of very interesting biological properties.

The porosity of our gels, measured by AFM and environmental electron microscopy, is found to be between 200 nm and 500 nm, thus allowing free diffusion of most molecules, even large macromolecules. However, this precludes any transfer of microorganisms including cells, fungi or bacteria. This observation is particularly important and explains why, as in living extra-cellular matrices, there is no chance of observing physical colonization of the material by cells. On the contrary, it is possible to see it being invaded by a neo-formed extra-cellular matrix. Thus, our concept of decoy concerns both the chemical structure and the morphology of the material.

When such a decoy is subjected to a living medium such as chondrocyte cells, favourable interactions arise between the surface of the decoy material and the cell surroundings. These interactions cause the formation of a first interface responsible for a second series of biological responses. In our case, the latter lead to the production by the cells of a neo-formed extra-cellular material which invades the gel matrix. Some responses also correspond to enzymatic hydrolysis, allowing a more or less rapid resorption of the material, which is easily controlled by the DA value. This parameter also controls the nature of the interactions and thus of the biological responses. The interaction between the decoy material and the biological medium is thus regulated by the chemical and physical structures, but the surface of the material plays a major role.

A divided form of the hydrogel is thus of particular interest for some applications in tissue engineering. Ground gels exhibit a fractal contour corresponding to a high surface area, thus allowing the formation of a maximum interfacial area. For in-vitro generation of cartilage, micro-particles of chitosan hydro-gels constitute a system that allows numerous interactions with cell surroundings, etc. These systems represent a model of reverse cell encapsulation. This

geometry has the advantage of preserving the cell freedom, contrarily to what happens when cells are sequestered in a scaffold such as a sponge of collagen.

Conclusion

Physical hydrogels based on polymers of the series of the copolymers of glucosamine and N-acetyl glucosamine are highly interesting as tools for physicochemical studies but also for their typical biological properties.

[1] S. Hirano, Y. Ohe, *Agric. Biol. Chem.* **1975**, *2*, 1337.
[2] S. Hirano, R. Yamaguchi, *Biopolymers* **1976**, *15*, 1685.
[3] G.K. Moore, G.A.F. Roberts, *Inter. J. Biol. Macromol.* **1980**, *2*, 78.
[4] L. Vachoud, N. Zydowicz, A. Domard, *Carbohydr. Res.* **1997**, *302*, 169.
[5] P. Sorlier, A. Denuzière, C. Viton and A. Domard, *Biomacromolecules* **2001**. *2*, 765.
[6] L. Vachoud, N. Zydowicz, A. Domard, *Carbohydr. Res.* **2000**, *326*, 295.
[7] L. Vachoud, N. Zydowicz, A. Domard, *Inter. J. Biol. Macromol.* 2001, *28*, 93.
[8] A. Domard, M. Domard, in: *"Polymeric Biomaterials"*, S. Dimitriu, Ed., M. Dekker Press, New-York, 2001, p. IV/187.