SOLUTION PROPERTIES OF CHITOSAN

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Abstract

Some of the most recent works on the solution properties of chitosans are contained in two PhD-thesis from this university by Marit Walbye Anthonsen (1993)[1] and by Mette Halvorsen Ottøy (1996)[2]. The main conclusions by Anthonsen were that both homogeneously and hetereogeneously deacetylated chitosans had random (Bernoullian) distribution of acetylated and deacetylated residues. Chitosan samples with fraction of acetylated units, F_A, from 0 to 0.6 were studied by viscometry at different ionic strengths, osmometry and total intensity light scattering. At all ionic strengths the exponent, a, in the MHKS-equation $[\eta]=K \cdot M^a$, increased with increasing F_A suggesting a stiffer backbone structure of the highly acetylated chitosans possibly because of hydrogen bonding between the carbonyl function in the acetyl group and OH(6) in the neighbouring units. Light scattering revealed negative second virial coefficients most probably caused by a small fraction of the chitosan entering into a reversible aggregation reaction. This made molecular weight determination by static light scattering very difficult. The problem of compositional heterogeneity was studied in more detail by Ottøy. She found that hetereogeneously deacetylated chitosan had a bimodal compositional distribution with one acid insoluble fraction with F_A from 0.88 to 0.95 and another acid soluble fraction with lower F_A and a random distribution of the two units. Two chitosan samples with F_A 0.15 and 0.52 were fractionated by preparative SEC and further studied by viscometry and SEC/LALLS. By operating with very low concentration of chitosan and by proper choice of buffer and column materials, aggregation phenomena could be avoided and molecular weights and molecular weight distributions could be determined. The MHKS-exponents obtained were in agreement with the results obtained by Anthonsen and other published data.

Keywords: Chitosan, viscosity, molecular weight, light scattering, compositional distribution.

Introduction

Studies of the solution properties of chitosan is of considerable interest both from a basic and an applied point of view. The solution properties reflect both the chemical composition and the sequence of acetylated and deacetylated units along and among the chains, as well as the chain conformations including the ability of the chains to form aggregates or other supramolecular structures. Viscosity, molecular weight and molecular weight distribution are key parameters which must be strictly controlled for any approval and use of chitosan in the pharmaceutical industry as for example as excipient in nasal drug delivery[3]. The reproducible processing of such well defined chitosans requiers both understanding and control of the important process parameters, and experimental methods used on a routine basis for chemical and physical characterisation of the products. In the present paper we first discuss data which give some insight into the deacetylation process, then we discuss how the fraction of acetylated units, F_A, in chitosans with a random distribution of acetyl groups affects viscosity and chain conformation, finally we discuss methods for both preparative and analytical determination of molecular weight and molecular weight distribution.

Composition and sequence

Chitosans can be made by deacetylation of chitin by alkali either in a heterogeneous process (high concentration of sodium hydroxide, and high temperature) or in a homogeneous process (moderate concentration of NaOH and low temperatures)[4]. Kurita and co-workers[4,5] studied chitosans prepared with both methods and proposed that the homogeneously prepared chitosans had a random distribution of the acetyl groups whereas the heterogeneously prepared chitosans had a non-random (blockwise) distribution of the acetyl groups. In a series of papers Vårum and co-workers[6,7,8,9] used high field ^1H and ^{13}C NMR spectroscopy to determine acetyl sequences in both types of chitosans and concluded that all acid soluble chitosans had a Bernoullian (random) distribution of acetyl groups. The same conclusions were independently obtained by Sashiwa and co-workers[10,11] by studying the composition of oligomers after non-random degradation with nitrous acid. These apparently conflicting results were resolved by Ottøy et al[12] who fractionated commercial, heterogeneously, prepared chitosans with different F_A^- into acid-soluble and acid-insoluble fractions. The amount of soluble material increased and the F_A-values of these fractions decreased with the time of deacetylation whereas CP-MAS ^{13}C NMR-spectroscopy yielded F_A-values for the insoluble fractions virtually constant between $F_A = 0.88$ and 0.95. Both NMR-studies and fractional precipitation[13] of the soluble material (at high pH-values) did not violate the assumption of a random distribution of acetyl groups both along and among the chains. The diminishing amount of insoluble chitosan with a composition almost identical to unreacted chitin may be identical to the crystalline regions which Kurita et al.[4,5] referred to in their work as a block structure of chitosan. It seems, therefore, that some heterogeneous or uneven swelling and penetration of NaOH into the chitin particles may be the cause of the bimodal compositional distribution in the commercial chitosans, but that this heterogeneity in the process is on a larger scale than the size of the individual chains which have been fully surrended by NaOH when being deacetylated. If swelling is the rate determining step of the deacetylation process a somewhat broader compositional distribution curve for the fully acid soluble material should probably have been expected because different parts of the chitin particles have experienced NaOH at different length of time, but no fractionation experiments carried out so far have revealed this. From these studies it was concluded that all fully water soluble chitosans have a random distribution of acetyl groups. This means that both the sequence of units along the chains and the compositional distribution of F_A among chains may be adequately determined theoretically with a single value of F_A provided that the molecular weight and the molecular weight distribution of the sample is known[13].

Chain conformation

As discussed in earlier papers from this laboratory[14,15] including a paper[16] in the present series of proceedings, the solution viscosity and the chain conformation are strongly influenced by ionic strength and by F_A. From determination of molecular weight by osmometry (number average), the exponent, a, in the Mark- Houwink- Kuhn- Sakurada (MHKS)-equation $[\eta] = K \cdot M^a$ was found to decrease with increasing ionic strength as expected for polyelectrolytes, but at all ionic strengths the value a was found to be highest for the chitosan with the highest F_A indicating a stiffening of the chain by the presence of acetyl groups. Several reports[14,17,18] on determination of the stiffness parameter B introduced by Smidsrød and Haug[19] suggest that chitosans with high F_A-values have low B-values, corresponding to the most extended conformation in the unperturbed state. Based on measured values of intrinsic viscosities and number-average molecular weights, Anthonsen did an independent calculation of the persistence lengths[17,18] at high ionic strength where the electrostatic expansion of the chitosans should be low, and obtained the results as given in *Table 1*. Anthonsen had to make some assumptions about the molecular weight distribution in her calculations based on $\overline{M}_w/\overline{M}_n$-ratioes between 2 and 2.5 for the samples, and

Table 1. Persistance length, q*, of chitosans

Parameters obtained from molecular weight and intrinsic
viscosity data at 0.1 (M) and infinite ionic strength

F_A	$q_{0.1}$ (nm)		q_∞ (nm)	
	1)	2)	1)	2)
0.6	15	15	10	11
0.15	9	9	5	4
0	7	5	4	3

1) Use of FLORY-FOX viscosity function[21]
   (non-drained random coils)

2) Use of the YAMAKAWA-YOSHIZAKI model[22]
   (worm-like chains with hydrodynamic diameter)

$$q = \lim_{n \to \infty} \left\langle \frac{l_i}{l_1} \cdot \sum_{i=1}^{n} l_i \right\rangle$$

use of two different hydrodynamic models in the calculation yielded somewhat different results. However, a markedly higher persistence length for $F_A = 0.6$ compared to $F_A = 0.15$ and 0 is evident at both ionic strength 0.1 and infinite (an extrapolated condition), and hydrogen bonding between the carbonyl function in the acetyl group and in the neighbouring units seemed a possible reason. Some preliminary data by B.T.Stokke[20] from statistical mechanical calculation of chain extention in the rigid ring approximation and including potential functions for hydrogen bonding, also indicated a chain stiffening at high F_A-values. No influence of F_A on chain extension was found when hydrogen bonding was not included in the calculation. However, no competition from hydrogen bonding to water molecules was considered in the calculation, and it is therefore somewhat premature to discuss in detail the short range interactions around the glycosidic linkages in the chain leading to the observed experimental results.

A light scattering study of Anthonsen[15] failed to give further information on the chain conformation in solution due to a reversible aggregation phenomena affecting the weight average molecular weight and the Z-average radius of gyration and leading to large negative apparent second virial coefficients. By use of gel permeation chromatography coupled to an on-line low angle laser light scattering detector and differential refractive index concentration detector (HPSEC-LALLS-RI), a bimodal molecular weight distribution was observed in which about 5% of the sample had a very high molecular weight. Electron microscopy revealed the presence of some supramolecular structures. Anthonsen could not correlate the aggregation with the chemical composition of the chitosan. She concluded that static light scattering could not readily be used due to large difficulties in removing the aggregates with standard cleaning procedures, before measurements on true molecular dispersed chitosan samples could be performed.

Molecular weight distribution

Linear polysaccharides are in general widely polydisperse with polydispersity index $p.i.=\overline{M}_w/\overline{M}_n$ above 2, where $\overline{M}_w$ and $\overline{M}_n$ are the weight and the number average molecular weights, respectively. A $p.i.$-value of 2 would be expected from a random degradation of polymer chains and corresponds to the Kuhn-distribution or "the most probable distribution". To obtain fractions of a more narrow molecular weight distribution preparative size exclusion chromatography have been used. Ottøy et al.[23] studied the effect of different column materials, the effect of different concentrations of chitosan, and the influence of different buffer ions on aggregation and on column adsorption in both preparative and analytical SEC-experiments. In analytical HPSEC-LALLS-RI experiments use of very low concentrations of chitosans (~0.1mg/ml), ammonium acetate pH 4.5 as buffer[24], and a new type of column material based on hydrophilized, macroporous monosized polystyrene beads[25], eliminated both the aggregation phenomena[15] and the reversible absorption phenomenas observed with commercial Ultrapac TSK columns[23]. Molecular weight distributions and the different molecular weight averages was obtained on chitosans with F_A ranging from 0.20 to 0.52. *Figure 1* shows the separation of two different alginates, three different chitosans (F_A of 0.20, 0.23 and 0.52) and pullulan standards on the macroporous, monodisperse and hydrophilic polymer particles[25]. This column material appears to be well suited for characterizing alginates and chitosans with varying chemical composition, as the calibration curves ($\log \overline{M}_w$ versus V_e) are almost identical.

A combination of two columns with Sepharose CL-4B and Sepharose CL-6B run in series gave good separation of two high molecular weight chitosans with $F_A = 0.15$ and 0.52. A universal plot (*Figure 2*) based on measured intrinsic viscosities of the fractions suggests a marked reversible absorption of the chitosan with the highest charge density, most possibly due
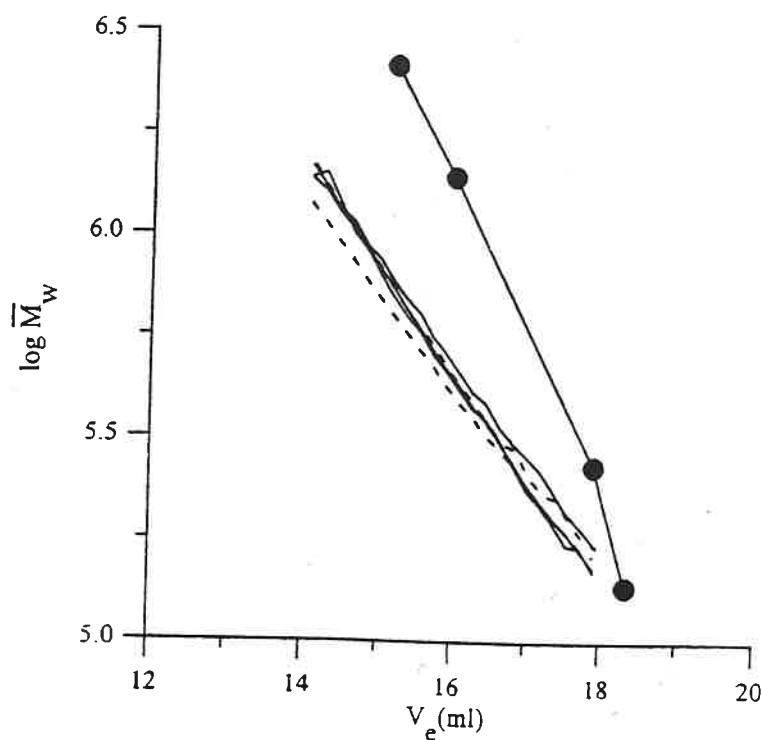

Figure 1 $\overline{M}_w$ determination of (—●—) Pullulan standards, (- - - -)alginate and (——)Chitosans with $F_A = 0.20$, 0.23 and 0.52 fractionated on column packing
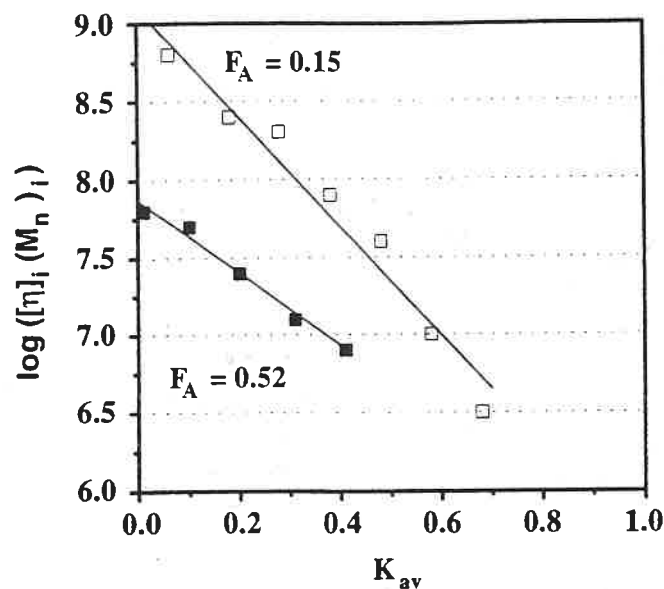
Figure 2 Semilogaritmic plot of the molar hydrodynamic volume ($[\eta] \cdot \overline{M}_n$) versus $K_{av} = \dfrac{V_e - V_o}{V_t - V_o}$ where V_e is the observed elution volume, V_o is the void volume and V_t is the total volume of the column. Buffer 0.02 M Na-acetate/acetic acid pH 4.5 containing 0.1 M NaCl. □ Chitosan with $F_A = 0.15$, ■ Chitosan with $F_A = 0.52$.
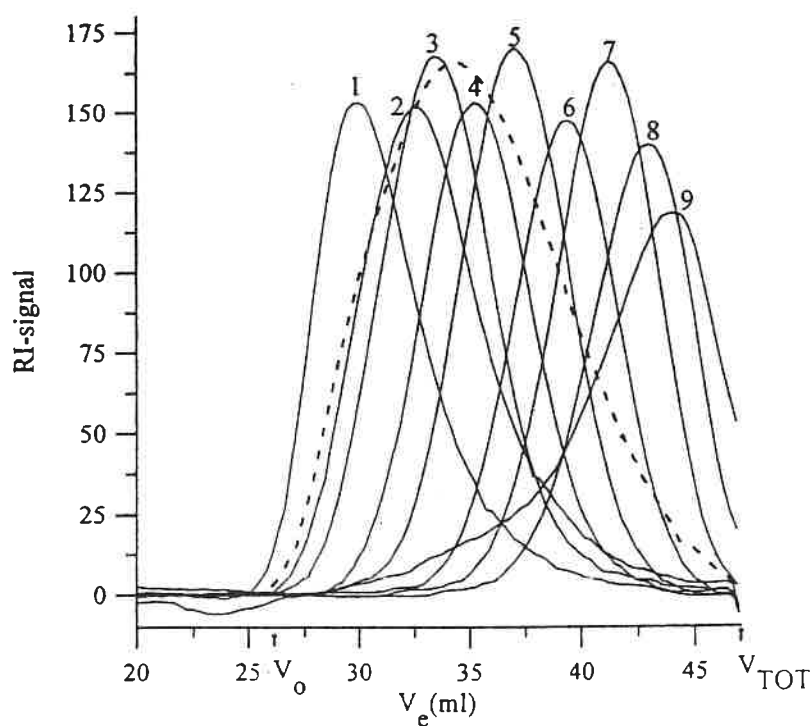

Figure 3 HPSEC-chromatograms of chitosan fractionated on anlytical TSK G 6000 PWXL and G 5000 PWXL in series. Buffer 0.2 M ammonium acetate/acetic acid, pH 4.5.

‑ ‑ ‑ ‑ ‑ ‑ ‑ ‑ Unfractionated chitosan with $F_A = 0.15$

———————— The nine fractions obtained from preparative SEC-fraction

to some fixed negative charges on the column material. Nevertheless, good fractionation was achieved as seen in *Figure 3* where the RI- signal from analytical SEC of unfractionated chitosan with $F_A = 0.15$ are given together with the results for 9 fractions obtained from the preparative column system.

Table 2
Fraction numbers (i), distribution coefficients [$(K_{av})_i$], weight fractions (W_i), intrinsic viscosities ([η]$_i$), molecular weights [$(\overline{M}_w)_i$ and $(\overline{M}_n)_i$] and polydispersity indices [$(\overline{M}_w/\overline{M}_n)_i$] of chitosan, F_A=0.15 fractionated on preparative SEC with Sepharose CL 6B - CL 4B.

i	$(K_{av})_i^1$	W_i	[η]$_i$(ml/g)^2	$(\overline{M}_w)_i \cdot 10^{-5}$(g/mole)^2	$(\overline{M}_n)_i \cdot 10^{-5}$(g/mole)	$(\overline{M}_w/\overline{M}_n)_i^2$
1	0.06	0.143	1310	7.2	4.9	1.5
2	0.18	0.161	1000	3.6	2.6	1.4
3	0.28	0.194	899	2.7	2.2	1.2
4	0.38	0.163	506	1.7	1.4	1.2
5	0.48	0.158	461	1.1	0.84	1.3
6	0.58	0.087	206	0.61	0.48	1.3
7	0.68	0.045	118	0.34	0.28	1.2
8	0.78	0.026	n.d.	0.26	0.20	1.3
9	0.88	0.017	n.d.	0.22	0.15	1.5
10	0.97	0.005	n.d.	n.d.	n.d.	n.d.

$$[\eta]_{calc} = \Sigma_{i=1}^7 (W_i [\eta]_i) = 720 \qquad (\overline{M}_w)_{calc} = \Sigma_{i=1}^9 (W_i (\overline{M}_w)_i) = 2.7$$

Unfractionated sample			833	2.7	1.3	2.1

[1] $K_{av} = (V_e - V_o)/(V_i - V_o)$
[2] n.d. = not determined

All the data from the fractionation experiment are given in *Table 2*. The unfractionated sample had a weight average molecular weight of 2.7 x 10^5 Dalton and a number average molecular weight of 1.3 x 10^5 Dalton corresponding to a *p.i.*-value of 2.1 near to the theoretical value for random degradation. The fractions had *p.i.*-values from 1.2 to 1.5 showing that fractionation had occurred in the full molecular weight range of the unfractionated sample giving fractions with $\overline{M}_w$ from 0.22 to 7.2 x 10^5 Dalton. By calculation of the weight average molecular weight of the weighted sum of all the fractions, $(\overline{M}_w)_{calc}$, a value of 2.7 • 10^5 Dalton was obtained, which was identical to the measured value for the unfractionated samples. It seems, therefore, that although some reversible absorption to the column material and possible aggregation in solution occur in preparative SEC-experiments, fractionation to more narrow molecular weight distributions is possible, but even a *p.i.*-value of 1.2 corresponds to a quite wide distribution as indicated in *Figure 3*, and preparation of monodisperse, high molecular weight chitosans would require a new and much better fractionation technique than available or even known today.

Based on the data in *Table 2* and similar data for a chitosan sample with $F_A = 0.52$, Ottøy et al.[23] obtained the following MHKS-equations in 0.02 M NaAc/HAC, 0.1M NaCl, pH 4.5:

$$F_A = 0.15: \qquad [\eta](ml/g) = 8.5 \cdot 10^{-3} \cdot \overline{M}_w^{0.92\pm0.07}, \qquad r = 0.9859 \qquad (1)$$

$$F_A = 0.52: \qquad [\eta](ml/g) = 1.1 \cdot 10^{-3} \cdot \overline{M}_w^{1.1\pm0.1}, \qquad r = 0.9750 \qquad (2)$$

The exponents in the MHKS-equations are in reasonable agreements with the results of Roberts and Domszy[26] (one F_A-value), Anthonsen et al.[15], Wang et al.[27] and Rinaudo[28] although the latter authors had lower values and smaller span in F_A (from 0.02 to 0.21) than in the study of Ottøy et al.. The values of K in the MHKS-equation are not easy to compare both because small differences in the exponent, a, affects the absolute value of K markedly, and because Anthonsen determined $\overline{M}_n$-values on samples with much wider molecular weigh distributions than the present samples. The intrinsic viscosities reflects the viscosity average, $\overline{M}_v$, of a sample which becomes identical to the weight average when $a = 1$ in the MHKS-equation

$$\overline{M}_w = \sum_i W_i \cdot M_i \text{ and } \overline{M}_v = (\sum W_i \cdot M_i^a)^{1/a} \tag{3}$$

where W_i is the weight fraction of polymer with molecular weight M_i, and a is the exponent in the MHKS-equation. The a-values of Ottøy are close to 1, and the condition for their determination will therefore yield molecular weights close to the weight average, even if the molecular weight distributions are broader than in the present case. Converting to number averages or other averages would require information about the molecular weight distributions. In many cases it seems, to avoid confusion, that it would be better to characterize a sample with the value of the intrinsic viscosity at a certain pH and ionic strength as such, instead of converting to molecular weights by one of the published MHKS-equations.

Determination of a correct molecular weight of any polysaccharide sample is still in 1997 a research project of a certain difficulty and some duration whereas determination of an intrinsic viscosity can be done rapidly in instruments well suited for routine operation. If the international community, and in particular the chitosan industry which is in an early phase of its development, could agree on some standard conditions for determination of intrinsic viscosities (for examples the condition given above), and use [η] as a relative measure of molecular weight, it would be beneficial both for the industry itself and its customers, including the scientific community.

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CHITOSAN INTERACTIONS

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Abstract

The chemical structures of chitin and chitosan are exactly the same and correspond to the copolymers of β, $(1 \rightarrow 4)$ linked glucosamine and N-acetylglucosamine. This particular architecture is characterized by the presence of various atoms and functions allowing all the known interactions in chemistry. In addition to the properties of the substrates, these interactions depend on numerous parameters which are either inherent to the structure or the crystallography of the considered copolymer, or belong to the environment in which it has been introduced. These external physico-chemical parameters are classically: the hydration, the dielectric constant of the media, pH, ionic strength and temperature. When the whole of these parameters is well controled, the behaviours of chitosan in a given situation can be understood and numerous interesting applications can be considered.

The aim of this paper is to try to demonstrate these relations through chosen examples.

Keywords : Interactions, ionisation, molecular mobility, hydrogen bonding, hydrophobicity, complexation.

Introduction

If we consider the chemical structure represented on figure 1, it corresponds to a linear copolymer of glucosamine and N-acetylglucosamine.
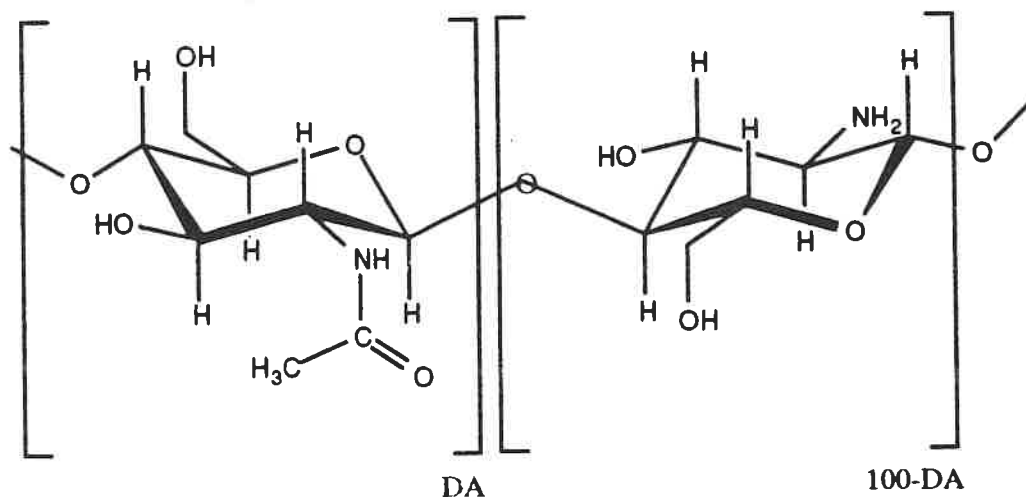

Figure 1 *Chemical structure of Chitin/chitosan*

This structure is the same for both chitin and chitosan and it bears various kinds of atoms and functions allowing approximately all the interactions known in physico-chemistry. The difference of properties which is at the origin of the two terms is only due to the difference in the balance between the two kinds of residues, characterized by the degree of acetylation well known under the symbole F_A for the ones (1) or DA for the others (2). Water is the most common media for the use of chitosan and the second important parameter tightly connected to the first is the charge density. It will be responsible not only of electrostatic interactions but also of the définition of the solubility of chitosan in aqueous media. These two parameters play an important role on the third one, *i.e* the molecular mobility which is at the origin of the kinetics aspects of the interactions and of their quantitative limits. The latter parameter is particularly important for the interactions in the solid state. These 3 fundamental parameters are intercorrelated and then, most of the interactions do not vary monotonously with one of them. The first behaviour to be understood and controled is certainly the solubility and the hydration of chitosan. It will lead to consider two kinds of interations, wether they are obtained in solution or in the solid state. Since water has been shown to be the only media allowing a possible solubilisation of chitosan under particular conditions (3), the study of this problem is somewhat restricted.

I   Interactions in aqueous media

a - Solubility of chitosan

If we consider polymer chains, their dissolution is classically weakly favoured for entropic reasons and only a favourable balance between the interactions corresponding to the polymer chain segments together and the chain segments and the solvent molecules gives the possibility to the enthalpic term to favour the dissolution(4). The entropic term depends essentially on the concentration and the molecular weight of the polymer. The interaction parameter is sensitive to structural and environmental variables. In fact, the major variable is the charge density which depends on DA and environmental parameters such as pH, ionic strength and the dielectric constant of the media. The role of the charge densisty is simply illustrated by the Katchalsky equation (5)

$$pK_a = pH + \log (1-\alpha)/\alpha = pK_0 - \varepsilon\Delta\Psi(\alpha)/KT \qquad (\text{I})$$

where pKa is the apparent acidity constant, α, the degree of neutralization of the $-NH_3^+$ functions, pK_0, the intrinsic pK of a

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charge supposed isolated and the last right term of the equation is an electrostatic parameter which depends essentially on the charge density of the polymer i.e. the number of cationic charges per length unit along the chain axis. The solubility of chitosan is observed when the electrostatic repulsions corresponding to cationic charges are more important than the attractive interactions due to low energy interactions such as hydrogen bondings or hydrophobic interactions. This solubility is also favoured by the hydration of various sites, mainly those who are charged. As a consequence, the ratio $-NH_3^+/-NH_2$ which is directly related to the charge density of the polymer is very important. Equation I shows clearly that it depends on pH, α and pK_0. The later term is specific to the structure and depends on the chain length, DA and the dielectric constant of the solvent. The chain length is particularly important in the case of the oligomers. Thus, for the first terms of the series (6), the intrinsic pK varies from the value of the monomer close to 7.7 to attain rapidely 6.5, the value of the fully deacetylated polymer (7). This value does not change when DA's vary within 0-25% (7). Although it has never been verified, it should increase up to the value of the monomer when the DA goes to 100%. Indeed for DA's over 50%, the polymer chains lose their polyelectrolyte character and must be regarded as coplymers of low DP oligomers of glucosamine and acetylglucosamine.

If we consider now the solubility, it can be explained as follows. The oligomers of glucosamine are water soluble whatever the pH up to DP 7 (8). In this case, two parameters are important : the charge density, due to higher values of pK_0, is high in a large range of pH and the entropic parameter is low compared to the polymer. Nevertheless, it has been shown that the dissolution of the oligomers isolated in the free amino form was conditioned by the memory of the crystalline arrangement in the solid state with preservation of some supra-molecular organizations corresponding to assemblies of oligomer chains (9). In the case of the polymer, the chain length does not play the major role. If we consider fully deacetylated chitosans, the insolubility occurs generally over pH 6 when the cationic charges become insufficient to counterbalance the attractive interactions. The latter can be attributed either to hydrogen bondings or to hydrophobic interactions involving free amine groups. When DA increases, the solubility range increases for two reasons. First for the increase of the steric hindrance related to the increase of the number of acetyl groups and then, possibly for an increase of the value of the intrinsic pK. As a consequence, chitosans with acetyl contents near 50% are water soluble whatever the pH. The role of DA and molecular weight on the solubility and aggregation of chitosan has been extensively studied by Smidsrød and coworkers (10).

In addition to pH, external parameters can affect the solubility by their influence on the value of pK_0 or on the effective charge density. We can mention the effect of ionic strength. This effect is particularly well illustrated by the precipitation of chitosan in the salt form, for example in the hydrochloride form, when the pH is lowered below 2. In this case, the ionic strength brought about by the high concentration of protons ($>10^{-2}$ M.) but also by the polyelectrolyte itself leeds to a screening of the charges beared on the polymer chains. The consequence is a decrease of the effective charge density favouring the depletion of the chain and then the interactions between chain segments responsible for the precipitation. This behaviour is classically used by those who want to precipitate polyelectrolytes in their salt form in water. The same reason allows us to explain the insolubilty of chitosan (in the free amino form) in strong acids such as hydrochloric acid for concentrations of the acid over 0.1M. Nevertheless, the salt is formed and this behaviour allows an easy preparation of salts in the solid state with the minimum steps and then a low cost (11). Another possibility to reduce the solubility of chitosan ammonium form concerns the addition of a water soluble solvent to a solution of chitosan. For particular concentrations of the solvent, we observe the formation of gels (12), or an important increase of viscosity (13). This behaviour must be related to the formation of local hydrophobic contacts, or hydrogen bondings, responsible for a more or less important physical reticulation of chitosan chains. This situation is due to the decrease of the dielectric constant of the solvent responsible for an important change of the value of the pK_0 of chitosan but also of the pK of the acid used to form the salt, in favor of an increase of the proportion of free amine groups. This behaviour is favoured by the use of chitosan salts of weak acids such as acetic acid (14) and can be used to do chemical modifications on the amine groups like reacetylation of chitosan in a highly solvated form (15).
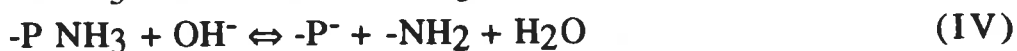
b - Electrostatic interactions

If we consider the chemical structure shown on figure 1, and equation I, the polyelectrolyte constituted by chitosan chains is obviously a weak polyelectrolyte due to the pK_0 value of the ammonium form but also to the relatively low charge density. Indeed, if we consider the crystallographic length of a glucosidic unit (3), and a fully deacetylated chitosan, this means that for this optimal situation, we will have at maximum 1 cationic charge every 0.514 nm. This value will increase rapidly with the DA and the neutralization degree. This is certainly the reason why chitosan has not found significative applications as ion exchanger or as a stationary phase for ion exchange chromatography.

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The particular polyelectrolyte character of chitosan is much more interesting when we consider the second kind of interactions of polyelectrolytes represented by polyanion/polycation complexes (PA/PC complexes). Polycations are rare in nature and chitosan is the only case in the family of polysaccharides. It will be thus highly solicited for this kind of interactions and we can remember that both chitosan and PA/PC interactions are at the origin of the colloidal titration developped to titrate polyanions (16). The PA/PC interactions depend on the charge density of each kind of polymer and on the pK_0 of the charged sites involved in the interaction. If we represent chitosan by $-NH_3^+$ functions, the optimal conditions for the formation of such complexes can be simply schematized by:

$$-P^-, A^+ + -NH_3^+, B^- \Leftrightarrow -P\,NH_3 + A^+, B^- \qquad (II)$$

where A^+ and B^- are the counterions of the polyanion and polycation respectively. Two other reactions must be considered :

$$-P\,NH_3 + H^+ \Leftrightarrow -PH + -NH_3^+ \qquad (III)$$
$$-P\,NH_3 + OH^- \Leftrightarrow -P^- + -NH_2 + H_2O \qquad (IV)$$

Therefore, the complex formation is maximum when the charge densities are maximum, *i.e.* when the ionization of each kind of polymer is maximum. In this case, as written in equation II, the complete formation of the complex will be achieved when the total amount of negative charges will be exactly neutralized by the same amount of ammonium functions. As a consequence, the media will contain only the complex which is neutral and then generally insoluble in water and the stoichiometric amount of AB salt liberated during the interaction (17). In relation with equations III and IV, the stability of the complex depends on the pK_0 values of the anionic and cationic sites. Thus, when the anionic site corresponds to a weak carboxylic acid, the complex is easily destroyed by a decrease of pH. and, on the contrary, the complex is only slightly formed when chitosan in the salt form is added to a solution containing the acidic form of the polyanionic polymer. This is the case, for example, of the electrostatic interactions between chitosan and collagen (18). On the contrary, when the anionic sites correspond to strong acids such as sulfates, whatever the pH, these sites are always protonated and the complex is formed and stable at all pH's <6.5 (17). An interesting case corresponds to moderately strong carboxylic acids like hyaluronic acid for which the pK_0 is found close to 2.9 (19). In this case, the complex is also formed and stable even in acidic media (17) and we can observe a decrease of pH during the formation of the complex due to a progressive deprotonation of the carboxylic sites. Thus, addition of

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a solution of chitosan hydrocloride which pH is close to 4.3 to a solution of hyaluronic acid in the free acidic form initially at pH 3.85 induces a continuous decrease of pH up to 3.61 where the complex is completely achieved. This is possible simply because the pH remains always over the pK_0 of hyaluronic acid and then the residual uncomplexed sites are at a pH sufficiently over this pK_0 to be easily deprotonated. When the pK_0 continue to increase we can have an intermediate situation for which, in relation with the pK_0 but also the concentration of the polymers, only a part of the carboxylic sites initially in the free acidic form can be deprotonated in the course of the complex formation (17).

Another kind of electrostatic interactions concerns the behaviour of chitosan in the presence of dispersions. These dispersions can be of mineral or organic nature. Thus according to the experimental conditions, chitosan is known to be an excellent flocculant or dispersant of various systems such as kaolin particles, lipidic miceles and living cells. This particular phenomenon is to be related to the fact that in a general manner, the surface charge of particles constituting a dispersion as those mentioned above is negative. As a consequence, the cationic character of chitosan in the ammonium form provides this polymer a very large field of applications. The electrostatic interactions will be thus essential and the same parameters as those enumerated above will be considered. Therefore, the charge density will play the major role. In addition, the dimensions of the particles and the polymer will also have an important influence on the mechanism. In the case of kaolin particles, their average dimension can be considered as very important compared to that of classical chitosan chains even for the highest molecular weights we can test. Therefore, the interactions which generally occur between several chitosan chains and one particle. The consequence is a progressive neutralization of the net charge of the particle leading to a juxtaposition of negative and positive domains on the same particle. Then, the flocculation is maximum when this charge becomes zero and the mechanism of flocculation is of mosaic type *i.e.* a process in which particules collapse together by means of interactions between domains of opposite charges of different particles (20). In the case of kaolin, the charge density of the mineral particles is very low and the flocculation is maximum for relatively low amounts of chitosan which are of the order of the percent. The sorption process generally obeys to Langmuir laws (21) and, depending on the molecular weight of chitosan chains but in relation with the low charge density of the surface of kaolin particles, the chitosan chains are more or less weakly in interaction with the surface of kaolin. Thus, for the longest chains, only a few points of anchorage on chitosan chains are obtained and the flocculation is

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obtained sooner (21). The situation is more complex when both the charge density of the particles is higher and their dimensions smaller. This case is encountered when chitosan is contacted with organic particles, in particular lipidic aggregates more or less highly ordered like aggregates of fatty acids or liposomes of phospholidids. In this case, the problem is complicated by the stability of these structures and external parameters such as pH, ionic strength concentration and temperature play a much important role compared to the previous case. Thus, high charge densities, high ionic strength and temperature are factors which destabilise this kind of dispersions and the pK of the ionic sites beared by the hydrophylic head of the lipid is very important. As a consequence, the most complex situation is encountered with simple carboxylic fatty acids like undecylenic acid (22). In this case, the most stable dispersions are observed for the lowest ionic strength and pH's near the pK of the carboxylic sites. Nevertheless, the highest interaction between chitosan and this kind of lipid corresponds to a pH close to 5.8 which is a compromise between this pK and the pK_0 of chitosan. The charge density of these particles is much higher compared to that of kaolin and the amount of chitosan necessary to observe the flocculation process is relatively important compared to the previous case. It is shown that it only depends on the charge number beared by the particles and those brougth about by chitosan chains. Thus, for a given situation of lipidic dispersions (pH, ionic strength, concentration and temperature), the number of charges necessary to observe the maximum flocculation is imposed and does not vary in the range where the pK_0 of chitosan is constant i.e. for DA's located between 0 and 25% (23). This means that for a given molecular weight of chitosan, the more the DA is high, the more the amount of chitosan necessary to observe the maximum flocculation increases. Contrarily to the previous case, the particle dimension remains largely below the micron meter and then, the role of the molecular weight on the mechanism of flocculation is very important. Thus, for chitosans having the same DA value, the amount of chitosan necessary to observe the maximum flocculation decreases with the molecular weigth to reach a limit value where an inversion of this law is observed. This behaviour can be simply related to the evolution of the mechanism of flocculation when the molecular weight increases. Thus, for a first range of molecular weights, the mechanism is essentially of mosaic type but, the number of anchorage points decreases as the molecular weight of the polymer increases. As explained above, the zero charge of the particle is more rapidly achieved as the molecular weight of the polymer increases and then needs a lesser amount of chitosan. For a critical value, the chain length becomes sufficiently high to allow the second mechanism of flocculation of intraparticular type to occur. Then, the amount of chitosan necessary to observe the

maximum flocculation increases suddenly. It is very interesting to associate this behaviour to the elicitation process observed when chitosan is placed in the presence of living cells. Indeed, these cells are similar to charged particles and chitosan is well known to induce their agglutination *i.e.* their flocculation where the maximum elicitation is generally observed. The same role of the molecular weight as above is observed on the elicitation process when some plant cells are subjected to chitosan chains (24). As expected by the theory (20), when the intraparticle mechanism of flocculation becomes preponderant, the flocs are fractal systems corresponding to highly branched structures where particles of lipid aggregates are interconnected by means of chitosan chains (25).

c - low energy interactions

The family of interactions regrouping hydrogen-bonding, Van der Waals and hydrophobic interactions plays an important role in numerous circumstances where chitosan chains are subjected to chemical structures allowing this kind of interactions. Certainly due to the difficulty of their identification and quantification, no much works were devoted to their study. In addition, in numerous circumtances, they are involved together. We can remember the mechanism of aggregation of chitosan in the salt form (13), the precipitation when increasing pH or the interaction with some non-ionic dyes (3). We can also mention the case observed when chitosan is contacted with collagen (26). In this case, for weak proportions of chitosan, the interaction is purely electrostatic and the triple helix conformation of the protein is entirely preserved. When a great excess of chitosan is added , we observe a partial deprotonation of chitosan followed by a strong interaction with collagen certainly by means of hydrogen bonding and hydrophobic interactions (26). The interaction is so strong that we observe a denaturation of the protein into single α helixes. These low energy interactions play also an important role when chitosan is reacetylated in an hydro-alcoholic media (15,27).

II Interaction in the solid state
We will avoid to consder the crystallographic aspect of the problem which has been extensively discussed in the litterature (3). The solid state is essentially refered to chitosan chains where the amino groups are mainly in the free amino form. This function is also very sensitive to the presence of numerous substrates and the interaction the most widely studied in the litterature concerns the complexation with metals. If we consider chitosan dispersions in water, in the presence of metals in solution, the interaction must be studied in terms of kinetics and thermodynamics. Numerous parameters have an influence on these two aspects of the problem.

They are physical or chemical. It is also very important to know the physico-chemistry of the metals to which chitosan is subjected. Unfortunately, due to the difficulty of the problem but thanks to the improvement of calculations, only recent papers have discussed this very important problem (28, 29) thus allowing to avoid some misinterpretations due to metal precipitation or to an unexpected form of the metal. The most important parameters to take into account are the chain accessibility and mobility. They depend on various terms which can be discussed as follows.

a - crystallinity and morphology of the polymer

It is well known that crude chitosans are obtained from chemical treatments performed in heterogenous media. This is the reason why this chitosan is highly crystalline, generally around 35-40% (30). Only the amorphous domains of these systems are available for possible interacions. In this case, the amorphous domains are represented essentially by crystallinity failures present in the native organisation or produced during the various treatments leading to chitosan. These amorphous domains are not easily accessible and the chitosan particles have strong mechanical properties and have their specific surface area which is not modified in contact with water even under heavy stirring. This parameter depends on the total contact area corresponding to the amorphous domains *i.e.* to the sum of the area represented by the contour of the particle and the internal area represented by the pores. The only possibility to increase this area is to decrease both the crystallinity and the particle size. The first possibility consists to grind the crude particles and to select the smallest size particles (31). Nevertheless, this method is limited by the fact that it is not possible to easily obtain particles below a certain limit of size and also by the fact that it has no significative influence on both the total internal area and the crystallinity. The problem can be solved by the use of particles of low crystallinity and very high specific area. This kind of particles is represented by lyophylisates of chitosan in the salt form. This solid form has a crystallinity which is both weak (10-15%) and corresponds to relatively small crystallites. The very expanded form of the lyophilisate has also the advantage to have very poor mechanical properties. When this particles are placed in water, at a pH where chitosan is not soluble, the dispersion is maximum and, as a consequence, the hydration is maximum leading to an important decrease of the crystallinity which becomes certainly very close to zero (30).

b - Hydration: molecular mobility

The interaction between chitosan dispersions and metal ions depends on diffusional and interactive parameters. Water plays a

major part on the two aspects of the problem. Indeed, hydration of chitosan is very important since it plastifies the chains thus allowing the amorphous domains to be over their glass transition temperature and then to have a maximum mobility favouring both the diffusional and interaction parameters. This water is also the vector of the metal species and the agent of the hydration of the interaction sites necessary to the complexation. As a consequence, the hydration of chitosan, in particular the kinetics of hydration of chitosan, plays an important role on the mechanism of the metal uptake. It is important to consider that it needs at least 10 hours for the most accessible form represented by a lyophilisate. As a consequence, the kinetics of metal uptake obtained after this hydration time are very rapid on this form and show that the diffusional parameter is not the limiting factor. On the contrary, when hydration is unsufficient, the kinetics reflect essentially the hydration kinetics instead of the kinetics of the metal uptake (30).

c - Structural and configuarational parameter

The stuctural aspect of the interaction is very important. It is first important to know the exact chemical structure of the metal ions present in solution in the experimental condition. Some softwares allow to know relatively well the diagramme of repartition (28, 29). This knowledge allows also, for a given metal, to define the best conditions of pH and ionic composition for an optimal complexation. For numerous metals, it is clearly shown that the only sites of complexation on chitosan chains are the free amino functions. For numerous metals such as U, Cu, Al, Fe, Ag the complexation mechanism is very similar. For example, the maximum uptake of uranyl or silver ions is the same and corresponds to 1 metal atom for 2 amino functions. This behaviour let also to suppose that the complexation constant can be considered as infinite (29).

Conclusion

The only use of the two parameters represented by the charge density and the degree of acetylation of chitosan allows us to obtain all the interactions known in physico-chemistry. In all the situations, the biological properties of chitosan are quite preserved. It seems important to have in mind this potenty before to do some onerous chemical modifications which generally completely modify the biological behaviour of chitosan.

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Determination of Mark-Houwink-Sakurada Equation Constants for Chitosan

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Abstract

The intrinsic viscosity of a polymer solution is generally correlated with the molecular weight of the polymer through the Mark-Houwink-Sakurada (MHS) equation, which strictly holds for monodisperse polymers. For polydisperse polymers such as chitosan, a numerical method with a polydispersity correction factor would be more appropriate. Twenty eight chitosan fragments with degree of acetylation of 25 ± 3 % were prepared by several chemical and physical methods in the molecular weight range of 2.2×10^4 - 2.9×10^6 Daltons. We obtained the following equation from intrinsic viscosity and size exclusion chromatography data : $[\eta] = 2.80\times10^{-5}\ M_v^{0.93}$, where $[\eta]$ is the intrinsic viscosity in 0.1 M acetic acid / 0.02 M NaCl (in $dL.g^{-1}$) and M_v is the viscosity average molecular weight. The MHS constants were compared with the literature values.

Keywords: Chitosan, Mark-Houwink-Sakurada equation, intrinsic viscosity, molecular weight, polydispersity.

Introduction

The intrinsic viscosity of a polymer solution varies with the polymer molecular weight for a homologous series according to the Mark-Houwink-Sakurada (MHS) equation[1]

$$[\eta] = K\,M^a \tag{1}$$

where K and a are constant for a given solute-solvent system and temperature. The equation is strictly valid for monodisperse polymer samples. If the polymer is polydisperse, the intrinsic viscosity is related to an average molecular weight, appropriately termed the viscosity average by Flory[2], and equation (2) must be used:

$$[\eta] = K\,M_v^{\ a} \tag{2}$$

where $[\eta]$ is the weight-average intrinsic viscosity and M_v is the viscosity-average molecular weight. In general, M_v is not experimentally accessible. Therefore, the evaluation of constants K and a requires the knowledge of the molecular weight distribution (MWD). For instance, the use of polydispersity correction factor has been proposed[3], leading to the following form of the MHS equation:

$$[\eta] = K\,q_{MHS}\,M_w^{\ a} \tag{3}$$

The polydispersity correction factor, q_{MHS}, can be evaluated[4] according to equation (4):

$$q_{MHS} = (M_w / M_n)^b\,(M_z / M_w)^c \tag{4}$$

where M_n, M_w, M_z are the number-average, weight average and z-average molecular weight, respectively ; and b and c are empirical constants. Combining equations (3) and (4), one obtains:

$$[\eta] = K\,M_w^{\ a}\,(M_w / M_n)^b\,(M_z / M_w)^c \tag{5}$$

Equation (5) shows that the intrinsic viscosity, [η], correlates with M_w through polydispersity parameters. Constant c depends only on a according to[5]:

$$c = 0.113957 - 0.844597\,a + 0.730956\,a^2 \qquad (6)$$

Constant b depends on a and (M_z / M_w):

$$b = A1 + A2[\,(M_z / M_w) - 1\,]^{A3} \qquad (7)$$

where constants A1, A2 and A3 depend on a through the following least square polynomials:

$$A1 = 0.048663 - 0.265996\,a + 0.364119\,a^2 - 0.146682\,a^3 \qquad (8)$$
$$A2 = -0.096601 + 0.181030a - 0.084709\,a^2 \qquad (9)$$
$$A3 = -0.252499 + 2.31988a - 0.889977\,a^2 \qquad (10)$$

The method was tested for polystyrene samples having different MWD[6], as well as for various polymers with different MWD[4] (M_w/ M_n up to 200 and M_z/ M_w up to 9).

Viscometric constants for chitosan have been reported in various solvents[7-12]. However the constants were generally determined in the medium-high molecular weight range. Wide range of values for both constants, a and K, have been reported, even in the same solvent and for chitosan with similar degree of acetylation (DA). Furthermore, the polydispersity of chitosan samples have not been taken into consideration in many reports.

The objective of this work was to determine the values of a and K by the empirical polydispersity correction factor method, for chitosan fragments prepared by various chemical and physical methods, in a wide molecular weight range encompassing 3 orders of magnitude (10^4 - 10^6 Da).

Materials and methods

Materials

Shrimp-shell chitosan, with a nominal DA of 20 %, was purchased from Nova- Chem. Ltd. (Halifax, Nova Scotia, Canada) and was purified. Pullulan standards ($5.88 \leq M_w \leq 1660$ kDa; $1.06 \leq M_w/M_n \leq 1.19$) were purchased from the American Polymer Standards Corporation (Mentor, Ohio). Acetic acid (HAc) and sodium acetate (NaAc) were of HPLC grade, sodium nitrite and NaCl were of analytical grade and all other chemicals were of reagent grade.

Fragmentation

Fragmentation was carried out by various chemical (hydrolysis with HCl, oxidation with $NaNO_2$ or H_2O_2) and physical (sonication) methods. Hydrolysis was performed in a spherical flask equipped with a vertical condenser at 65°C for 5 h. with different HCl concentrations in the range 0.1-1.5 N. Oxidation with sodium nitrite was carried out at 23°C for 4 h. using different molar ratios of $NaNO_2$/glucosamine, in the range of 0.01-0.06. Oxidation with hydrogen peroxide was performed at 23°C for 24 h., using different molar ratios of H_2O_2/glucosamine, in the range of 6.6-13.2. Sonication was performed with an ultrasonic liquid processor (Model XL 2020, Heat Systems, Inc., New York, USA) operating at a frequency of 20 kHz. Different fragments were prepared by changing the chitosan concentration (between 0.2 and 1.0%), ultrasound power (between 70 and 120 W) and solution temperature between (5 and 35°C).

After fragmentation, the solution was neutralized with 1.0 N NaOH to precipitate the chitosan and the resulting polymer was centrifuged, washed with deionized water and lyophilized.

Viscometry

Intrinsic viscosities of chitosan fragments were measured at 25°C in a capillary viscometer (Model AMV-200, Paar Physica USA Inc., Edison, N.J.) in 0. 1 M HAc / 0.02 M NaCl.

Size Exclusion Chromatography

The MWD and average molecular weights of the fragments were determined by size exclusion chromatography (SEC) on a HPLC/SEC instrument (Hewlett-Packard, Model 1050) fitted with a refractive index detector. Separation was achieved at 35°C using a Toso Haas-TSK gel column (GMPW$_{XL}$, 30 cm × 7.8 mm) with 0.25 M HAc / 0.25 M NaAc as the eluent at a flow rate of 0.4 ml.min^{-1}. Monodisperse pullulan samples were used as standards. A calibration curve of pullulan molecular weight *vs* elution volume was used in order to convert the elution volume scale for chitosan samples into a molecular weight scale. Average molecular weights (relative to pullulan) were calculated from the resulting chromatograms.

Degree of acetylation

Elemental analysis of the fragments prepared from various methods was performed using a Carlo-Erba 1108 Elemental Analyzer (Model EA 1109, CHN, FISONS). The degree of acetylation of the chitosan fragments was calculated from the C/N ratio. The degree of acetylation for the original chitosan and chitosan fragments was 25±3 %. The observed standard deviation is within the experimental uncertainty, indicating that no significant change in substitution occurred during fragmentation.

Polydispersity correction factor

An iterative procedure was used to calculate the polydispersity correction factor, q$_{MHS}$ (equation 4). An initial value was first computed for each sample using equations 6 to10, assuming *a* is equal to 1. The (log [η] - log q$_{MHS}$) values were plotted against of log M$_w$, which yielded a straight line whose slope provided a new estimate for *a*. The latter was used to calculate a new value of q$_{MHS}$. The procedure was repeated until arriving at two successive values for *a* which differed by less than 0.001.

Results and Discussion

The average molecular weights, M$_n$, M$_w$ and M$_z$, the polydispersity parameters, (M$_w$/M$_n$) and (M$_z$/M$_w$), the intrinsic viscosity in 0.1 M HAc / 0.02 M NaCl at 25°C, [η], and the polydispersity correction factor, q$_{MHS}$, for 28 chitosan fragments are given in Table 1. Figure 1 shows the plot of (log [η] - log q$_{MHS}$) versus log M$_w$. The resulting MHS equation for chitosan in the M$_w$ range of 2.2×10^4 - 2.9×10^6 Da was :

$$[\eta] = 2.80 \times 10^{-5} \, q_{MHS}. \, M_w^{0.93} = 2.80 \times 10^{-5} \, M_v^{0.93} \tag{11}$$

where [η] is expressed in dL.g^{-1}. The polydispersity correction factor was nearly unity suggesting that our samples have a relatively narrow molecular weight distribution (Table 1).

Table 1 : Average molecular weights, polydispersity correction factor, q_{MHS}, and intrinsic viscosity ([η]) in 0.1 M HAc / 0.02 M NaCl at 25°C.

Sample	M_n (kDa)	M_w (kDa)	M_z (kDa)	M_w/M_n	M_z/M_w	q_{MHS}	[η] (dL/g)
1	37.6	74.9	117.9	1.97	1.57	0.975	0.89
2	48.1	105.6	188.3	2.20	1.78	0.968	1.27
3	48.0	104.7	188.0	2.18	1.80	0.967	1.18
4	492.9	993.0	1628	2.01	1.64	0.972	10.91
5	820.9	1733	2880	2.11	1.66	0.971	21.02
6	35.9	70.2	111.5	1.95	1.59	0.974	0.92
7	59.2	117.4	183.9	1.98	157	0.975	1.45
8	34.3	65.1	101.6	1.90	1.56	0.975	0.90
9	12.5	22.1	34.0	1.77	1.54	0.976	0.33
10	31.5	58.5	88.2	1.86	1.51	0.977	0.78
11	101.1	189.4	282.2	1.87	1.49	0.978	2.08
12	52.4	103.1	158.8	1.97	1.54	0.976	0.95
13	147.7	294.2	441.5	1.99	1.50	0.977	2.54
14	34.6	64.1	96.5	1.85	1.51	0.977	0.90
15	411.8	882.6	154.6	2.14	1.75	0.969	10.32
16	610.6	1367	2423	2.24	1.77	0.968	15.45
17	1046	2435	4212	2.33	1.73	0.969	21.85
18	1096	2492	4275	2.27	1.72	0.969	22.69
19	1164	2908	5494	2.50	1.89	0.964	23.60
20	579.4	1270	2233	2.19	1.76	0.968	11.88
21	294.2	563.5	878.8	1.92	1.56	0.975	5.84
22	317.4	605.3	977.3	1.91	1.61	0.973	5.95
23	744.4	1676	2984	2.25	1.78	0.967	15.51
24	785.0	1671	2820	2.13	1.69	0.970	14.21
25	564.9	1169	1961	2.07	1.68	0.971	15.11
26	654.2	1456	2523	2.23	1.73	0.969	16.23
27	618.8	1385	2454	2.24	1.77	0.968	16.05
28	623.5	1345	2272	2.16	1.69	0.970	14.53

Chitosan fragments were prepared by hydrolysis (samples 1-5) ; oxidation with hydrogen peroxide (samples 6-8) ; and sodium nitrite (samples 9-14) ; and sonication (samples 15-28).

Table 2 compares the values of MHS equation constants, a and K, obtained in this work with the values published in the literature [7-12]. The values for a ranges from 0.59 to 1.26 in the literature compared to 0.93 obtained in this work: Similarly, the values for K ranges from 2.0×10^{-1} to 3.0×10^{-5}, compared to our value in the order of
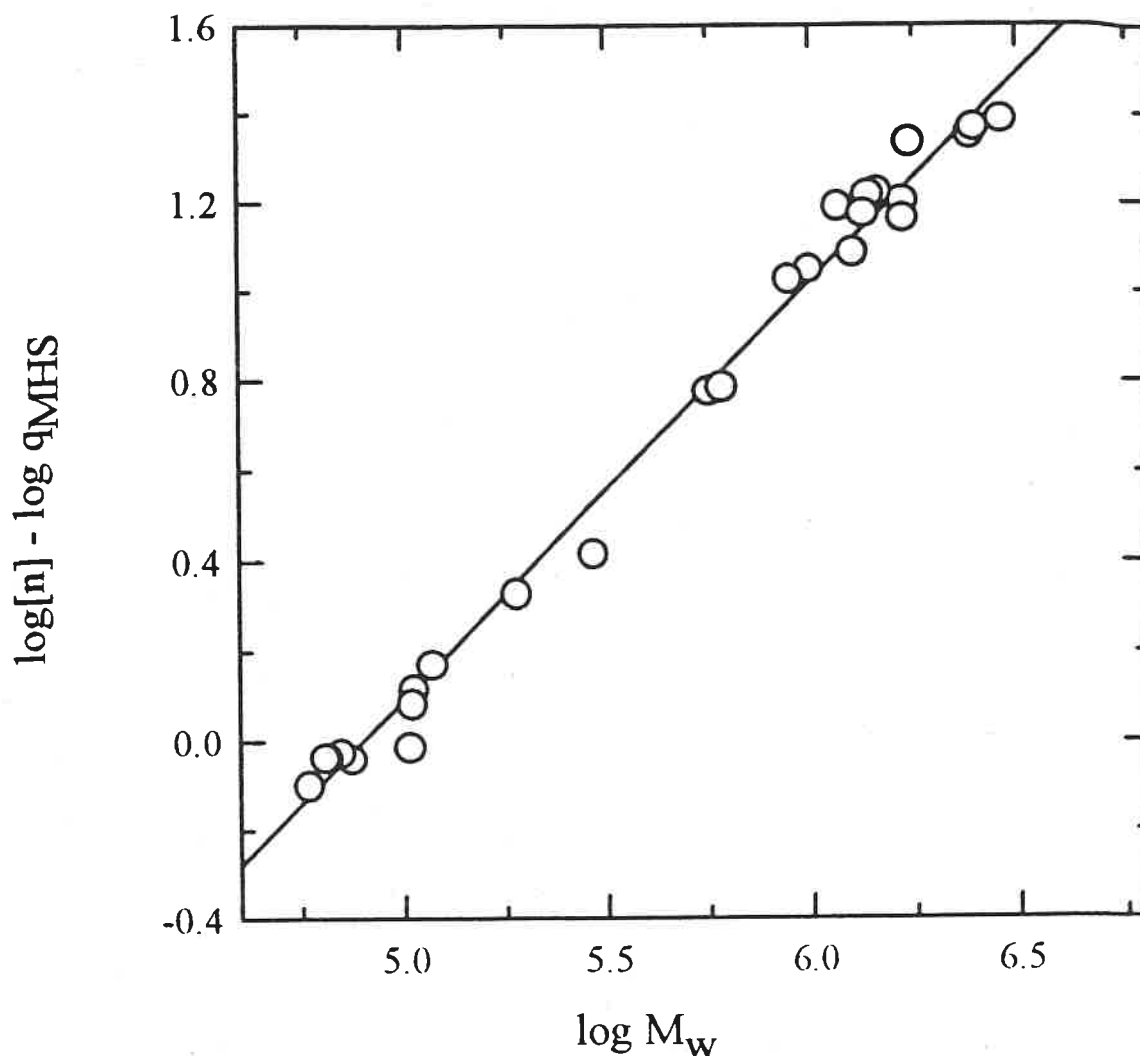
Figure 1: (log [η] - log q_{MHS}) as a function of log M_w for chitosan fragments in 0.1 M HAc / 0.02 M NaCl at 25°C.

10^{-3}: The values of both constants K and a depend on the nature of polymer, the solvent, and the temperature. In the literature, there is scarcely any information about the polydispersity of the chitosan samples used. Reported values of a vary with the ionic strength of the solvent and DA of the polymer, as shown in Figure 2. Generally, a decreases with an increase in ionic strength. It also exhibits a slight increase when DA increases[10]. Both effects are due to the polyelectrolyte nature of chitosan. The polymer conformation is sensitive to its charge density, which depends on DA, the pH as well as the ionic strength of the solvent. An increase in acetylation and/or a decrease in ionic strength reduce electrostatic repulsions, facilitating intermolecular hydrogen bonding. As a result, the conformation of chitosan would be of a linear stiff chain. A more detailed discussion of the influence of charge effects on molecular conformation of polyelectrolytes can be found elsewhere[13-15].

Table 2: MHS equation constants for chitosan in solvents of varying ionic strength, μ.

Solvent	T (°C)	DA (%)	μ (M)	K (cm^3.g^{-1})	a	Molecular weight range (kDa)	Ref.
0.1 M HAc 0.02 M NaCl	25	25 ±3	0.02	2.80×10^{-3}	0.93	22-2900	this work
0.2 M HAc 0.1 M NaCl 4M urea	20	9	0.1	8.93×10^{-2}	0.71	163-492	7
0.1 M HAc 0.2 M NaCl	25	≈ 20	0.2	1.81×10^{-3}	0.93	48-630	8
0.1 M HAc 0.02 NaCl	25	≈ 20	0.02	3.04×10^{-5}	1.26	48-630	8
2% HAc 0.2 M NaAc	25	15 ±3	0.2	1.38×10^{-2}	0.85	61-150	9
0.2 M HAc 0.1 M NaAc	30	31	0.1	1.04×10^{-4}	1.12	477-2510	10
0.2 M HAc 0.1 M NaAc	30	16	0.1	1.42×10^{-3}	0.96	536-1850	10
0.2 M HAc 0.1 M NaAc	30	9	0.1	6.59×10^{-3}	0.88	211-1260	10
0.2 M HAc 0.1 M NaAc	30	0	0.1	1.68×10^{-2}	0.81	194-937	10
0.5 M HAc 0.5 M NaAc	25	29.5	0.5	1.99×10^{-1}	0.59	115-1590	11
0.3 M HAc 0.2 M NaAc	25	2	0.2	8.20×10^{-2}	0.76	100 - 600	12
0.3 M HAc 0.2 M NaAc	25	10.5	0.2	7.60×10^{-2}	0.76	100 - 600	12
0.3 M HAc 0.2 M NaAc	25	21	0.2	7.40×10^{-2}	0.76	100 - 600	12

In polydisperse systems, the measured intrinsic viscosity relates to the viscosity average molecular weight, M_v. Since the polydispersity correction factor of our chitosan samples is close to unity (Table 1), M_v is almost equal to M_w. Should q_{MHS} be much lower than unity, *i.e.* high degree of polydispersity, then the constant K would be different. Since this information is not available in most studies, it is difficult to compare our value for K with others in the literature.
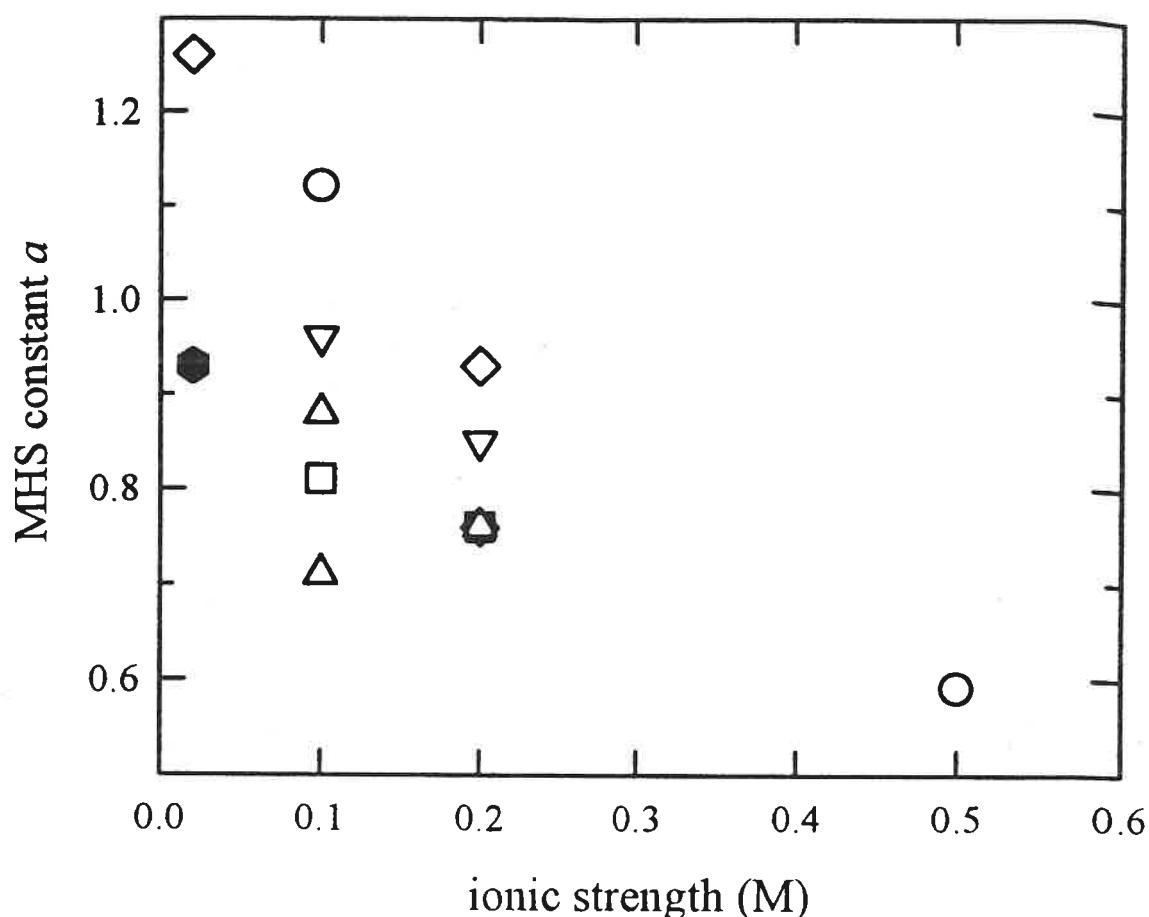
Figure 2: Exponent *a* as a function of the ionic strength of the solvent for chitosan having an approximate DA equal to zero (square), 10% (upside triangle), 15% (downside triangle), 20% (lozenge), 25% (diamond) and 30% (circle). Open symbols are for literature results, while the full symbol refer to this work.

However, there is at least one study by Roberts and Domszy[8] which takes into account the polydispersity value of chitosan. Our study and that of Roberts and Domszy used chitosan in a similar range of molecular weight, of a comparable DA and in the same solvent system. There appears to be a discrepancy between our values and those of Roberts and Domszy for both constants *a* and *K*. Roberts and Domszy reported a value of 1.26 for *a*, compared to our value of 0.93. A cursory evaluation would reveal that a value of 1.26 corresponds to a stiff linear conformation whereas a value of 0.93 indicates a conformation which is more flexible. This disparity could be due to differences in the origin of chitosan and the preparation of the fragments. Furthermore, different methods were used to determine the molecular weights and to take into account the polydispersity. Roberts and Domszy determined the number-average molecular weight by end-group analysis, whereas we used SEC which is a relative method. In addition Roberts and Domszy used a statistical function to describe polydispersity, assuming that fragmentation of chitosan occurred by random scission. It will be desirable to obtain a SEC universal calibration curve, [η]M *vs*

elution volume. Alternatively, a combination of two methods such as light scattering to obtain the absolute molecular weight and SEC to provide information on the molecular weight distribution could yield more reliable data for MHS equation constants.

Conclusions

The viscometric constants, a and K, for chitosan were determined in 0.1 M acetic acid / 0.02 M sodium chloride at 25°C. Twenty eight chitosan fragments with DA of 25+3% were prepared by various chemical and physical methods in the molecular weight range of 2.2×10^4 to 2.9×10^6 Da. The following Mark-Houwink-Sakurada equation :

$$[\eta] = 2.80 \times 10^{-5} . M_v^{0.93}$$

was obtained, where the intrinsic viscosity $[\eta]$ is expressed in $dL.g^{-1}$.

Acknowledgments

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Physico-chemical characterization of chitosan in dilute solution

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Abstract

Three commercial samples varying in degree of acetylation (DA) were characterized in terms of the average molar masses M_n, M_w, the second virial coefficient B, the radius of gyration $\langle R_g^2 \rangle_z^{1/2}$, and the intrinsic viscosities [η] by membrane osmometry, static light scattering, and capillary viscometry in acetate buffer of pH 4.5. Using the model for wormlike chains with excluded volume and a special logarithmic molar mass distribution for the interpretation of data, the persistence length was determined as $L_P = 6$ nm.

The samples were also frationated on Sepharose CL-2B for subsequent light scattering and viscosity measurements to establish the relationships between M_w and [η] and $\langle R_g^2 \rangle_z^{1/2}$, respectively, which were found to be irrespective of the DA for the bulk of molecularly dispersed chitosan. Model calculations led to an increasing excluded volume effect with increasing molar mass and steadily increasing polydispersities for the fractions with increasing elution volume. The level-off behaviour in the [η] - M_w relation as well as the light scattering measurements (not shown in all detail here) indicated the presence of minor amouts of particulate matter in the samples the existence of which was assigned to the chemical heterogeneity of the material regarding the distribution of the DA.

Keywords: chitosan, conformation, wormlike chain model, molar mass, static light scattering, membrane osmometry, gel permeation chromatography

Introduction

State of art

The conformation of chitosan macromolecules in acidic aqueous solution has been the subject of several studies. Summing up the results published, one can say, that there is agreement in that the molecules form relatively flexible single-stranded chains. The interpretation of the experimental data in terms of the wormlike chain model has led to persistence lengths somewhere between 5 and 25 nm [1-4]. Central point of the current debate is whether or not a varying degree of acetylation causes any changes in the expansion and stiffness of chains either for sterical or charge effects [3,5,6]. The latter can be investigated only at rather low ionic strength. At sufficiently high ionic strength, the electrostatic repulsion between the charges along the polymer chain is largly suppressed due to the screening effect of the added salt. Then the electrostatic contribution to chain stiffness becomes small. Consequently, the sterical effect of acetyl groups can be studied selectively.

As a contribution to that topic, we have studied three commercial samples varying in degree of acetylation. Our theoretical approach and some of our results are presented in brief in the following. The detailed version has been submitted for publication in Carbohydrate Polymers.

Theoretical concept

The basic equation we made use of has been developed for a monodisperse system of wormlike chains under Θ conditions:

$$R^2_{g,\theta} = \frac{LL_P}{3} - L_P^2 + \frac{2L_P^3}{L} - \frac{2L_P^4}{L^2}\left(1 - e^{-L/L_P}\right) \tag{1}$$

Herein the persistence length L_P as expression for the stiffness of chains, the contour length L, and the square radius of gyration $R^2_{g,\Theta}$ of the molecules in solution are related to each other.

The contour length L is defined as the length of all units or bonds laid end-to-end. It cannot be measured directly but can be obtained by dividing the experimentally available molar mass M by the mass per unit length M_L. As we know both the length (by analogy to other polysaccharides as $l_o = 4.9\text{Å}$) and mass of the monomer units, which build up the macromolecular chain, we can easily calculate the value for M_L which correspondingly depends on the average degree of acetylation of the chitosan under study.

Both the molar mass and radius of gyration can be measured directly by static light scattering technique which is an absolute technique and does, consequently, not require any reference substances. Static light scattering is *the only method* which provides the radius of gyration. For polydisperse systems such as chitosan, it provides the z-average mean square radius of gyration and the weight average molar mass or weight average contour length instead. As these values represent different average values over a distribution, they cannot simply be correlated according to the equation (1). They are related to each other according to the equation (2)

$$\langle R^2_{g,\theta}\rangle_z = \frac{1}{L_w}\int_0^\infty L\ R^2_{g,\theta}(L)\ p_w(L)dL \tag{2}$$

where $p_w(L)$ means the normalized weight distribution of contour lengths. Knowing the polydispersity - expressed by M_w and M_n - and the type of distribution, one can estimate the persistence length by means of a combination of the equations (1) and (2). M_n denotes the number average molar mass.

The validity of the first equation is confined to an ideal solvent where the excluded effect becomes zero or, in other words, where the second virial coefficient B is equal to zero. (Then the unperturbed dimensions are obtained which are needed in order to assess the effect of acetyl groups on the stiffness of chains.) In practice, however, we normally use good solvents (B » 0) for several reasons. That is why the experimentally obtained radius of gyration has to be corrected for the contribution of the excluded volume. For doing so, we followed the well-known theory of Flory [7]

$$\alpha^2\ R^2_{g,\Theta} = R^2_g \quad \text{and} \quad \alpha^5 - \alpha^3 = (134/105)z$$

with α - the expansion factor. α is related to the excluded volume ß via the z-parameter (see, e.g., ref. (8)). Then we used Odijk's expression [9] for the electrostatic term of the excluded volume

$$\beta_{el} = 8\pi\lambda_D L_P^2$$

with λ_D - the Debye-Hückel screening length implying that, for highly charged polyelectrolytes with hydrophobic backbones, the electrostatic term of the excluded volume is dominant.

Starting an iterative procedure with a persistence length under neglection of the excluded volume effect from the first two equations, one finally obtains the correct persistence length.

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Experimental approach

In order to obtain the desired set of data, we carried out the following experiments:

- M_w, $R^2_{g,\theta}$, and the 2nd virial coefficient B_{SLS} by Static Light Scattering
- M_n and the 2nd virial coefficient B_{osm} by Membrane Osmometry
- the **type of distribution** by comparison of GPC elution lines with theoretical distribution curves

Material and methods

Three commercial products with average degrees of acetylation as 25, 22.5 and 7 %, respectively, were used without any prepurification or chemical modification (see Table 1). 0.02M acetate buffer of pH 4.5 (adjusted to the ionic strength of I = 0.12 moles/1 by added sodium chloride) was used as solvent. If indicated, solutions were ultracentrifuged (Beckman preparative ultracentrifuge L-70, fixed angle rotor Ti 70.1, 90 minutes, 40,000rpm, 20 - 25 °C). *Viscosity measurements* were carried out in an Ubbelohde type (Schott-Geräte, Germany) capillary viscometer (Lauda, Germany) at 25.0°C. The flow time for pure water was nearly 8.5s. The intrinsic viscosity was obtained by extrapolating η_{rel} /c to zero concentration (with c - polymer concentration in g/ml).

Membrane osmometry experiments were performed in 0.02M acetate buffer/ 0.1M NaCl, pH 4.5, at 30°C using a commercial osmometer (Osmomat 090, GONOTEC, Germany) equipped with two-layer membranes (cutt-off: ~ 5kD, made of cellulose acetate, supplier: GONOTEC, Germany) and concentrations up to 0.25g/100g solvent. The number average molar mass was calculated according to $M_n = R*T/(\pi/c)_{c=0}$ where $(\pi/c)_{c=0}$ - the reduced osmotic pressure extrapolated to zero concentration (linear regression), R - the universal gas constant, T - temperature. The osmotic second virial coeffcient B was obtained from the slope π/c versus c.

Static light scattering measurements were performed at 22-23°C in cylindrical cells using a SOFICA instrument (FICA, France), model 42000, equipped with a 5mW helium/neon laser at an operating wavelength of λ_o = 632.8nm. The scattered light intensity was measured at 31 positions between 30 and 150°. The Zimm procedure (see, e.g., ref.(10)) was used to derive M_w, $R_{g,z}$, and B by plotting $K*c/R_\theta$ versus q^2 + kc, where $q = (4\pi/\lambda)(\sin \theta)$ with θ - the angle of observation, K - the optical constant (see below), and k - an arbitrary constant.

For the same wavelength, the specific refractive index increment on chitosan A was obtained as $\delta n/\delta c$ = 0.203ml/g in both solvents after equilibrium dialysis (Brice Phoenix differential refractometer, UK; cutt-off of the dialysis tube used: 5kD). This value has been taken throughout this report for the calculation of the optical constant K.

All membrane filters used for clarification were made of cellulose acetate (Sartorius-Membranfilter-GmbH, Germany).

Gel Permeation Chromatography. The GPC equipment consisted of a Pharmacia column (2.6cm diameter, 95cm length) filled with about 500ml Sepharose CL-2B, a differential refractometer RID-6 (Shimadzu, Japan), a peristaltic pump (P1), a ReCyChrom valve for the sample injection and an UltroRac sample collector (all LKB Produkter, Sweden).

A sample volume of about 16ml (~2mg/ml) was injected into the ascendent flow stream of about 15 ml/h. Degassed 0.02M acetate buffer with 0.1M NaCl, pH 4.5, was used as solvent and eluant. If not mentioned otherwise, the fractions of ~16ml each were filtered directly (pore size: 0.45µm) into the dust-free measuring cell in the reverse order of their appearance for off-line SLS and, afterwards, viscosity measurements.

Tab. 1: Degree of acetylation; intrinsic viscosities; molar masses and 2nd virial coefficients from osmometry

Sample	DA (%)	$[\eta]$ (ml/g)	M_n (g/mol)	$B_{osm}*10^3$ (ml.mol.g^{-2})
chitosan A	25	333	31075	4.92
chitosan B	22.5	150	20400	9.50
chitosan C	7	650	57400	6.76

Fig. 1: Distribution curves
(M_w = 63,830 g/mol; M_n = 31,075 g/mol)

— logarithmic distribution
- - - Schulz-Zimm distribution
● experimental data (Chitosan A)

Tab. 2: Results of the characterization and interpretation in terms of the model for wormlike chains with excluded volume effect

Sample	M_w (g/mol)	$B_{SLS}*10^3$ (ml.mol.g^{-2})	$R_{g,z}$ (nm)	M_{GPC} (g/mol)	M_w/M_n	σ	M_L (g.mol^{-1}.nm^{-1})	L_w (nm)	α	$\langle R_{\vartheta\theta}\rangle_z$ (nm)	L_P (nm)	$B_{calc}*10^3$ (ml.mol.g^{-2})
chitosan A	63830	3.55	33.0	60000	2.05	0.85	349	182	1.18	27.9	6.5	7.4
chitosan B	39260	3.88	31.4	36200	1.92	0.81	347	113	1.13	27.7	12.5	8.2
chitosan C	147000	6.76	59.3	184000	2.56	0.97	335	439	1.26	47.1	6.0	6.6

$$B_{calc} = \frac{N_A N_K^2 \beta}{2M^2} h(z) \text{ with } N_A \text{ -Avogadro's number (see, e.g., ref. (8))}$$

Results and discussion

The results of our experiments are collected in the Tables 1 and 2 where also the corresponding values for the DA are given. At a glance, all data appear quite reasonable: when $[\eta]$ and M_n go down, so does M_w. The actually measured 2nd virial coefficients are all in the order of magnitude as 10^{-3} ml.mol.g^{-2} as generally predicted by the theory for linear flexible polyelectrolytes in aqueous solution. They agree well with those finally predicted on the basis of our own data (column on the very right - using the persistence lenght of $L_P = 6$ nm). Even the resulting polydispersities M_w/M_n seem not unlikely for a polymer with a history like that of chitosan. Consequently, the interpretation of our data according to the paragraph above seemed to be justified. In order to choose an appropriate distribution function, we have plotted experimental data from conventional GPC on Sepharose CL-2B together with a Schulz-Zimm distribution and also a special logarithmic distribution function which is given by $\dfrac{M_w}{M_n} = e^{\sigma^2}$

with σ - the polydispersity parameter (Fig. 1). In our judgement, the latter fits the real conditions sufficiently well and was used for all subsequent calculations. The results are listed on the right sight of the Table 2 . The probably most interesting column contains the values for the persistence length L_P. Obviously, two of the values are almost identical - with L_P of about 6 nm - whereas the third one is about 12 nm - *all this without any obvious relation to the average degree of acetylation* in Table 1. We must admit that the limited number of data for only three samples at present is no really satisfying situation. Anyway, for the lack of more data we have decided in favour of the value of 6 nm for some more model calculations which might, in turn, give little support to our choice.

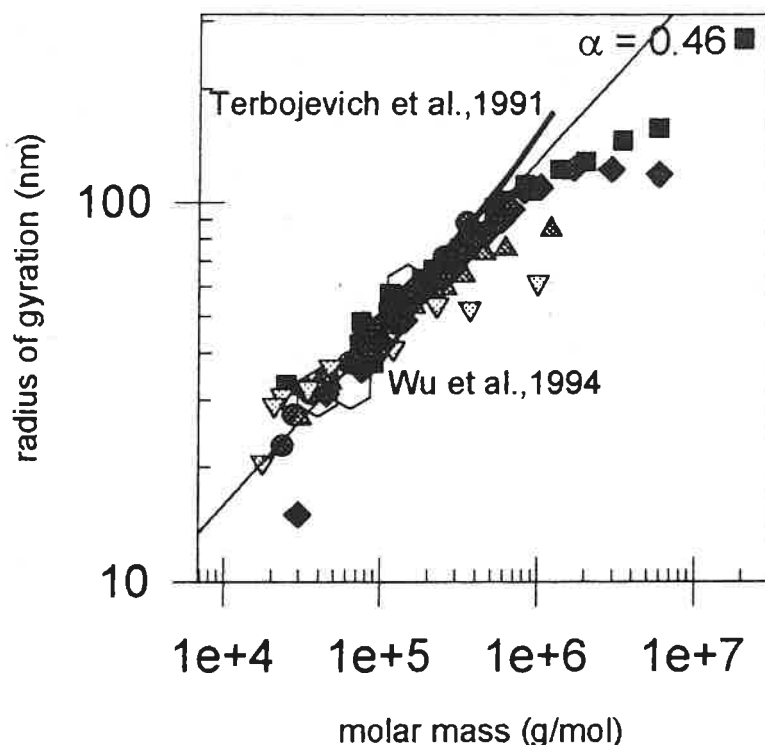In theory, the radius of gyration versus the molar mass is expected to give a straight line with a slope as high as 0.6 when both quantities are plotted in logarithmic scale. In fact, when we studied GPC fractions we obtained the plot in Fig. 2 - uncorrected for excluded volume and polydispersitiy effects - with slopes for the individual samples and GPC runs *significantly lower* than what is predicted by the theory. In previous studies on other very stiff polysaccharides such as xanthan [11] and related compounds [12] we had already been faced with similar findings and could explain them in terms of varying polydispersities within the fractions. Thus we used in principle the same approach as before and the same set of equations as above, but this time with a fixed value of 6.2 nm for the persistence length uniquely for all three samples and with

Fig. 2: Relation between molar mass and radius of gyration for the individual chitosan samples and several GPS runs

the aim to calculate the polydispersity instead. The results obtained on our three samples are summarized in Table 3.

Table 3: Results of model calculations on GPC fractions for the three chitosans varying in degree of acetylation; L_P =6.2nm

sample	elution volume (ml)	M_w (g/mol)	$R_{g,z}$ (nm)	L_w (nm)	z	α	$\langle R_{g,\theta}\rangle_z$ (nm)	M_w/M_n
A	328.4	3.323e5	64.2	952	1.30	1.31	49.0	1.25
A	344.8	2.545e5	59.3	729	1.14	1.29	46.0	1.43
A	361.2	2.116e5	58.9	606	1.04	1.28	46.0	1.73
A	377.7	1.612e5	53.0	462	0.90	1.26	42.1	1.90
A	394.1	1.144e5	47.1	328	0.76	1.23	38.3	2.21
A	410.5	8.917e4	42.7	256	0.67	1.21	35.3	2.42
A	426.9	6.358e4	37.7	182	0.57	1.19	31.7	2.77
A	443.3	4.726e4	33.5	135	0.49	1.18	28.4	3.01
B	366.5	2.249e5	53.5	648	1.07	1.28	41.8	1.34
B	382.4	1.192e5	41.2	344	0.78	1.23	33.5	1.63
B	398.4	7.645e4	39.4	220	9.62	1.20	32.8	2.47
B	414.3	4.712e4	36.9	136	0.49	1.18	31.3	3.67
B	430.2	3.389e4	32.3	98	0.42	1.16	27.8	4.02
B	446.2	2.315e4	30.9	67	0.34	1.14	27.1	5.71
C	230.0	8.064e5	107	2407	2.06	1.40	76.4	1.18
C	246.4	6.641e5	96.0	1982	1.87	1.38	69.5	1.19
C	279.2	5.531e5	89.5	1651	1.71	1.36	65.8	1.28
C	312.1	4.198e5	81.1	1253	1.49	1.34	60.5	1.43
C	344.9	3.422e5	74.3	1021	1.34	1.32	56.3	1.53
C	361.4	2.869e5	68.7	856	1.23	1.30	52.8	1.61
C	394.2	1.908e5	59.8	570	1.00	1.27	47.0	1.90
C	443.5	1.569e5	55.0	468	0.91	1.26	43.7	2.02
C	492.8	9.663e4	43.2	288	0.71	1.22	35.4	2.17
C	542.0	7.536e4	36.1	225	0.63	1.20	30.1	2.03

It can be seen that
- α - the expansion factor - decreases along with the molar mass M_w and
- the polydispersity increases with the increasing elution volume.

These findings appear to be quite reasonable.

This will finally say that all our experimental data were found to be self-consistent and have not given any serious hint for a significant sterical effect due to a varying content of acetyl groups along the polymer chains. This is in agreement with findings of some others [1,2].

If the polydispersity and excluded volume effects were not taken into account, the resulting values for L_P would be in the range between 15 and 20 nm.

It is fair to mention that the light scattering experiments on chitosan had been the proper problem. In order to get Zimm plots of the quality shown in Fig. 3 and the values given in Table 2, special care upon clarification was necessary. A combination of ultracentrifugation and membrane filtration revealed to be the method of choice where the filtration mode and the
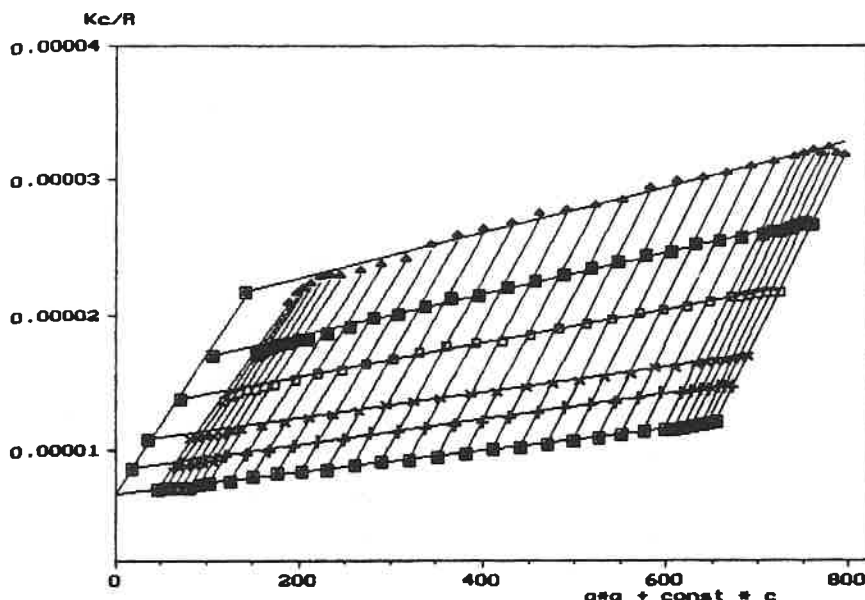
Fig. 3: Zimm plot for Chitosan C (M_w = 147,000)

minimum pore size had to be adapted to the sample under study. This is due to minor amounts of more compact particulate matter which also makes that

- the [η] - M_w plot subsequent to GPC fractionation shows a level-off behaviour for particularly high molecular weight fractions and
- that even relatively low-molecuar chitosan preparations of relatively high average degree of acetylation occasionally give relatively "flat" [η] - M_w plots
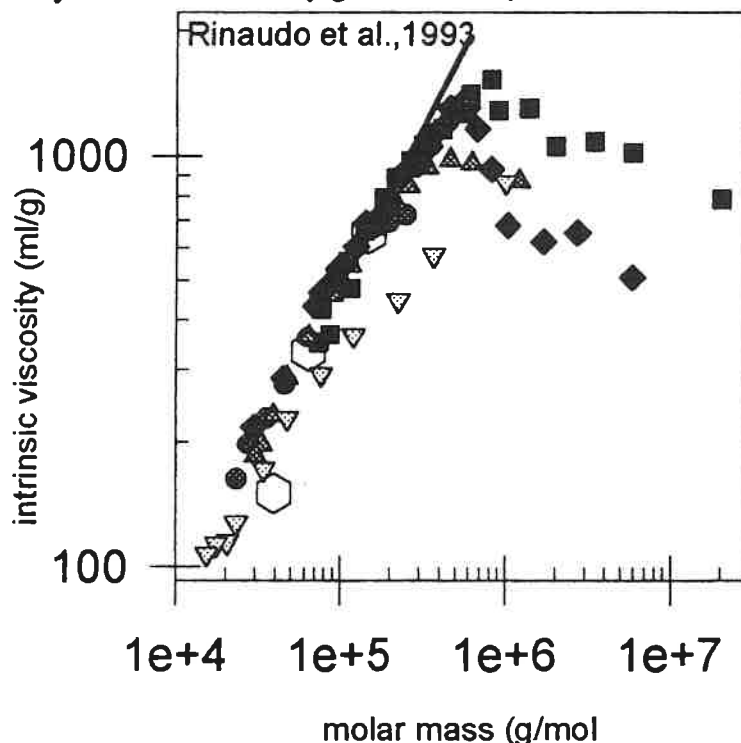

Fig. 4: Mark-Houwink-Kuhn-Sakurada plot for the individual chitosan samples and several GPC runs

as is shown in Fig. 4. We think these effects should be seen in context with *the chemical heterogeneity* of commercial chitosans. It is very likely that these particles represent a sort of biological debris or, in other words, more "chitin-like" components whose degree of acetylation is significantly higher than that of the bulk of the sample (see, e.g., G. Roberts' lecture in this volume). The solution behaviour of these particles is quite different from that of the molecularly disperse major proportion which has been described so far.

Conclusion

Further studies on chemically more homogeneous samples in a broader range of average DA

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values are to be performed to verify the conclusions drawn.

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Effects of ultrasonic-heating and heating only on changes of intrinsic viscosity, degree of deacetylation, and maximum melting point temperature of treated chitosan in acetic acid solution containing 4 M urea

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Abstract

Intrinsic viscosity [η] of treated chitosan in acetic acid solution with 4 M urea decreased with treatment time for both ultrasonic-heating and heating only treatments. The extent of decrease in [η] was more pronounced for chitosans treated by ultrasonic-heating than those by heating only, which indicates that degradation reactions of chitosan molecules was faster by ultrasonic-heating treatment than by heating only. Degree of deacetylation (DD) of treated chitosan also decreased with treatment time by both types of treatment. The effect of different DDs of chitosan used on the extent of decrease in DD caused by the above treatments was different. Lower DD chitosan decreases were less pronounced than those of higher DD ones regardless of whether the treatment method was ultrasonic-heating or heating only. This may be due to different preferential glycosidic cleavage during treatments and sucessive fractionation during dialysis. Maximum melting point temperature (MMPT) of treated 49% DD chitosan increased slightly with treatment time. However, MMPT of treated 67% or 74% DD chitosan decreased with treatment time by both types of treatment. The extent of decrease in MMPT of 67% and 74% DD chitosans was more pronounced with ultrasonic-heating than with heating only treatment.

Keyword: chitosan, ultrasonic, heating, degree of deacetylation, intrinsic viscosity, maximum melting point temperature.

Introduction

Chitosan is an abundant and widely distributed biopolymer [1]. It can be applied in food processing, agriculture, biomedicine, waste water treatment, membranes and microcapsules, etc. [1-8]. Physical and chemical properties of chitosan such as rheological properties [9,10], antimicrobial activity [11,12], immunoadjuvant activity [13,14], wound healing and blood coagulation [15], enzyme-binding activity [16], metal binding activity [17], film and gel forming properties [15], and mechanics and porosity of membranes [4,5,18-20] depend on the molecular weight and degree of deacetylation of the chitosan .

Polysaccharides are, in general, susceptible to a variety of degradation mechanisms such as oxidative-reductive, free radical depolymerization, acid, alkaline, or enzymically catalyzed hydrolysis, as well as heating degradation [21]. Ultrasonic treatment was reported to produce non-random degradation [22], and it has been employed to degrade chitosan to obtain different molecular weight products [10,23,24]. However, the degree of deacetylation (DD) varies with ultrasonic treatment and the results do not accord with each other [10,23]. Chen et al. [25] reported the effect of ultrasonic conditions such as

solution concentration, solvent characteristics, ultrasonic time, and reaction temperature on changes of the molecular weight decrease rate of treated chitosan.

Studies on the effects of acid hydrolysis on chain degradation of chitinous materials are numerous. Rutherford & Austin [26] reported that chitin is a sensitive material which may be degraded by hydrolytic depolymerization. Terbojevich et al. [27] reported that after reflux in 1% acetic acid at 80 °C for 64 h, the [η] decreased from 64 dl/g to 2.1 dl/g. However, studies of the effect of acid hydrolysis on deacetylation of chitinous materials are rare, although amides of chitinous materials may be hydrolyzed under either acidic or basic conditions [28]. Muzzarelli & Rocchetti [23] report that sonification of chitosan solution at extremely low pH results in detectable deacetylation after prolonged periods of treatment. Deacetylation as the result of sonification or acid hydrolysis has not been elucidated. Studies on the effect of DD and molecular weight of chitosan on its MMPT are rare [29,30]. Furthermore, the DD and molecular weight of chitosan depend on the conditions of ultrasonic or acid hydrolysis applied as mentioned. Besides the contradiction about the effect of ultrasonic treatment on DD, studies on the effect of ultrasonic treatment on the [η] and crystallinity of treated chitosan are very rare. A similar contradiction is the effect of urea on the stability of polymers. Frangou et al. [31] reported that an increased urea concentration in a xanthan solution resulted in an increasing conformation transition temperature. This was attributed to urea stabilizing the hydrogen bond of xanthan molecules to maintain its ordered conformation, therefore, the conformation transition temperature increases. However, Watase et al. [32] reported that adding urea to neutral agarose shifted the endothermic peak to a lower temperature. This was attributed to urea breaking the hydrogen bonding so, therefore, the melting point of the gel decreases. Urea was reported to destroy hydrogen bonds and affect rheological parameters such as relative viscosity [33], [η] [34], and persistence length [35]. Chen et al. [36] reported the effect of ultrasonic-heating and heating only on [η], DD, and MMPT of chitosan in acetic acid solution without 4 M urea.

The effect of ulrasonic-heating and heating only on the changes of [η], DD, and MMPT of treated 1% chitosan with different DDs in 10% acetic acid with 4 M urea were compared.

Materials and Methods

Chitosan preparation

Chitin was prepared from shrimp (*Solenocera prominentis*) waste by a modified method of Stanley et al. [37] and Chen et al. [2,38]. Chitin powder was alkali deacetylated (50% NaOH) at 60 °C, 100°C, and 140 °C for 3 h to got 49%, 67%, and 74% DD chitosan. The obtained product was washed and dried at 50 °C to get the final products.

Conditions of ultrasonic-heating or heating only treatment

Ultrasonic-heating treatment was performed by ultrasonic radiation (Crest 915D) of 1% chitosan (49%, 67%, and 74% DD) in 10% acetic acid solution with 4 M urea at 200 W at 60 °C for the time indicated. For heating only treatment, the 1% chitosan solution mentioned above was heated at 60 °C for the time indicated. After treatment, the solution

was dialyzed with distilled water using a seamless cellulose tubing, to remove small molecules such as urea etc. The residuum were lyophilized to preserve the samples for characterization their physico-chemical properties such as $[\eta]$, DD, and MMPT.

Degree of deacetylation determination

The colloid titration method of Toei and Kohara [39] was followed. An aliquot of 0.50 g of chitosan was dissolved in 99.50 g 5% (v/v) acetic acid. One gram of chitosan-acetic acid solution was mixed well with 30 ml deionized water. Two to 3 drops of indicator of 0.1% toluidine blue were added and the solution was titrated with N/400 PVSK (potassium polyvinyl sulfate, $(C_2H_3O_4SK)n$. n = 1500 or above). Degree of deacetylation was calculated with the following equations:

Degree of deacetylation = $[(x/161)/(x/161 + y/203)]$ x 100
x = 1/400 * 1/1000 * f * 161 * V;   y = 0.5 * 1/100 - X
V = milliliters of N/400 PVSK used in titration
f = factor of N/400 PVSK solution.

Intrinsic viscosity

The treated samples were adjusted to concentrations of 0.01%, 0.025%, 0.050%, 0.100%, and 0.200%. Five milliliters of the prepared solution was placed into the reservoir of a Cannon-Fenske # 100 capillary viscometer. The viscometer was placed in a water bath (Tanson, TMV 40, Sweden) maintained at 30 ± 0.5 °C. The measured flow time was used to calculate the relative, specific, and reduced viscosity. The reduced viscosity was plotted against solution concentration. The intercept obtained by extrapolating the reduced viscosity to zero concentration is the intrinsic viscosity.

Maximum melting point temperature

The energy of fusing chitosan is proportional to the crystallinity in the chitosan [40]. The maximum melting point temperature was measured by differential scanning calorimetry (Du Pont TA 2000, D.S.C. 10, U.S.A.). The point at which chitosan melts is termed the maximum melting point temperature.

Results

Effect on the intrinsic viscosity ($[\eta]$)

Table 1 shows that before treatment, $[\eta]$ of chitosan is larger for lower DD chitosans than it is for higher ones. $[\eta]$ of treated chitosan decreased along with treatment time. The extent of decrease is slightly larger for chitosans treated by ultrasonic-heating than those treated by heating only. The effect of different DD of chitosan used on the extent of decrease in $[\eta]$ caused by the above treatments is not significantly different.

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Table 1. Effect of ultrasonic-heating and heating only on changes of the intrinsic viscosity of chitosan with different degrees of deacetylation

Time(h)	49%DD		67%DD		74%DD	
	U60 °C	60 °C	U60 °C	60 °C	U60 °C	60 °C
0.0	4.51	4.51	4.16	4.16	3.89	3.89
1.5	4.09	4.40	3.68	4.10	3.58	3.83
3.0	4.35	4.37	4.02	4.07	3.47	3.76
6.0	4.22	4.30	3.93	4.05	3.35	3.72
12.0			3.87	4.03	3.27	3.67

U-60 °C represents ultrasonic-heating treatment on 1% chitosan in 10% acetic acid solution with 4 M urea with ultrasonic radiation at 60 °C for the time indicated.

60 °C represents heating only treatment on 1% chitosan in 10% acetic acid solution with 4 M urea with heating at 60 °C for the time indicated.

Effect on degree of deacetylation (DD) of chitosan

Table 2 shows DD of treated chitosan in acetic acid solution with 4 M urea decreased along with treatment time. The extent of decrease is more pronounced for chitosans treated by ultrasonic-heating than those treated by heating only. The effect of different DD of chitosan used on the extent of decrease in DD caused by the above treatments is different. The decrease of lower DD chitosans was less pronounced than that of higher DD ones regardless of whether the treatment method was ultrasonic-heating or heating only.

Table 2. Effect of ultrasonic-heating and heating only on changes of the degree of deacetylation of chitosan with different degrees of deacetylation

Time(h)	49%DD		67%DD		74%DD	
	U60 °C	60 °C	U60 °C	60 °C	U60 °C	60 °C
0.0	49.70	49.70	66.52	66.52	74.73	74.73
1.5	48.22	48.54	64.99	61.92	70.81	70.90
3.0	47.25	47.38	61.42	60.10	67.99	68.86
6.0	43.52	46.65	55.24	56.39	61.72	60.75
12.0	35.37	38.56	48.61	46.69	55.68	53.27
24.0	29.32	33.29	37.16	39.63	45.13	46.36

U-60 °C and 60 °C are the same as in Table 1.

Effect on the maximum melting point temperature (MMPT)

Table 3 shows that before treatment, the MMPT of chitosan is larger for higher DD chitosans. MMPT of treated 49% DD chitosan increased slightly with treatment time by both ultrasonic-heating and heating only. However, MMPT of treated 67% or 74% DD chitosan in acetic acid solution with 4 M urea decreased with treatment time. The extent of decrease in MMPT of 67% and 74% DD chitosans was more pronounced by ultrasonic-heating than by heating only treatment. The extent of decrease in MMPT of 67% DD was smaller than 74% DD chitosan regardless of the treatment method of ultrasonic-heating or heating only.

Table 3. Effect of ultrasonic-heating and heating only on changes of the maximum melting point temperature of chitosan with different degrees of deacetylation

Time(h)	49%DD		67%DD		74%DD	
	U60 °C	60 °C	U60 °C	60 °C	U60 °C	60 °C
0.0	205.86	205.86	235.43	235.43	237.72	237.72
1.5	205.95	206.89	222.30	233.84	226.25	236.36
3.0	205.95	207.28	222.30	231.86	226.25	227.77
6.0	206.58	206.69	207.66	222.33	211.43	226.25
12.0	207.29	207.03	198.22	221.59	204.46	218.93
24.0	209.87	210.03	210.85	222.02	206.78	217.44

U-60 °C and 60 °C are the same as in Table 1.

Discussion

Effect on intrinsic viscosity

Results in Table 1 show that intrinsic viscosity ($[\eta]$) of treated chitosan in acetic acid solution with 4 M urea decreased with treatment time for both treatment methods of ultrasonic-heating and heating only. This was due to chitosan being degraded by either method. The effect of different DD of chitosan used on the extent of decrease in $[\eta]$ caused by the above treatments is not significantly different. However, Chen et al. [36] reported the extent of decrease in $[\eta]$ of chitosan in acetic acid solution without 4 M urea to be more pronounced for chitosans lower in DD. They attributed this to the molecular weight and chain flexibility of lower DD chitosan being larger and smaller, respectively, than those of higher DD chitosan. Before ultrasonic-heating and heating only treatment, values of $[\eta]$ of those chitosans in acetic acid solution with 4 M urea were larger than their respective chitosans in acetic acid solution without 4 M urea reported in Chen et al. [36]. This may be attributed to urea destroying intra-molecular hydrogen bonds of chitosan which causes the relative viscosity [33], $[\eta]$ [34], and persistence length [35] to increase. The results in Table 1 indicate the effect of urea on the extent of decrease in $[\eta]$ to overwhelm the effect of different DD of chitosan used.

The extent of decrease in $[\eta]$ of chitosan in acetic acid solution with 4 M urea is slightly larger for chitosans treated by ultrasonic-heating than those treated by heating only, which indicates that molecular degradation is more effective by ultrasonic-heating than by heating only treatment. This may be the result of breakage of the β-1, 4 linkage of chitosan in acetic acid solution with 4 M urea by ultrasonic-heating being more effective than by heating only treatment, because both ultrasonic radiation and heating exert energy to break the glycosidic bonds. Basedow & Ebert [41] reported that when dextran was ultrasonified at 82 °C in 0.6 M phosphoric acid, the rate constant was proportional to the molecular weight raised to the power of 4/3 when the molecular weight is higher than the limiting value. However, the rate constant is proportional to the molecular weight raised to the power of 5/6 for molecular weights below the limiting value. This may due to combined effects of mechanical stress and acid hydrolysis [27]. In other words, mechanically strained dextran molecules are more easily degraded by acid hydrolysis than are molecules not subjected to mechanical stress. Chen et al. [36] reported similar results

for chitosan in acetic acid solution without 4 M urea.

Effect on the degree of deacetylation (DD)

Table 2 shows DD of treated chitosan in acetic acid solution with 4 M urea decreasing along with treatment time. The extent of decrease is more pronounced for chitosans treated by ultrasonic-heating than those treated by heating only. However, Chen et al. [36] reported that DD of chitosan in acetic acid solution without 4 M urea did not change with treatment time for both treatments. They reasoned that ultrasonic radiation at 200 W caused no deacetylation reaction on the treated chitosan in 10% acidic acid solution. Their results are in accord with the report of Wang et al. [42], who reported that ultrasonic degradation of chitosan did not result in a significant change in DD of treated chitosan. However Muzzarelli & Rocchetti [23] reported that sonification led to detectable deacetylation after prolonged treatment, especially at pH 1.0. Amides of chitinous material may, in principle, be hydrolyzed in either acidic or basic conditions. The use of acidic deacetylation is precluded because of the susceptibility of the glycosidic links in chitinous material to acidic hydrolysis [28]. Effects of acid hydrolysis at elevated temperatures on changes of DD of treated chitinous materials are dependent on the type and concentration of acid used, solution temperature, and duration of hydrolysis [23,43]. However, results in Table 2 show DD of treated chitosan decreased with treatment time by both methods. This may be due to different specific cleavage of the four different glucosidic linkages (A-A, A-D, D-A and D-D) [44] but similar stability of amide group in chitosans during both treatments in 10% acetic acid solution with 4 M urea. After dialysis, the degradated products contained higher proportion of A-units was remained however, those contained higher proportion of D-united was removed due to different solubility (chemical composition and degree of polymerization dependency) [44] and thus resulted in decreasing DD of the product. The reason that the extent of decrease in DD is more pronounced for chitosans treated by ultrasonic-heating than those treated by heating only may be due to molecular degradation is more effective by ultrrasonic-heating than by heating only treatment. The decreases in DD shown in Table 2 is parallel to the decreases in [η] shown in Table 1 by both treatments supported the above speculation.

Table 2 also shows that the effect of different DD of chitosan used on the extent of decrease in DD caused by the above treatments is different. Decrease in DD of 49% DD chitosan was less pronounced than was that of 67% or 74% DD chitosans, but there was no different between 67% DD and 74% DD ones regardless of treatment method of ultrasonic-heating or heating only. This may be due to higher susceptibility of glycosidic cleavage of higher DD chitosans than that of 49% DD chitosan and successive preferential fractionation during dialysis.

Effect on maximum melting point temperature (MMPT)

Results in Table 3 show that the MMPT of untreated chitosan increased with increasing DD of chitosan used. Although the molecular weight of 74% DD chitosan was lower than that of 67% or 49% DD chitosan, however, the MMPT of 74% DD chitosan was higher than that of the other two chitosans. This may be due to the chain flexibility of 74% chitosan being higher than that of the other two chitosans. Higher chain flexibility

facilitates intra-molecular hydrogen bond formation and, in turn, the crystallinity formed, thus resulting in higher MMPT. Chen et al. [2] reported similar results. After ultrasonic-heating or heating only treatment, MMPT of 49% DD chitosan in acetic acid solution with 4 M urea increased slightly. However, MMPT of 67% DD or 74% DD chitosan in the same solvent decreased. This indicates that the original amphorous structure becomes slightly crystalline while the original crystalline structure becomes more amphorous after either treatment. The extent of decrease in MMPT of 67% DD and 74% DD chitosan was larger by ultrasonic-heating than by heating only treatment, which indicates that the decrease in crystalline structure and increase in amphorous structure are more pronounced by ultrasonic-heating than by heating only treatment. Therefore, the extent of crystallinity destruction is more severe for chitosans subjected to ultrasonic-heating treatment than those subjected to heating only treatment. Therefore, the decrease in MMPT is larger for ultrasonic-heating treatment than for heating only treatment.

Conclusions

1. [η] and DD of chitosan in 10% acetic acid with 4 M urea decrease with treatment time for both ultrasonic-heating and heating only treatments.
2. MMPT of treated 49% DD chitosan in acetic acid solution with 4 M urea increases slightly; however, MMPT of 67% DD and 74% DD chitosans in the same solvent decrease with treatment time by bothtreatments.
3. Ultrasonic-heating treatment is more effective than heating only treatment in lowering DD and MMPT of treated chitosans.

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EFFECT OF pH, PARTICLE SIZE AND CROSS-LINKING ON SORPTION ISOTHERMS OF MOLYBDATE BY CHITOSAN FLAKES AND GEL BEADS

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Abstract

The adsorption of molybdate ions on chitosan was studied at various pH values. The influence of the physical form (flakes or gel bead), the chemical cross-linking and the size of the particles were investigated. Isotherm have shown that the form has little effect on the maximum uptake capacity except for cross-linked flakes. In the latter case, the cross-linking of the polymer by glutaraldehyde seemed to have prevented the diffusion of molybdate ions. In all events, the shape of the isotherm was correlated with the molybdenum speciation diagram. It seemed that the adsorption of molybdate was efficient only when metal was in the polyoxyanion form. The concentration at which the polyoxyanions were formed depends greatly on the pH value and isotherms can take an unexpected sigmoïdal form.

Keywords: chitosan, chemical cross-linking, flakes, gel beads, molybdate, adsorption isotherms, pH effect, uptake capacity

Introduction

Chitosan proved to be an efficient sorbent for metal ion recovery (1-12). Molybdenum is a metal commonly used in electrical and optical manufacturing and catalyst production. Although there is little international agreement for molybdenum discharge levels, industrial plants are subjected to local regulation. The molybdenum recovery by uptake and desorption processes can reduce the lost of raw material in catalyst production line. The traditional technologies of precipitation are not efficient for this type of metal at low concentrations. Some studies have shown the affinity of molybdenum for chitosan (1, 17). A preliminary work emphasized the role of several parameters (pH value, equilibrium concentration, size of adsorbent particles, conditioning of chitosan in the form of beads or flakes and cross-linking) on adsorption capacity. The aim of this work is to quantify these effects and to determine the optimum conditions of molydenum adsorption on chitosan. The maximum uptake capacities and the shapes of the isotherms obtained at various conditions are compared.

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Materials and methods

Material

Chitosan was supplied by ABER-Technologie (France). Its deacetylation percentage measured by IR spectrometry was closed to 87 % (13-14). Glutaraldehyde solution (25 % w/w) was obtained from Acros. The other reagents (sodium hydroxide, ammonium heptamolybdate, acetic and sulfuric acids) were supplied by Prolabo as analytical grade quality.

Flakes conditioning

The chitosan was ground and sieved in three particle size fractions (Table 1). These non cross-linked fractions (FiNCL, i = 1 to 3) were used to produce cross-linked fractions (FiCl) of chitosan flakes. Chemical cross-linking of chitosan flakes was performed using 2.6 g of the substrate mixed with 1 liter of glutaradehyde solution at a final concentration of 25 g/l. The mixture was then incubated at room temperature for 24 hours. Cross-linked flakes were washed in several demineralized water baths and finaly air dried.

Beads formation procedure (9-12)

A 4 % (w/w) chitosan solution in 4 % acetic acid was poured dropwise from an hypodermic needle in a 2.5 M solution of sodium hydroxyde. The beads remained in the alkaline bath during 16 hours and were washed. To reduce the size of the gel beads, an air flow was provided around the needle. Three diameters of beads were obtained: BiNCL with i = 1 to 3 (Table 1). 2.6 g of chitosan beads (measured in dry weight) for each diameter were cross-linked in the same way as flakes. The cross-linked beads were designated BiCL. Table 1 presents physical characteristics of the adsorbents used in the study.

Table 1: Physical caracteristics of the chitosan adsorbents

Product	Diameter d (mm)	Dry weight % or mg/bead
F1 NCL	d<0.125	90
CL	d<0.125	88
F2 NCL	0.125<d<0.250	90
CL	0.125<d<0.250	87
F3 NCL	0.250<d<0.500	90
CL	0.250<d<0.500	88
B1 NCL	1.01 ± 0.06	4
CL	1.03 ± 0.05	6
B2 NCL	1.47 ± 0.08	0.070
CL	1.46 ± 0.08	0,100
B3 NCL	2.76 ± 0.16	0.360
CL	2.70 ± 0.08	0.505

Experimental procedure : metal ion adsorption isotherms

Amonium heptamolybdate was dissolved in demineralized water at the concentrations 100 or 200 mg.l⁻¹. Each point of the sorption isotherms was determined by contact of a volume of molybdate solution (0.1, 0.2, 0.5 or 1 liter) with a known weight of adsorbent (dry sorbent weights varying between 20 and 500 mg). All experiments were performed at 20°C with a constant pH control, achieved by adding micro-volume of molar sodium hydroxide or sulfuric acid solutions. Samples were collected and separated by filtration after 72 hours agitation on a adjustable reciprocating shaker. The metal ion content C (mg.l⁻¹) was determined by ICP spectrometry and the metal solid content at equilibrium Q_{eq} (mg.g⁻¹) was determined by the mass balance equation:

$$Q_{eq} = \frac{(C_o - C_{eq}))V}{m}$$

where C_o and C_{eq} are respectively the initial and equilibrium concentrations expressed in mg.l⁻¹, V the solution working volume (l), and m the dry mass of sorbent (g). All metal ion concentrations are expressed as total molybdenum (Mo) content and do not depend on its ionic form (free or hydrolyzed species).

Molybdate speciation

The distribution diagram of molybdate species was obtained from thermodynamic data presented by Baes and Mesmer (15) and calculations were performed with the program HYDRAQL (16). Figure 1 shows the variation of the percentage of each chemical species as a fonction of pH and total metal ion concentration.

Results and discussion

Effect of pH and equilibrium concentration

pH has a double effect on the chitosan/molydenum system; it modifies the protonation of the chitosan amino groups (positive charge under the pKa = 6.3, (2)) and it influences molybdate speciation (Figure 1). Therefore sorption performances may change as a function of the pH value.

pH effect has been studied with four types of sorbents: F2NCL, F2CL, B3NCL and B3CL, for which eight pH values ranging from 1 to 8 were used. The influence of the pH is independant of the sorbent used. Obviously, pH has a high effect on adsorption isotherms (as shown on Figure 2 with F2NCL). The following effects were observed:

- for all the sorbent, optimum pH value for adsorption lies between 3 and 4,
- adsorption occurs at pH 2 to 5 and maximum uptake capacities are similar at these pH values for a given sorbent; low adsorption occurs at pH 1 and 6; and it decreases at pH higher than 5, while no adsorption was observed at pH 7 and 8,

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- because of the poor efficiency of F2CL, we can not conclude about this sorbent. For the three others, we can observe that the shape of the isotherms depends on the pH. Between pH 2 and 6, the adsorption starts at various equilibrium concentrations and are best described by both sigmoïdal and classical concave-shaped curves depending on the pH values.
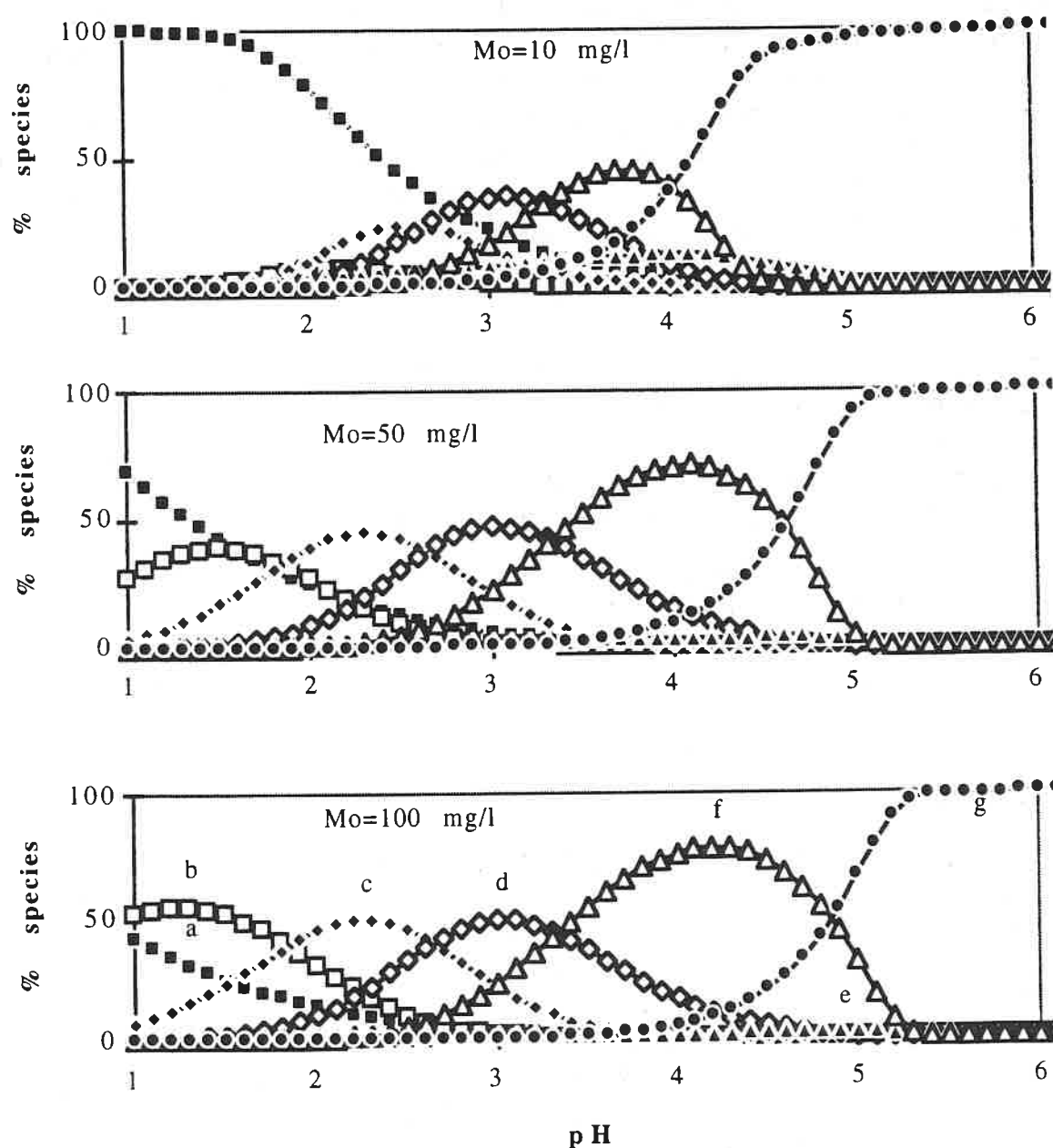

Figure 1: Molybdate speciation as a fonction of pH and molybdenum concentration
a)H_2MoO_4 b)$MoO_7O_{21}(OH)_3^{3-}$ c)$Mo_7O_{22}(OH)_2^{4-}$ d)$Mo_7O_{23}(OH)^{5-}$ e)$HMoO_4^-$ f)$Mo_7O_{24}^{6-}$ g)MoO_4^{2-}

These three statements can be explained using the distribution diagram of Mo species presented on Figure 1. At any molybdate concentration used, polynuclear species are predominant between

pH 3 and 4. At pH 1 or 2 it is necessary to reach high concentrations of Mo to find these species. At a pH higher than 5, there is no polynuclear species when Mo concentration is lower than 100 mg.l^{-1}; consequently, MoO_4^{2-} is the most prevalent ionic form and; the ionic charge of the chitosan become neutral. Therefore, adsorption efficiency is reduced by the presence of mononuclear species or by neutral or negative charge of the chitosan. We observed positive adsorption under the following conditions: pH and concentrations compatible with the presence of polyoxyanions. That explains the original sigmoïdal shape of some isotherms.

Figure 2: pH effect on molybdate adsorption by F2NCL

All the following experiments on the evaluation of the influence of particle size, chitosan cross-linking and its geometry on adsorption efficiency were performed at pH 3.

Effect of particles size

Influence of particle size is different for chitosan flakes and gel beads (Figures 3-5).

For every particle size considered, experimental points obtained with BiNCL lie on the same isotherm curve. Maximum uptake capacity reached 1000 mg.g^{-1} The same results were obtained with BiCL with a maximum uptake capacity of 700 mg.g^{-1}. It shows that molybdate ions can diffuse in the whole bead.

In the case of flakes, particle size has specially a noticeable effect on adsorption capacity of the cross-linked forms. Therefore molybdate can diffuse in the whole particle in spite of its poor porosity, whereas Mo is adsorbed only on the surface of the cross-linked flakes.

Effect of chitosan cross-linking

Figures 3 to 5 show the important effect of cross-linking on the adsorption isotherms of flaked adsorbent, reducing their maximum

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uptake capacities: for example, F2NCL adsorbs 800 mg.g^{-1} of molybdate ions whereas F2CL adsorbs only 200 mg.g^{-1}. Cross-linking can fill up the weak porosity of the polymer network of chitosan flakes.

On the contrary, cross-linking of chitosan beads has a little effect on maximum uptake capacities: 700 mg.g^{-1} for BiCL and 1000 mg.g^{-1} for BiNCL. The difference between these two results can be explained by the dry mass of sorbent: the dry mass of BiNCL is made of chitosan whereas a part of the dry mass of BiCL is constituted of glutaraldehyde that does not adsorb. Furthermore, amino groups that reacted with glutaraldehyde can no longer attract Mo ions.

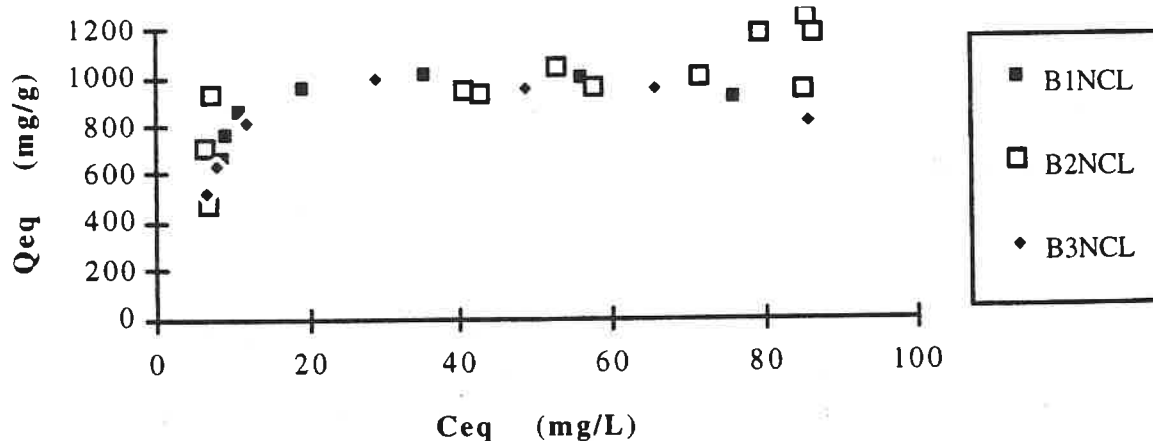**Figure 3:** Chitosan conditioning effect on molybdate adsorption on BiCL

Figure 4: Chitosan conditioning effect on molybdate adsorption on BiNCL

Effect of chitosan physical form

In the case of cross-linked material, maximum uptake capacities vary from 150 mg.g^{-1} for F1CL to 700 mg.g^{-1} for BiCL.

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Higher capacities were obtained in the case of non cross-linked sorbent: between 700 and 1000 mg.g^{-1} of Mo ions were adsorbed on this more fragile material.
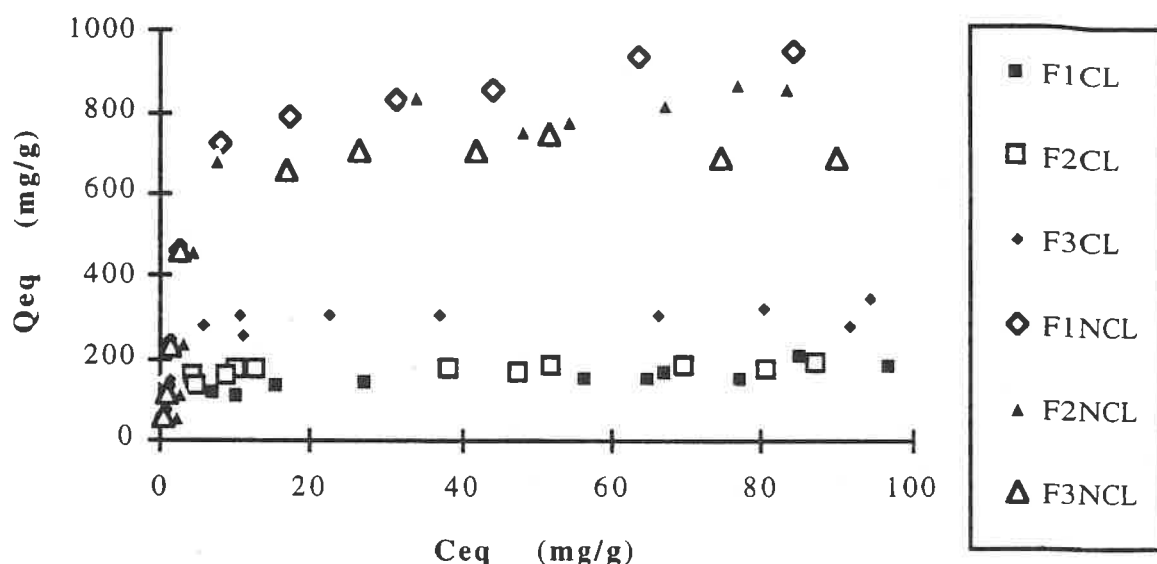

Figure 5:   Chitosan conditioning effect on molybdate adsorption on Fi

Conclusion

Study of isotherms of molydenum adsorption on chitosan beads or flakes have shown that:
- chitosan demonstrates high adsorption capacity for Mo removal,
- the shape of the adsorption isotherms depends on the pH value because of the speciation of the molybdate ions: all but polyoxyanions are not adsorbed on chitosan Thus optimum adsorption pH was determined between 3 and 4; at other pH values, isotherms present a novel sigmoïdal shape,
- the maximum uptake capacity also depends of the conditioning of chitosan (size, flakes or beads, cross-linking) as presented on Table 2 which summarizes the results obtained at pH 3.

Table 2:   Maximum uptake capacities of molybdenum on chitosan (mg.g^{-1})

	Flakes Fi		Beads Bi	
i	NCL	CL	NCL	CL
1	850	300	1000	700
2	800	200	1000	700
3	750	150	1000	700

The choice of the adsorbent type for such process as wastewater treatment will depend on the water quality to treat, dynamic flows, the type of reactors used and the pressure drop allowed in fixed bed systems.

The desorption is an important step for recycling process. This last aspect of the problem will be studied in future work on both sorbents: chitosan flakes and beads, either cross-linked or not. The kinetics of adsorption and desorption process will also be studied.

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Acknoledgements