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Chitosan behaviours in a dispersion of undecylenic acid

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Chitosan is able to flocculate or stabilize aqueous dispersed media containing undecylenic acid. These flocculation/dispersion phenomena were studied by potentiometric, UV spectrometric and quasi-elastic light scattering techniques. Parameters such as pH, ionic strength of the media and total lipid concentration have a great influence on the interactions occurring between chitosan and the lipid particles. Adequate pHs, ionic strengths and a lipid concentration above a certain limit are required to obtain flocculated or dispersed systems. High ionic strengths, as well as dilution, tend to increase the particle sizes and destabilize the dispersions. On the other hand, high lipid concentrations yield smaller chitosan coated particles. A mechanism of adsorption of chitosan chains onto lipid particles is proposed, which explains the influence of the various parameters.

INTRODUCTION

Chitosan and its oligomers are now well known for their numerous biological properties, which have led to very diverse applications. Among them, we can mention those concerning cosmetics (Lang & Clausen, 1989), pharmaceutics (Balassa, 1975; Hirano *et al.*, 1988; Balassa & Prudden, 1989), biomaterials (Malette *et al.*, 1986; Muzzarelli *et al.*, 1989; Ito, 1991), biotechnologies (Chandy & Sharma, 1989), food (Hirano *et al.*, 1990) and seeds (Pearce & Ride, 1982; Conrath *et al.*, 1989).

Though the various consequences of the presence of chitosan in biological media are beginning to be well known, there is still much to investigate in order to fully understand the mechanisms of interaction of chitosan with living matter. Such interactions induce a biological signal which is often the first step in a cascade of events.

Lipids, particularly fatty acids and phospholipids, play an important part in many biological processes (Elias & Brown, 1978; Zwaal, 1978; Ward & du Reau, 1991). There are few results reported in the literature about the behaviour of chitosan in the presence of lipids. Some papers have shown the hypolipidic effect of chitosan, especially concerning cholesterol and triglycerides (Sugano *et al.*, 1978; Kobayashi *et al.*, 1979; Nagyvary *et al.*, 1979; Sugano *et al.*, 1980, 1988; Jennings *et al.*, 1988). Other works have pointed out the interesting role of chitosan on the flocculation of oil-in-water emulsions which were obtained from soya bean oil dispersions, stabilized with anionic surfactants (Axberg *et al.*, 1980). Finally, studies have shown the existence of interactions between chitosan and plant cell suspensions via the lipid membrane, inducing a biological response (Kauss *et al.*, 1989).

The aim of our research was to study the behaviour of chitosan in the presence of lipids, in particular fatty acids and phospholipids. In this work, we focused our investigations on the characterization of the interactions which may occur between chitosan and a fatty acid in the dispersed form. We chose to use an unsaturated fatty acid to easily obtain micelles, and, more specifically, undecylenic acid for the solubility of its sodium salt in aqueous media.

Former studies allowed us to show that chitosan interacts with solutions of this acid by means of a purely electrostatic charge neutralization mechanism. No interaction involving complexation, for example, could be shown (Domard & Demarger-André, 1992). In the present work, the various parameters influencing the stability of lipid dispersions (pH, concentration, ionic strength, etc.) were investigated by different techniques such as spectrometry (UV-visible, Fourier Transform

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infra-red), quasi-elastic light scattering and chromatography.

EXPERIMENTAL

Materials

Following the method described by Domard and Rinaudo (1983), fully deacetylated, purified chitosan (average viscosimetric degree of polymerization, $\overline{DP_v} = 3850$) was obtained by *N*-deacetylation of partially deacetylated chitin (Abertechnologies, batch no. BGL25; acetylation degree = 2·45%, $\overline{DP_v} = 5430$). Stock solutions of 5×10^{-2} eq/litre chitosan hydrochloride were then prepared by dissolving chitosan flakes in a stoichiometric amount of HCl (0·05 M), and stored at −18°C. The concentration of such solutions was verified by potentiometric (pH) titrations with 0·1 M NaOH.

Sodium undecylenate was purchased from Sigma (approximate purity: 98%), and used without further purification. Aqueous stock solutions of concentration 5×10^{-2} M were obtained by dissolving the fatty acid in water, and they were controlled by potentiometric (pH) titrations with 0·1 M HCl.

The buffer solutions were prepared by dissolving sodium acetate in water, according to the equation: $pH = pK_a + \log \frac{[salt]}{[acid]}$, and then adding acetic acid until the desired pH was obtained.

Unless otherwise specified, experimental conditions were as follows: temperature = 25°C, lipid concentration = 5 mM, ionic strength = 0·15 M, pH = 5·8.

All reagents were of reagent grade and all aqueous solutions were prepared with deionized water.

Methods

Potentiometry (pH)
All pH measurements were carried out on a TACUSSEL pH-meter (Minisis 8000), equipped with a standard calomel electrode and a glass electrode. The values were read after 1 min stirring of the solutions followed by 1 min rest.

Steric exclusion chromatography (SEC)
The measurement of chitosan concentrations in supernatants by chromatography was performed on a 200 SW Protein-Pak type column (Waters), with an online differential refractometer (Waters 410, Waters-Millipore).

The eluent was a solution of ammonium acetate 0·15 M/acetic acid 0·2 M of pH 4·5, which was introduced into the column by means of a Spectra-Physics IsoChrom LC pump. A calibration curve was obtained with samples of chitosan hydrochloride of different concentrations. It could then be used to determine the concentration of unknown samples.

Fourier Transform infra-red spectroscopy (F.T.i.r.)
F.T.i.r. measurements were carried out on a Perkin Elmer 1760 spectrophotometer; the resolution and the number of scans were $4\,cm^{-1}$ and 10 respectively. Solutions were analysed by the attenuated total reflection technique, while freeze-dried samples were pelleted with KBr and analysed using the transmission technique.

In our studies, chitosan was characterized by a band near 1090 cm^{-1} (ether group), very strong for chitosan-rich samples, and thus relatively sensitive to the presence of low polysaccharide concentrations. This absorption band was also convenient since neither undecylenic acid nor sodium undecylenate absorb in that region.

UV spectrometry
Turbidimetry is one of several methods used in the determination of the critical micellar concentration of surfactants (Hunter, 1987), as well as for the analysis of dispersed systems (Fujii *et al.*, 1978; Axberg *et al.*, 1980; Terrassin, 1986; Washington, 1990). To study the micellisation of undecylenic acid or determine lipid concentrations, we measured the absorbances of the samples at 230 nm (maximum of absorbance of the lipid band), on a Varian 634 UV-Vis spectrophotometer. For the study of the dispersion/flocculation phenomena, we measured absorbances at 270 nm rather than 230 nm, in order to avoid a too strong absorption (saturation) of highly dispersed systems. These samples were analysed after settling for approx. 24 h at 25°C. Depending on the sample turbidity, 1 cm or 1 mm Quartz Suprasil (HELLMA) cells were used. In order to compare the results obtained with the two types of cells, the absorbances measured with 1 mm cells were multiplied by a factor of 10. The solvent contained in the dispersions (water or a buffer solution) was taken as the blank.

Quasi-elastic light scattering (QELS)
Dispersions can be analysed using QELS (Wang & Audebert, 1987). Our experiments were performed on a Coulter Nano Sizer (Coultronics), after settling of the samples for approx. 24 h at 25°C.

RESULTS AND DISCUSSION

General mechanism

Figure 1 shows the typical behaviour obtained when chitosan hydrochloride is added to various UA dispersions in water (UA = undecylenic acid molecules, being in the acid —COOH or carboxylate —COO^- form). Such UA dispersions were initially set at different pH

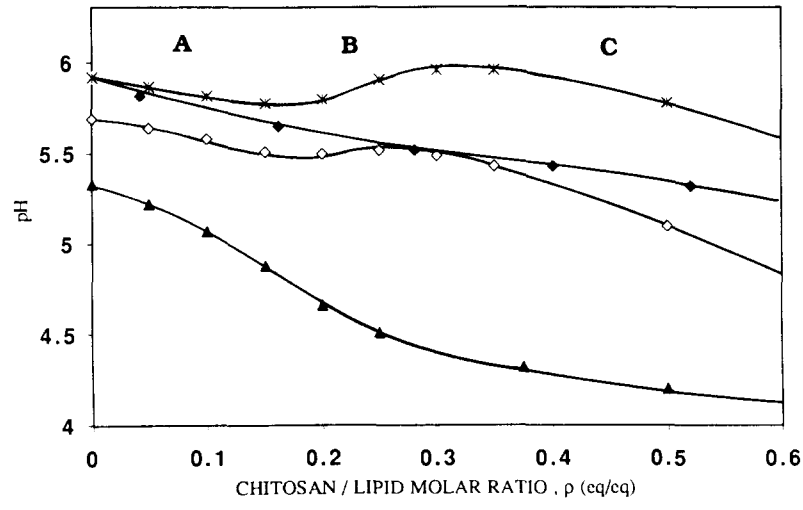

Fig. 1. pH variations obtained when chitosan hydrochloride (5×10^{-2} eq/litre) is added to dispersions of 5 mM sodium undecylenate initially set at various pHs (*, ◇, ▲) and to a dilute solution of sodium undecylenate (0·5 mM) initially set at pH ≈ 5·8 (◆)

values (pH_i) by means of hydrochloric acid. For a $pH_i > 5{\cdot}5$, the curves in Fig. 1 show 3 parts.

In the first, (A), we observed a slight decrease in pH, corresponding to partial neutralisation of the lipid $-COO^-$ charges by the chitosan $-NH_3^+$ functions. This was accompanied by a flocculation process which became maximal when pH values reached the first slope change. In other equivalent systems, it has been shown that the electrokinetic potential of the particle is zero at that point (Wang & Audebert, 1987). A similar study seems difficult in our case since the flocs stuck to the cell surface. In part (A) of Fig. 1, the surface potential therefore rapidly became zero, and flocculation withdrew many non-neutralized charges from the medium.

The study of chitosan in the flocculation supernatants by SEC (Domard & Cartier, 1992) and F.T.i.r. allowed us to show that the flocculation supernatants contained no chitosan, whatever the initial pH of the lipid dispersion. The flocculated chitosan was therefore not in equilibrium with free chitosan, and all added chitosan was consumed during the flocculation process. This is not the case in the flocculation of mineral particles such as kaolin (Domard *et al.*, 1989), where the interaction, mainly electrostatic, is much weaker since the charge density at the surface of mineral particles is weaker than that of UA particles.

The lipid concentrations in the flocculation supernatants were measured by UV spectrometry at $\lambda = 230$ nm (Table 1). We noticed that the flocculation supernatants contained UA, but in concentrations which considerably decreased upon addition to chitosan. Chitosan is thus entirely used up to flocculate most of the lipid.

In part (B) of Fig. 1, while the pH increased, we observed a redispersion of the fatty acid, which was completed approximately when the pH curve reached the second slope change. This stage corresponds to a progressive adsorption of chitosan onto the lipid particle surface, which would lead to a net positive surface charge. Redispersion of the medium occurred, rendering accessible some charges which had been withdrawn in the flocculation. In this domain, the dispersion appeared much more turbid than initially. The medium contained more suspended aggregates with a high local charge density of $-COO^-$ groups (in spite of the resultant positive surface charge), which gave them a polyelectrolyte character. The apparent pK of such charges being much higher than that of an isolated charge (Katchalsky, 1954; Rinaudo & Domard, 1975), we observed a pH increase.

Finally, in part (C) of Fig. 1, the pH decreased monotonously, due to the acidity of the polymer which was now in excess.

In Fig. 1, we noticed that, when the initial pH of the lipid dispersion was below 5·5, the flocculation/redis-

Table 1. UV spectrometry analysis of the lipid in flocculation supernatants

Total lipid concentration of samples (C_{TL}) (mM)	Concentration of added chitosan (meq/litre)	Concentration of lipid in the flocculation supernatant (mM)	Molar ratio UA mol/ glucosamine residue in the flocs
5	1	2·8	2·2
5	1·25	2·2	2·24
3	0·25	2·6	1·6
3	0·375	2·45	1·47
3	0·5	1·9	2·2
3	0·625	1·85	1·84
2	0·125	1·8	1·6
2	0·25	1·42	2·32

Samples were obtained by adding chitosan hydrochloride (5×10^{-2} eq/litre) to sodium undecylenate dispersions, in acetate buffers (pH 5·8, ionic strength 0·15 M).

persion mechanism described above was not observed. Indeed, UA dispersions only occurred for a particular equilibrium between molecules in the $-COO^-$ and in the —COOH forms. Figure 2 shows that UA micelles are not formed below pH 4·9. This allows us to explain the behaviour of the curve with $pH_i \approx 5{\cdot}3$ (Fig. 1), where the initial dispersion is rapidly destroyed by the pH decrease.

It seems very interesting to compare the flocculation/redispersion curves (Fig. 1), to those obtained when chitosan hydrochloride is added to dilute solutions of sodium undecylenate (0·5 mM) initially set at the same pH. In this case, the UA is essentially in the carboxylate form, no micelles are present and the chitosan $-NH_3^+$ functions simply titrate the lipid $-COO^-$, inducing a pH decrease. We noticed that the $pH_i = 5{\cdot}8$ flocculation/redispersion curve showed higher pH values than the chitosan hydrochloride ('reference') curve, which implied that, during flocculation, the neutralization of the lipid $-COO^-$ functions by the chitosan $-NH_3^+$, was incomplete compared to the 'reference' case.

This is consistent with an adsorption giving rise to only few $-NH_3^+/-COO^-$ contact points per chitosan chain, according to a mechanism probably of the mosaic type as proposed by Gregory (1973).

In addition to the pH, the ionic strength is also known to have an influence on lipid dispersions. (Hunter, 1987). This is why, in the following experiments, the pH or the ionic strength, or both were kept consant.

In order to work with a well-defined medium, we studied the micellization of undecylenic acid at constant ionic strength (0·15 M) with varying pH, and at constant pH (5·8) with varying ionic strength. The observed phenomena were characterized by UV spectrophotometry.

Influence of the physicochemical parameters

Influence of the pH

The pH is a very important parameter in lipid dispersions (Washington, 1990). As shown in Fig. 2, the critical micellar concentration of sodium undecylenate is multiplied by a factor of 10 when the pH increases from 5 to 6. This behaviour may be related to the pK_a of undecylenic acid, which is close to 5 (Sprague *et al.*, 1983). Figure 3 shows the variations of absorbance (A) obtained when a chitosan hydrochloride solution is added to lipid-containing media of pHs between 5·2 and 5·9. Such a narrow range was chosen because the dispersions are unstable below pH 5 and chitosan precipitates above pH 6·1 (Domard, 1987).

Figure 3 points out a clear flocculation phenomenon,

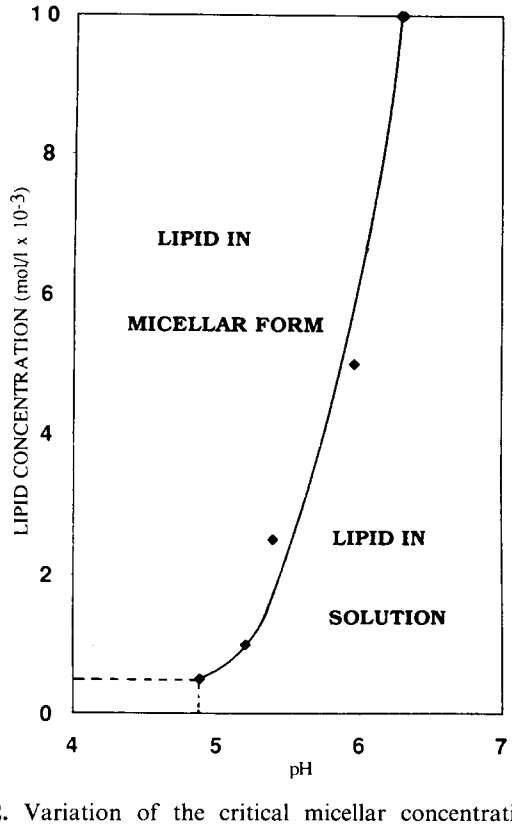

Fig. 2. Variation of the critical micellar concentration of sodium undecylenate with pH, in water, obtained by UV-spectrophotometry at 230 nm.

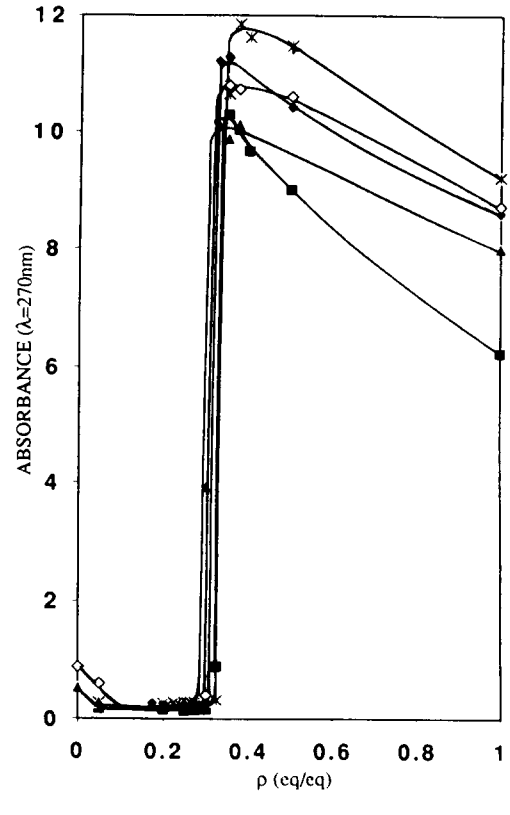

Fig. 3. Variation of the absorbances ($\lambda = 270$ nm) obtained when chitosan hydrochloride (5×10^{-2} eq/litre) is added to dispersions of sodium undecylenate (5 mM), in 0·15 M acetate buffers of the following pHs: 5·9 (▲), 5·8 (◇), 5·6 (◆), 5·4 (*) and 5·2 (■).

characterized by very low absorbances of the flocculation supernatants. Flocculation occurs at the first chitosan addition and tends to a maximum for a ratio ρ of glucosamine residues to UA moles close to 0·3 (mol/mol). Above this ratio, the flocculation is followed by a sharp redispersion. It corresponds to the destruction of the flocs, which is complete slightly after the absorbance maximum, i.e. for ρ close to 0·42. At that stage, the absorbance reaches values approximately 10 times higher than the initial values, which implies that the medium is now in a much more dispersed state. This large increase could also be due to a change of the intrinsic constants of the absorbance, brought about by the presence of chitosan.

The pH does not seem to have a great influence on the mechanism. We noticed, however, that the redispersions occurred according to the following order of pH: 5·9, 5·8, 5·6 and 5·4 $\approx$ 5·2. This phenomenon should be correlated to the increase of the $-COO^-$ surface charge density of the UA particles at higher pH, leading to a stronger interaction with chitosan, but this is counterblanced by the chitosan cationicity which decreases in the same conditions. The major effect is probably due to the fact that, for a given lipid concentration, less micelles will be present at higher pHs (Fig. 2). Consequently, less chitosan is needed to redisperse the medium. In the domain of redispersion, pHs ranging from 5·4 to 5·8 yield the most dispersed (turbid) systems. At pH 5·9 and 5·2, for opposite reasons, the dispersion is less favourable. In the former case, the high concentration of $-COO^-$ charges favours the solubilization (high critical micellar concentration), whereas in the latter case, the solubility is minimal but the dispersion is also much poorer in surface charges and thus may undergo a phase separation process.

After the absorbance maximum, all curves showed a stability decrease which may be assigned to a progressive neutralization of the lipid $-COO^-$ functions by the chitosan $-NH_3^+$, leading to weaker electrostatic repulsions between the aggregates. Another possible reason would be the screening effect between the aggregates, brought about by a possible excess of chitosan in the medium. We therefore centrifuged (57000*g*, 30 min) dispersions prepared at $\rho < 0{\cdot}5$, and analysed the supernatants (freeze-dried) by F.T.i.r. spectroscopy. No chitosan was present in these supernatants, implying that for ρ values <0·5, all added chitosan was adsorbed on the dispersed particles and thus no screening effect could be provided by an excess of chitosan. On the other hand, this supernatant contained solubilized molecules of lipids (not particles). Under our conditions, the absence of free chitosan revealed a very great selectivity of this polymer toward the dispersed form of lipid molecules. This observation is very important if we consider the applications of chitosan in living media.

We also noticed an effect of the ionic strength on the stability of dispersions; these could be obtained at pH 5·2 in the presence of salts, whereas in the absence of electrolyte it was not possible below pH 5·4.

In order to investigate possible kinetic effects, we studied the evolution of the samples with time. We showed that, even after storage for a week at a temperature of 25°C, the absorbance curves were analogous to those reported in Fig. 3, in both flocculation and redispersion domains. In addition, a dispersion obtained with lipid and chitosan in a molar ratio ρ close to 0·5 at pH 5·8 (acetate buffer), showed no sign of evolution after 6 months of storage, without protection against light, both at room temperature and at 4°C. Without chitosan, a similar dispersion gradually tends to a phase separation at room temperature after a few weeks, and crystallizes at 4°C. Chitosan thus also improves the stability of lipid dispersions upon storage.

The UV spectrometry curves (Fig. 3) show an absorbance maximum for molar ratios between 0·37 and 0·39. A study of the particle size by QELS reveals that optimal dispersion which should correspond to the minimal particle size (Becher, 1965) is slightly shifted from those values (Fig. 4). As previously reported by Fligner *et al.* (1988), for other systems, we notice that the turbidity maximum actually corresponds to a heterogeneity of the medium, where the dispersion always contains suspended flocs.

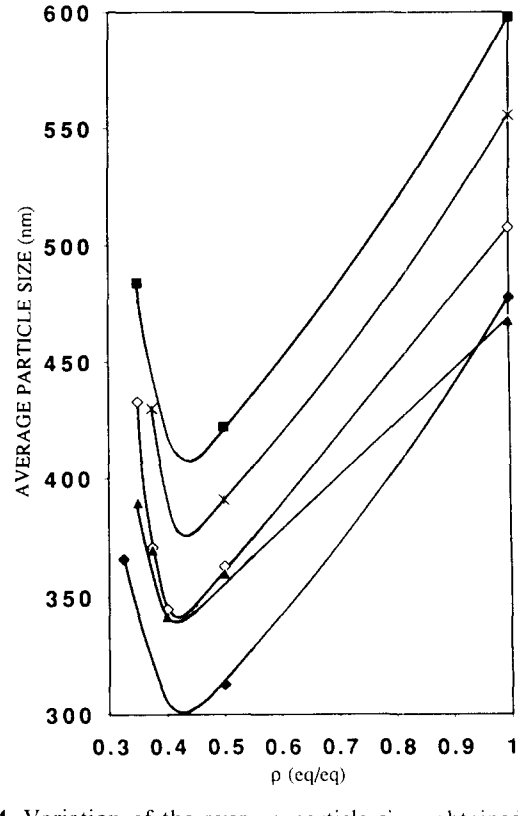

Fig. 4. Variation of the average particle sizes obtained when chitosan hydrochloride (5×10^{-2} eq/litre) is added to dispersions of sodium undecylenate (5 mM), in 0·15 M acetate buffers of the following pHs: 5·6 (■), 5·9 (*), 5·4 (◇), 5·8 (▲) and 5·2 (◆).

Considering the curves in Fig. 4, we observed that, generally, after redispersion had begun, the particle size reached a minimum before increasing again when chitosan was added to the medium. According to Stoke's law, the minimal particle size should correspond to a maximal stability of the system (Becher, 1965), related to maximal interparticular electrostatic repulsions. For our system, whatever the pH, an optimal mixture was obtained for ρ values between 0·42 and 0·43. As demonstrated above, the size increase after this point may correspond to further neutralization of the aggregates by chitosan (i.e. further adsorption of the polymer) rather than a screening effect between the aggregates, brought about by an excess of poly(glucosamine) chains in solution.

Considering now the influence of pH on the particle size, it seems that pH 5·8 corresponds to an optimal pH value, although the aggregates are larger than those at pH 5·2. The latter case could correspond to an exception to Stoke's law as suggested by Fligner *et al.* (1988), where infant formulae dispersions exhibited small particles, yet were not stable. We can note that pH 5·8 corresponds to the isoelectric pH of our system since the lipid pK_a is close to 5, and that of chitosan is 6·5 (Domard, 1987).

From the study of the influence of pH, we can conclude that this parameter has a considerable effect on the redispersion of flocculated systems. We can also define optimal conditions for the dispersed state as being a pH between 5·4 and 5·8 with $\rho = 0{\cdot}42$, when the UA concentration is 5 mM and the ionic strength 0·15 M.

Influence of the ionic strength

The ionic strength is a parameter known to influence greatly the stability of lipid dispersions (Washington, 1990). In the case of UA, we showed that the critical micellar concentration decreased with an increase of ionic strength from 0 to 2 M, for an acetate buffer of pH 5·8 (results not shown). For the higher ionic strengths, the dispersions became unstable and tended to undergo phase separation, which is why we limited our range of study to ionic strengths below 1 M. Figure 5 shows the influence of the addition of chitosan to lipid dispersions, in acetate buffers of pH 5·8, at various ionic strengths. We observed the same flocculation process as described above, with a redispersion by chitosan which occurred sooner, the higher the ionic strength. This agrees well with a purely electrostatic mechanism.

During redispersion by chitosan, the turbidity is also related to the ionic strength, and is maximal for an ionic strength of 0·15 M. It is important to note that, at an ionic strength of 1 M, suspended flocs remain in the dispersion until ρ reaches values close to 1. The QELS study of particle sizes (not shown) showed that a minimal size is achieved with the lowest ionic strength. In all

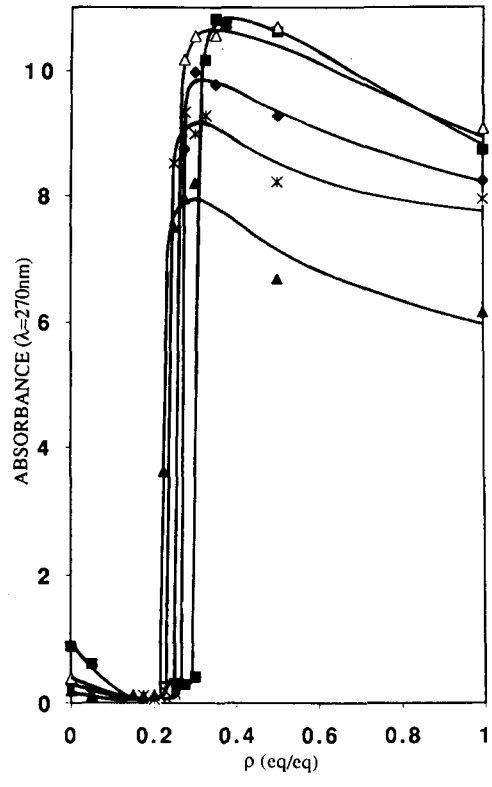

Fig. 5. Variation of the absorbances ($\lambda = 270$ nm) obtained when chitosan hydrochloride (5×10^{-2} eq/litre) is added to dispersions of sodium undecylenate (5 mM), in acetate buffers (pH 5·8) of the following ionic strengths: 0·15 M (■), 0·3 M (△), 0·4 M (◆), 0·5 M (*) and 1 M (▲).

the situations, we observed the behaviour described in Fig. 4, in particular a minimal particle size for ρ values slightly higher than those corresponding to the turbidity maximum, as well as an increase of the size as ρ increased again.

We can thus conclude that high ionic strengths, at constant pH, destabilize the dispersion mechanism, which agrees entirely with a purely electrostatic process. This has led us to consider that a buffered medium of pH 5·8 and ionic strength 0·15 M corresponds to optimal experimental conditions. In addition, this ionic strength has the advantage of being close to that of biological media; this may be related to results obtained with protoplast suspensions in similar conditions (Kauss *et al.*, 1989).

Influence of the lipid concentration

Having defined the optimal pH and ionic strength conditions for our experiments, we studied the influence of total lipid concentration (C_{TL}). Figure 6 illustrates the absorbance variations measured at various UA concentrations, upon addition of chitosan hydrochloride. We noticed that the redispersion of flocs occurred at higher ρ values when C_{TL} was increased, implying that the flocs became more stable. The lower the lipid concentration, the lower the percentage of lipid

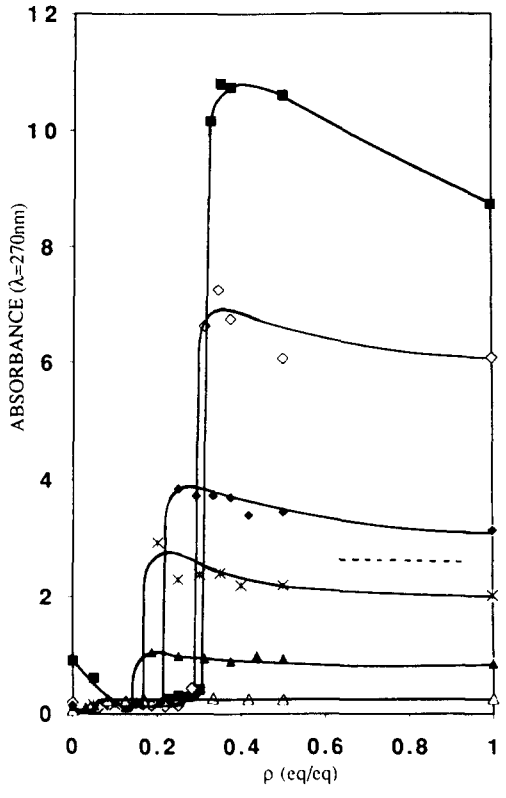

Fig. 6. Variation of the absorbances ($\lambda = 270$ nm) obtained when chitosan hydrochloride (5×10^{-2} eq/litre) is added to dispersions of sodium undecylenate of the following concentrations: 5 mM (■), 4 mM (△), 3 mM (◇), 2·5 mM (*), 2 mM (▲) and 1·5 mM (△), in acetate buffers (pH 5·8, ionic strength: 0.15 M). Concentrations below the dotted line (2·5, 2, 1·5 mM) are lower than the critical micellar concentration of undecylenic acid.

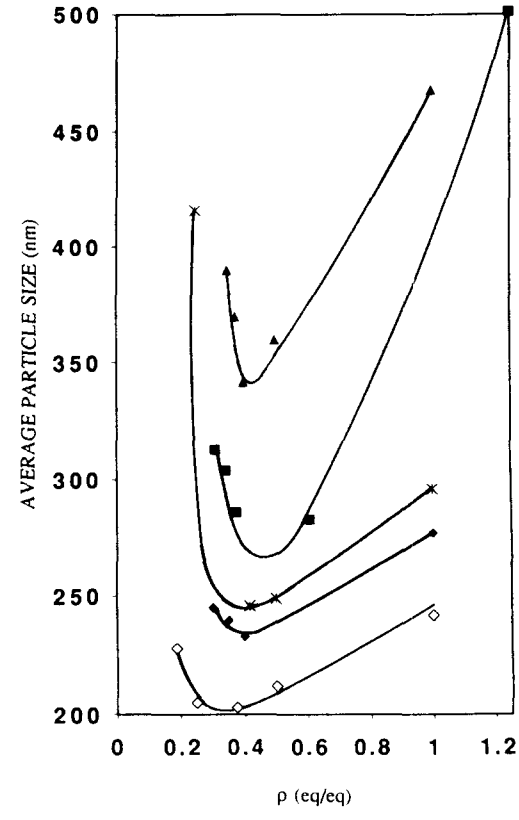

Fig. 7. Variation of the average particle sizes obtained when chitosan hydrochloride (5×10^{-2} eq/litre) is added to dispersions of sodium undecylenate of the following concentrations: 5 mM (▲), 4 mM (■), 3 mM (*), 2·5 mM (◆), 2 mM (◇), in acetate buffers (pH 5·8, ionic strength: 0·15 M).

in the dispersed form; and it is thus normal that the ρ value necessary to cause redispersion was lower. In the redispersion domain, we showed that the turbidity was almost proportional to C_{TL}, which suggests that the mechanism is the same whatever the lipid concentration.

The most important phenomenon in Fig. 6 is that chitosan is able to flocculate and redisperse media where C_{TL} is lower than the critical micellar concentration of UA, which is 2·8 mM in the same pH and ionic strength conditions (see below). Figure 7 shows that the particle size at the redispersion optimum decreased with decreasing C_{TL}. As in the previous experiments, we confirmed that the ρ value at the redispersion optimum agrees with the turbidity measurements, though slightly shifted from the value deduced from the UV absorbance maximum.

In the second part of this study, we looked at the influence of dilution on a UA dispersion in the presence of chitosan. Figure 8 shows the variation of the absorbances measured when a lipid dispersion or an equimolecular mixture of lipid and chitosan, was diluted. We showed that, in our experimental conditions, the critical concentration below which the particles are in solution was approximately 3 times lower in the presence than in the absence of chitosan. This critical concentration shows that chitosan can flocculate and redisperse UA at very low concentrations — about 200 ppm. The linearity obtained in the dispersion domain confirms that the mode of interaction is unique whatever the total lipid concentration.

Figure 9 illustrates the evolution of particle sizes obtained upon dilution of either equimolecular lipid/chitosan mixtures or a dispersion corresponding to the redispersion optimum. We noticed that in all 3 cases, dilution led to an increase of aggregate sizes, which is contrary to the result obtained when chitosan was added to media of decreasing lipid concentrations (compare Figs 8 and 9). Diluting a chitosan/UA dispersion of initial concentration C to obtain $C'(C < C')$ was therefore not equivalent to preparing the dispersion at the concentration C'.

Finally, as reported in Table 1, analysis of lipid in flocculation supernatants (by UV spectrometry) reveals that, whatever the initial lipid concentration, the molar ratio fatty acid/glucosamine residues in the flocs is generally close to 2. This confirms a unique mechanism of flocculation for all lipid concentrations.

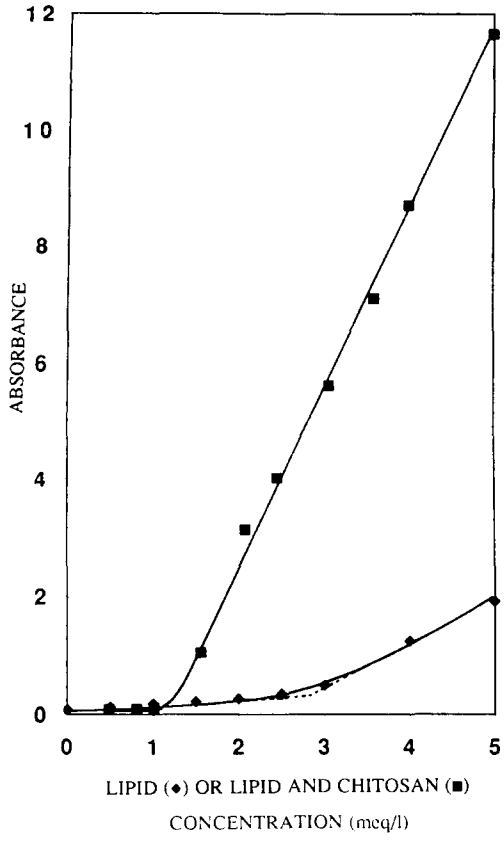

Fig. 8. Variation of the absorbances obtained when the following dispersions are diluted: 5 mM sodium undecylenate (◆) (absorbance measured at 230 nm), and 5 mM sodium undecylenate +5 meq/litre chitosan hydrochloride (■) (absorbance measured at 270 nm). The samples are prepared in acetate buffers (pH 5·8, ionic strength: 0·15 M).

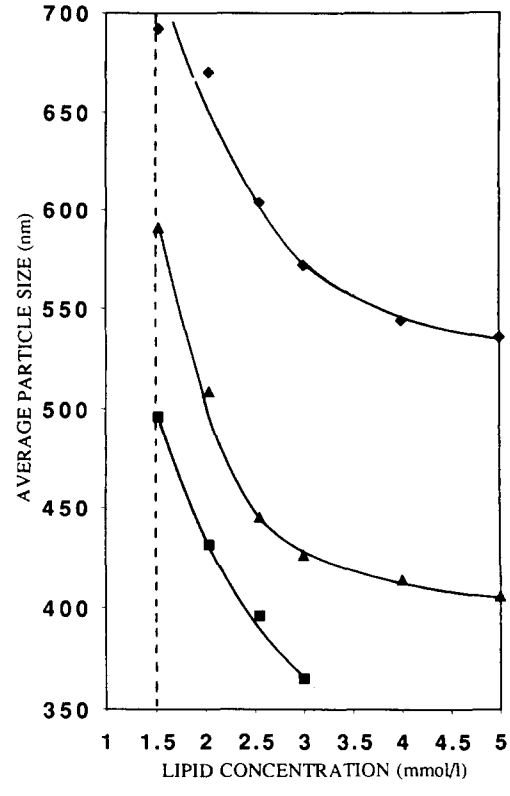

Fig. 9. Variation of the average particle sizes obtained when the following dispersions are diluted: 5 mM sodium undecylenate +5 meq/litre chitosan hydrochloride (◆), 3 mM sodium undecylenate +3 meq/litre chitosan hydrochloride (▲), and 5 mM sodium undecylenate +2·5 meq/litre chitosan hydrochloride (■). The samples are prepared in an acetate buffer (pH 5·8, ionic strength (0·15 M). The lower limit corresponds to the disappearance of the dispersed states.

Proposed mechanism

Let us consider a UA dispersion of concentration C_1 or C_2 above 1 mM ($C_1 < C_2$). We have seen that addition of small amounts of chitosan to such a dispersion leads to a flocculation process. This may result from an interparticular bridging phenomenon or a mosaic effect (Sato & Ruch, 1980; Terrassin, 1986; Guyot *et al.*, 1990). Bridging flocculation is due to the adsorption of one macromolecule onto many particles, forming a necklace-like structure. This process generally occurs with neutral polymers (Guyot *et al.*, 1990) or polyelectrolytes of the same charge sign as the particle (Sato & Ruch, 1980). With chitosan, electrostatic interactions between opposite charge signs leads to a strong adsorption of the polyelectrolyte onto the lipid aggregates (all chitosan is consumed during flocculation). Neutralization of the polymer and particle surface charges is incomplete (see discussion of Fig. 1, comparison of a flocculation/dispersion curve to a 'reference curve'). Therefore, positive and negative domains are formed which, upon collision, may interact to form larger aggregates called flocs (Guyot *et al.*, 1990). In a system of higher lipid concentration (C_2), more collisions will occur and the flocs will thus be larger than those obtained with a lipid dispersion of concentration $C_1 < C_2$. This situation is depicted in Figure 10.

Addition of more chitosan to such flocculated media leads to further adsorption onto the lipid aggregates, destabilizing the flocs by electrostatic repulsions which arise from the increase of positive surface charges. This results in the redispersion of the flocs, the new chitosan-coated aggregates being stabilized by electrostatic repulsions (Sato & Ruch, 1980).

In our system, it seems that the redispersion is not composed of isolated chitosan-coated lipid micelles, but rather aggregates of such structures. Indeed, the sizes of these aggregates (≈300–600 nm, see QELS studies) are much larger than those usually reported for micelles (≈10–20 nm) (Darling, 1982; Fevrier, 1990; Hofer *et al.*, 1991), and it has already been shown that micellar structures can form secondary stackings (Darling, 1982; Oakenfull, 1983; Theodoor & Overbeek, 1985). Thus the aggregation of lipid particles occurring during flocculation is very important in the mechanism of redispersion, in particular regarding the size of the redispersed particles.

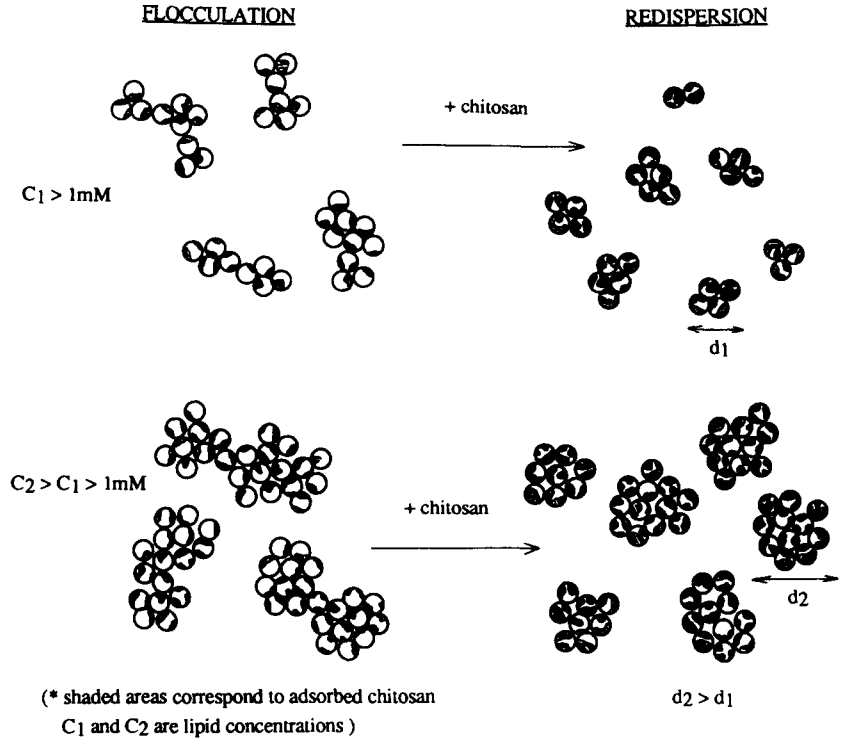

Fig. 10. Proposed mechanism for the adsorption of chitosan chains on lipid particle aggregates.

The above discussion may be illustrated by Fig. 10, where higher initial lipid concentrations lead to larger flocs, and in turn to larger redispersed chitosan-coated lipid aggregates. In dilution studies (Fig. 9) the reverse trend is obtained: dilution results in size increase of the aggregates. Addition of solvent to a dispersed lipid/chitosan system increases the distances between the aggregates, which leads to a decrease of the electrostatic repulsions existing between adjacent aggregates. Solvent adsorption also occurs onto the hydrophilic surface, causing an increase of the aggregate sizes. We can therefore easily understand that a lipid–chitosan dispersion directly prepared at a concentration of 3 mM shows smaller aggregate sizes than one prepared at 5 mM and then diluted to 3 mM. Indeed, a redispersion of initial concentration $C_i = 5$ mM contains larger aggregates than one of concentration $C_i = 3$ mM (see discussion of Fig. 10 above), and its dilution can only lead to a further size increase.

Figure 9 also shows that sizes corresponding to concentrations below 1·5 mM are no longer measurable on the Coulter Nano Sizer, because the dispersed state is lost. Correlating this result to the lipid/chitosan curve of Fig. 8, we may state that the redispersion collapsing which occurs upon excessive dilution, is mainly due to redissolving of the aggregates below a critical concentration, and not to the aggregate size, since it is possible to have stable aggregates of sizes 500–600 nm (5 mM equimolecular curve in Fig. 9), which are the 'limit' sizes for the other 2 curves (Fig. 9). This implies that, contrarily to the flocculated system, chitosan-coated aggregates in the redispersion domain are in dynamic equilibrium with the surrounding medium, and we are not in the presence of a true encapsulation of lipid particles by chitosan, where the mechanical properties of the polymer membrane would play an important part. A similar interpretation has been proposed in the case of the encapsulation of phospholipid liposomes by chitosan (Iwamoto *et al.*, 1991).

CONCLUSION

In this work, we have studied the flocculation and dispersion properties of chitosan in media containing undecylenic acid (this term refers to both the acid and the carboxylate forms). The relatively high size of the suspended particles in the presence of chitosan suggests that, in this case, lipid molecules are associated as aggregates of micelles rather than isolated micelles. From all our results, we may conclude that chitosan chains interact by electrostatic interactions at the surface of the lipid aggregates, thus stabilizing the dispersed state. Chitosan chains could be partly involved in the mechanism allowing easy changes of the shape and volume of the aggregates. This behaviour is quite different to a chemical encapsulation process by a continuous three-dimensional membrane, usually obtained in the case of polyanion–polycation interactions, for example.

Our results, which show that chitosan interacts very strongly with the surface of lipid particles, must be

related to those concerning some biological properties of chitosan. We can quote the example where addition of chitosan to diets leads to a decrease of the fatty acid metabolization (Sugano *et al.*, 1978; Kobayashi *et al.*, 1979; Nagyvary *et al.*, 1979; Sugano *et al.*, 1980, 1988; Jennings *et al.*, 1988). Another example concerns the eliciting properties of chitosan when it is added to suspensions of plant cells, in particular in the case of protoplasts. Other studies in progress should allow us to determine the influence of the nature of the fatty acid, the molecular mass and the acetylation degree of chitosan on the adsorption of this polymer onto lipid surfaces.

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