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Chitosan behaviours in a dispersion of undecylenic acid. Morphological aspects

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Electron microscopy is used to complement our study of the interactions occurring between chitosan and undecylenic acid dispersions. Our previous papers reported investigations regarding the influence of physico-chemical and structural parameters, and a mechanism of interaction was proposed. We now confirm these studies by analysing the microscopic structure of chitosan/undecylenate flocs, using various TEM and SEM techniques. Catenary, fractal structures are observed, in agreement with a theoretical model established for the flocculation of spherical particles by polymer chains. Finally asymmetrical membranes were prepared and characterized. They result from the association of a chitosan undecylenate salt layer and chitosan/lipid aggregates in the redispersed state, according to a 3-step mechanism which is proposed.

INTRODUCTION

This paper is our third report on the interactions occurring between chitosan and undecylenic acid in the dispersed state. In the previous papers, we showed in particular that the addition of chitosan to such a dispersion induces either a flocculation or a stabilization of the dispersion, depending on the conditions. In the first paper (Demarger-André & Domard, 1993), we studied the role of various physico-chemical parameters such as pH, ionic strength, lipid concentration, temperature and ageing of the dispersions. In the second paper (Demarger-André & Domard, 1994), we investigated the influence of the structural parameters related to chitosan, i.e. its charge density (acetyl content) and molecular weight. The main result was that the mechanism of flocculation/redispersion of undecylenic acid in the presence of chitosan simply depends on the number of protonated sites ($-NH_3^+$) contained in the medium, without polyelectrolyte effect. As a consequence, the more chitosan is deacetylated, the less is the amount necessary to redisperse the flocculate. We also studied the role of the molecular weight of chitosan on the mechanism of redispersion. Two domains leading to two different behaviours were identified. In the range of low molecular weights, chitosan chains are essentially adsorbed onto individual lipid particles, while above a relatively high molecular weight, interparticular bridging occurs.

In this work, we propose to confirm the mechanisms described previously by observation of various systems by means of electron microscopy.

MATERIALS AND METHODS

Fully deacetylated chitosan was prepared by *N*-deacetylation (Domard & Rinaudo, 1983) of commercial chitosan (AberTechnologies, batch BGL25, acetylation degree = 2.5%, viscometric average molecular mass $\overline{M}_v$ = 1 100 000 g/mol using the conditions of Roberts & Domszy (1982)).

Aqueous chitosan hydrochloride solutions of concentration 5×10^{-2} eql^{-1} were prepared by dissolving chitosan powder in a stoichiometric amount of 0.1 N HCl. pH titrations were performed with NaOH in order to control the concentration.

Aqueous sodium undecylenate solutions of concentration 5×10^{-2} M were obtained by dissolving the fatty acid sodium salt (Sigma, purity >99%) in deionized water, and the concentration was checked by pH titration with 0.1 N HCl.

Chitosan/undecylenate flocs were prepared by adding aqueous chitosan hydrochloride to an undecylenic acid dispersion (concentration 5×10^{-3} M and pH = 5.8), in the molar ratio ρ = glucosamine residues/lipid mole-

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cules = 0.3. The initial fatty acid dispersion was obtained by diluting aqueous sodium undecylenate in water, and lowering its pH to 5.8 by addition of HCl. Flocs were isolated simply after sedimentation of the medium and removal of the clear supernatant (no centrifugation or filtration).

Asymmetrical membranes were prepared by submerging a dialysis bag (regenerated cellulose, average pore diameter = 2.4 nm) filled with 50 ml of a 1×10^{-2} eql^{1} aqueous chitosan hydrochloride solution (pH = 5.8, ionic strength $\mu = 0.15$ M, acetate buffer), in 250 ml of undecylenic acid dispersion (concentration = 5×10^{-3} M, pH = 5.8, $\mu = 0.15$ M, acetate buffer). The system was slowly stirred and an asymmetrical membrane could be removed from the internal side of the dialysis bag after 48 h. The membrane was then simply rinsed with deionized water and dried in open air for 24 h.

FTir spectroscopy

FTir spectra were recorded on a Perkin Elmer 1760 spectrophotometer (10 scans, resolution = 4 cm^{-1}). Flocs were observed by attenuated total reflexion (ATR), using a horizontal Zn Se crystal. The chemical structure of the asymmetrical membranes was studied by ATR using a KRS-5 crystal inclined so that the angle of the incident beam was 45° with respect to the sample.

Transmission electron microscopy (TEM)

The sample was rapidly frozen in liquid helium (−270°C), and then included in an EPON resin. Ultrathin sections were laid on 600 mesh copper grids and positively contrasted with uranyl acetate and lead citrate. These samples were observed on a Hitachi H600 microscope.

The sample was 'sandwiched' between two copper cups, rapidly frozen in nitrogen slush (≈ −200°C), and placed in a cryofract where the sandwich was open and the frozen sample was, therefore, fractured so that its internal pattern was revealed. Both fractured planes were coated with a thin layer of platinum and carbon. Finally, the replica was isolated by dissolving the sample (water and organic solvent baths), and laid onto 600 mesh copper grids. These samples were observed on a Hitachi HU12A microscope.

Although specific antibodies have not yet been found for chitosan, it is possible to label the polymer. Indeed, Horisberger & Clerc (1988) reported that chitosan may be complexed with colloidal gold. Considering the latter work, and the usual techniques for the preparation of gold particles (Frens, 1973; Horisberger & Tecchini-Vonlanthem, 1983; Horisberger & Rosset, 1977), we set up the following method for the labelling of chitosan: 250 µl of a 4% aqueous solution of $HAuCl_4$ (Sigma) were dissolved in 100 ml deionized water and heated under reflux. Two millilitres of a 1% sodium citrate solution were then added, in order to reduce the gold. After 5 min, the reaction medium was cooled down to room temperature and could then be stored at 4°C. This method yields gold particles of diameter approximately 20–30 nm (checked by TEM). Chitosan was labelled by mixing 5 ml of this colloidal gold solution, and 5 ml of an aqueous chitosan solution (1 mg/ml, pH = 5.8) which had been previously filtered on a membrane of porosity 0.22 µm.

Chitosan labelled according to this method was used to study the structure of chitosan/undecylenate flocs (colloidal gold marking + freeze–fracture).

Scanning electron microscopy (SEM)

This technique was used to observe asymmetrical membranes and freeze-dried flocs. The sample was stuck onto a sample holder by means of a double sided adhesive tape, and plated with C and Pt. It was then observed on a Hitachi S800 microscope.

RESULTS AND DISCUSSION

Characterization of chitosan/undecylenate flocs

In the first part of this study (Demarger-André & Domard, 1993), we showed that the flocculation of undecylenic acid dispersions, in the presence of chitosan, is optimal for a pH close to 5.8. These conditions were used to prepare flocs with chitosan having a viscometric average molecular mass of 1.1×10^{6} g/mol. The flocs were collected at the flocculation maximum for such conditions (Demarger-André & Domard, 1993), namely for ρ (ratio of glucosamine residues to fatty acid mols) close to 0.3. The absence of chitosan in the flocculation supernatants, and hence its presence in the flocs, was revealed by steric exclusion chromatography and FTir analyses. Furthermore, if we consider the FTir spectrum of such flocs (Fig. 1), the presence of chitosan is confirmed by various characteristic bands at

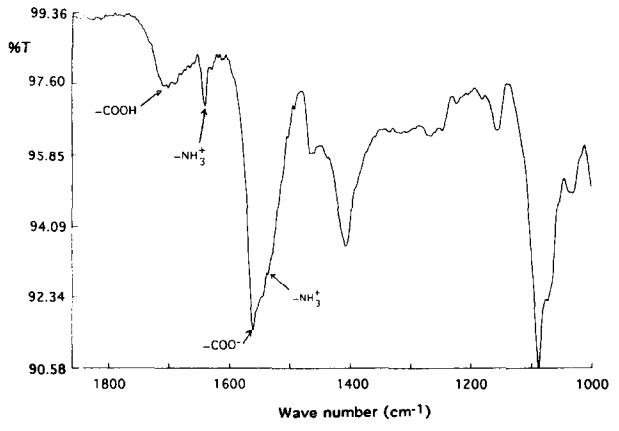

Fig. 1. FTir spectrum of chitosan/undecylenic acid flocs, using the ATR technique.

1620 and 1517 cm^{-1} for the $-NH_3^+$ group, and at 1080 cm^{-1} for the pyranose ring. The fatty acid may be detected at 1560 cm^{-1} (strong -COO^- band) and 1715 cm^{-1} (weak -COOH band). The proportion of chitosan in the flocs appears to be relatively weak. Indeed, this has been previously shown by UV spectrometry analyses of the flocculation supernatants; within a wide range of lipid and chitosan concentrations, the amount of lipid in the flocs was approximately twice that of chitosan (Demarger-André & Domard, 1993).

After characterizing the flocculation conditions as well as the chemical composition of the flocs, we were interested in studying their morphology. In order to observe such structures, we used both transmission (TEM) and scanning (SEM) electron microscopy.

Firstly, the initial lipid dispersions were observed by TEM after cryosubstitution (Fig. 2a) and freeze–fracture (Fig. 2b). The results presented in Fig. 2a seem to show that the initial dispersions contain complex aggregates of lipid particles. We distinguish a bimodal distribution of particle sizes, corresponding to dimensions between 400 nm and 1 μm for the largest population, and 20–50 nm for the smallest. The freeze–fracture micrographs reported in Fig. 2b show examples of large aggregates as a whole. It is important to notice that in all cases, whatever the mode of observation, no superstructure corresponding to long range associations of particles is observed. For characterization of the flocs, cryosubstitution was unsuccessful since it was not possible to obtain a clean microtomy of a floc, the latter being dispersed too heterogeneously in the medium. Nevertheless, freeze–fracture and SEM techniques provided many interesting results. Figure 3a shows the freeze–fracture micrograph of flocs and we clearly notice a catenary structure formed by nodules of sizes corresponding to those of the initial lipid aggregates. These nodules seem to be linked and coated by a substance which should certainly be chitosan. Furthermore, SEM micrographs (Fig. 3b) show branched structures leading to the fractal character of the objects constituting the flocs. In all cases, the dimensions of the nodules correspond approximatively to those of the initial lipid aggregates. SEM analysis of the flocs at various magnifications confirmed the interesting fact that the geometry of the flocs is fractal, i.e. the branches may be seen at all levels of observations. Finally, we looked further into the microscopic structure of the flocs and checked the presence of chitosan by labelling the polymer with colloidal gold before preparing the flocs with undecylenic acid dispersion. Standard freeze–fracture and TEM observations were then performed, a resulting micrograph being presented in Fig. 3c. A higher concentration of small gold particles (≈20 nm) may be noticed around the flocs, showing effectively that chitosan is present at the surface of the lipid particles.

It is important to note that the microscopic structure of the flocs which we have characterized, agrees quite well with the theoretical model described by Guyot *et al.* (1990), in particular regarding the fact that flocculation of spherical particles by a polymer depends on the size of this polymer. Indeed, the authors predicted that the flocs obtained with very long macromolecules would have a branched structure, similar to that observed in our case for chitosan of $\overline{M}_v = 1.1 \times 10^6$ g/mol. On the other hand, very small polymer chains should not lead to the flocculation of the system, since the layer of adsorbed polymer remains beneath the barrier of electrostatic repulsion of the particle. It is thus interesting to note that we have not been able to flocculate undecylenic acid dispersions with a chitosan sample of DP = 7.

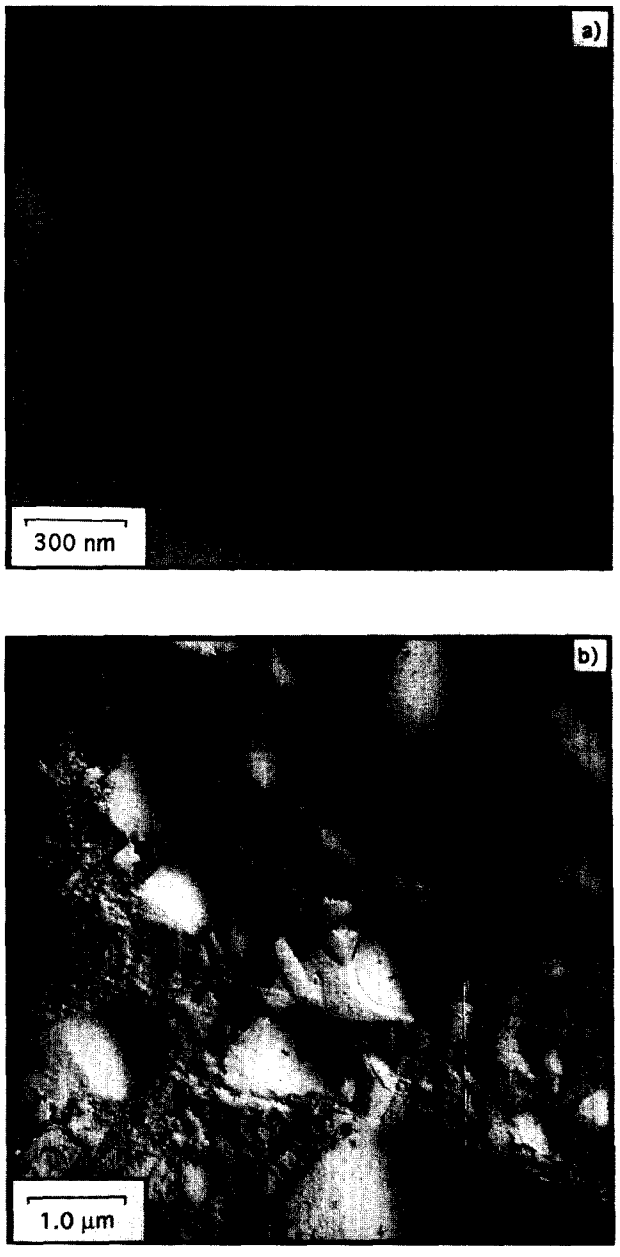

Fig. 2. TEM micrographs of 5×10^{-3} M undecylenic acid dispersions (pH 5.8) after: (a) cryosubstitution; and (b) freeze–fracture.

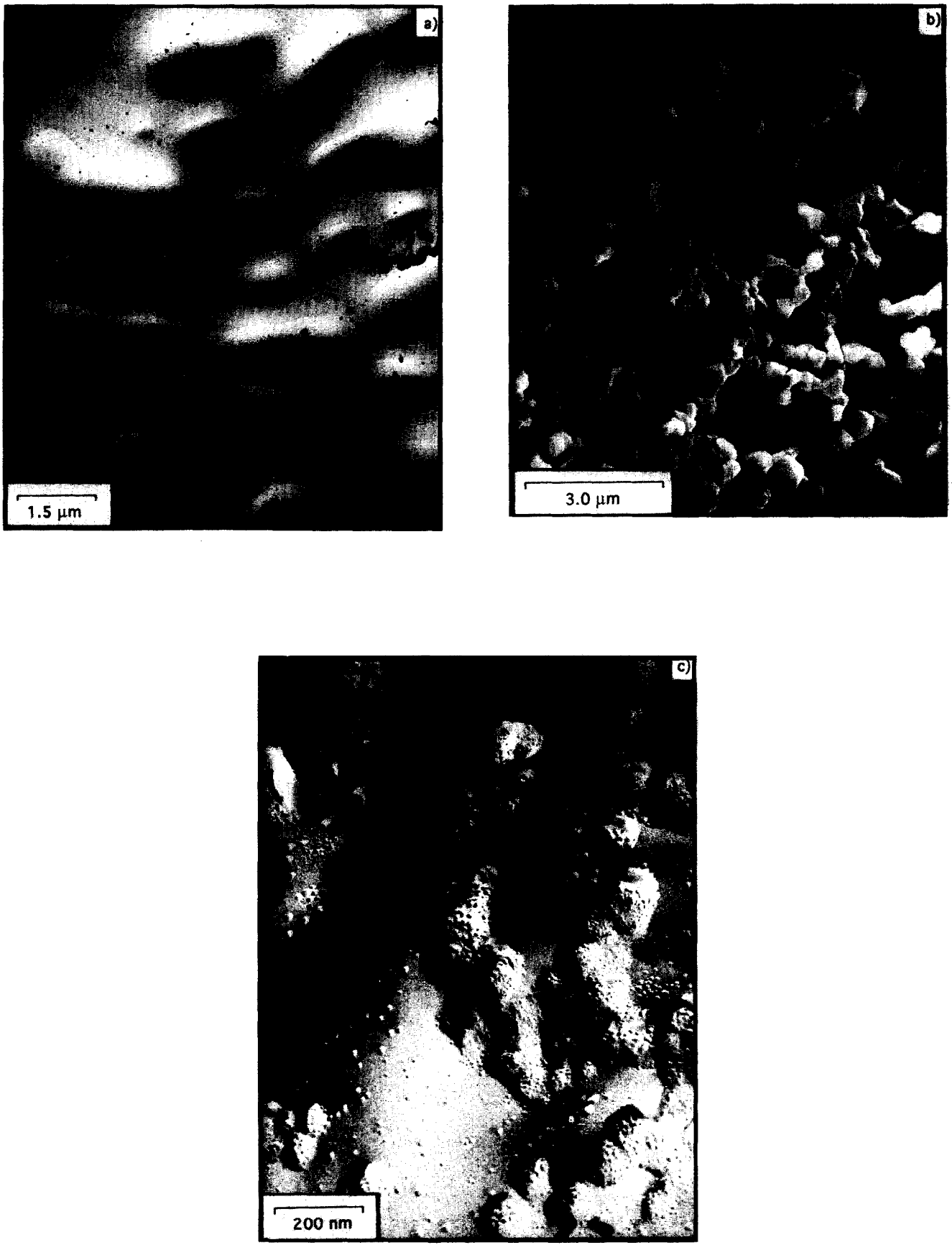

Fig. 3. Analysis of the microscopic structure of chitosan/undecylenic acid flocs: (a) TEM micrograph of a freeze–fractured sample; (b) SEM micrograph of a freeze-dried sample; c) TEM micrograph of a freeze–fractured sample, the flocs having been prepared with colloidal gold-labelled chitosan.

Characterization of asymmetrical membranes

In order to have another approach to the study of flocculation and redispersion of undecylenic acid by chitosan, we considered the interactions of the two components in a medium in which they would initially be separated by a semi-permeable membrane. We chose a cellulose dialysis bag with an average pore size of 2.4 nm, implying that neither chitosan molecules (average gyration radius greater than 10 nm) nor lipid aggregates could pass through it. Therefore, only small molecules, in particular isolated lipid molecules could cross the membrane.

The experiment was simply carried out by placing a dialysis bag containing a chitosan solution in a glass cylinder containing the undecylenic acid dispersion. After a few hours, the medium situated ouside the bag became clear, while the internal wall of the bag became opaque. This opacity was due to the formation of a thin membrane, easily removed from the wall. This membrane exhibited an asymmetrical appearance, the side which was in contact with the dialysis bag showing a shiny aspect, while the other side was relatively dull.

Figure 4 shows the time variation of the lipid concentration in the medium outside the dialysis bag, for a control and for a typical experiment. The control represents the situation where the bag contains no chitosan, and a mere diffusion of the lipid molecules is observed. The concentration decrease corresponds to the increase of the accessible volume due to the presence of the dialysis bag. This also allows us to demonstrate the absence of any adsorption of lipid molecules onto the cellulose membrane. The comparison of this control trial with the experiment for which chitosan was used, shows that in the latter, the lipid concentration in the external medium decreases considerably. This must be the consequence of an important interaction between chitosan and lipid molecules inside the dialysis bag.

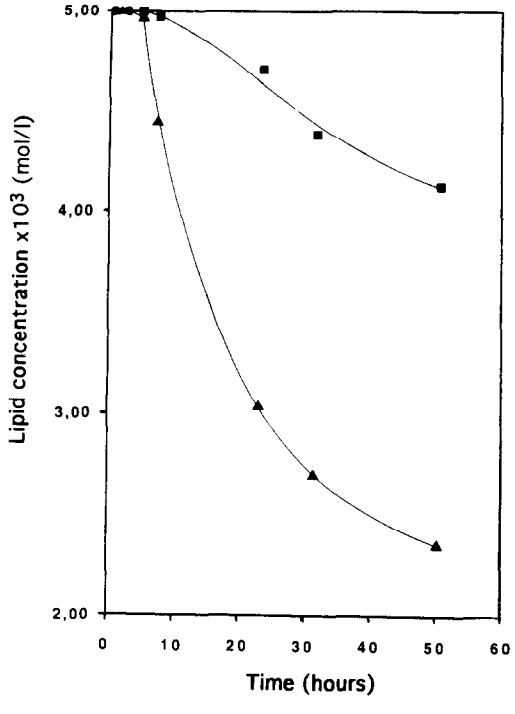

Fig. 4. Variation of the lipid concentration outside the dialysis bag as a function of reaction time. Control (■): the dialysis bag contains 50 ml of a 0.15 M acetate buffer, pH 5.8; experiment with chitosan (▲) : the bag contains 50 ml of a 1.10^{-2} eql^{-1} solution of chitosan hydrochloride in a 0.15 M acetate buffer, pH 5.8.

The asymmetrical membranes are generally 10–20 μm thick after the standard reaction time of ≈24 h, and this thickness may not be increased by extending this reaction time. Their mechanical properties are relatively poor in the wet state. They are largely improved in the dry state but remain lower than those of a pure chitosan film. Nevertheless, their chemical stability is quite good compared to that of chitosan. Indeed, they are not degraded in aqueous solutions of pHs between 3 and 12.4, even after several months of submersion.

In order to study the chemical composition of both sides of the asymmetrical membrane independently, we

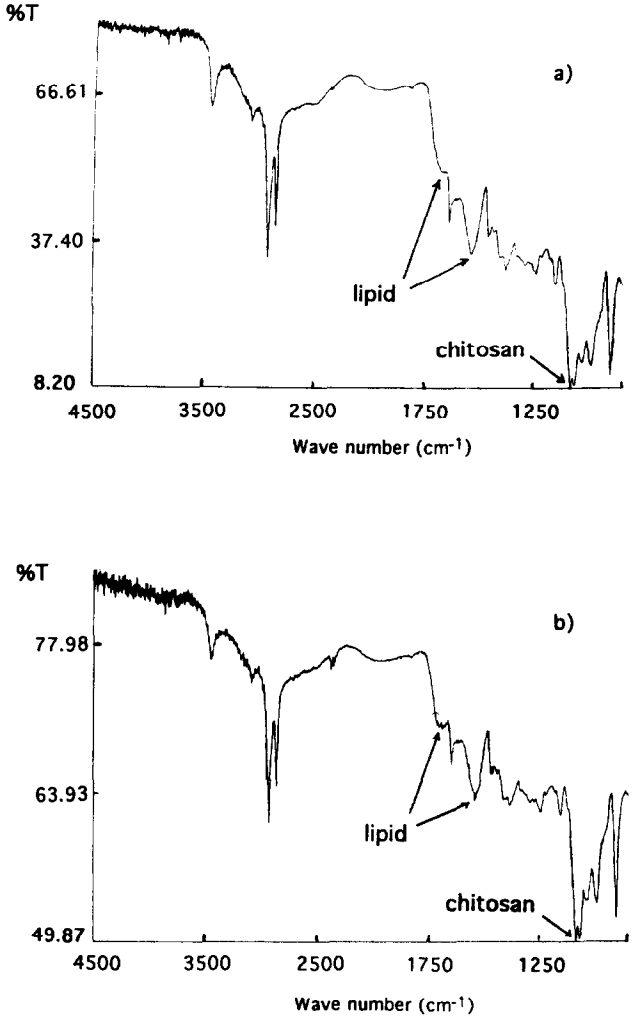

Fig. 5. FTir spectra of the (a) shiny; and (b) dull sides of a chitosan/undecylenic acid asymmetrical membrane.

used FTir spectroscopy with the ATR technique. Figure 5 shows the spectra corresponding to the shiny and dull sides of the membrane, both presenting the lipid and chitosan absorption bands. Unfortunately, the spectra are quite similar and do not allow us to conclude in favour of a possible asymmetry in the chemical composition of the two sides of the membrane. In fact, if we consider the ATR technique, the penetration of the sample by the infra-red beam depends on the wavelength and varies between 0.1 and a few micrometers (Romero & Domard, 1994). This implies that even if there is an asymmetry in the chemical composition, the membrane remains too thin (15–20 μm), and one of its two domains is probably too narrow compared to the other, to be identified by infra-red spectroscopy.

Figures 6 and 7 show SEM micrographs of the asymmetrical membrane for the shiny and dull sides, respectively. The former is relatively smooth and may be compared to the surface of a pure chitosan hydrochloride film. The internal (dull) side shows aggregates with a complex fractal structure. If we consider the cluster shape and the local dimensions, these structures very much resemble the aggregates of chitosan and lipid particles previously described (Demarger-André & Domard, 1993), after redispersion of the flocs.

These studies now allow us to propose a mechanism describing the formation of the asymmetrical membrane according to Fig. 8. The dialysis membrane is made from regenerated cellulose. As previously observed by Domard *et al.* (1989) and Roberts (1992), chitosan can interact with cellulose by means of hydrogen bonding. This mechanism is reinforced by electrostatic interactions occurring between some anionic charges present on cellulose and the ammonium sites of chitosan, in our experimental conditions. As a consequence, in an initial step (Fig. 8a) the chitosan chains cover the internal wall of the dialysis bag. This adsorbed layer is certainly very thin, in relation to the gyration radius of chitosan molecules which is of the order of a few tens of micrometres. When the dialysis bag is then immersed into the undecylenic acid dispersion, only the free lipid molecules may diffuse easily through the cellulose membrane. Ion exchange with the adsorbed chitosan layer then gives rise to the formation of the corresponding salt, yielding a thin membrane of chitosan undecylenate (Fig. 8b), which becomes less chemisorbed at the surface of the cellulose bag at the end of this step, and remains only weakly physisorbed. This chitosan undecylenate membrane is highly hydrated and offers a very large free volume, so that all the small molecules present in the medium can diffuse freely. Consequently, lipid molecules may cross this membrane and react with free chitosan chains at concentrations allowing a process of flocculation/redispersion to occur. The resulting lipid–chitosan aggregates are then adsorbed onto the chitosan undecylenate membrane initially formed. They will constitute the irregular and dull internal side of the asymmetrical membrane (Fig. 8c)

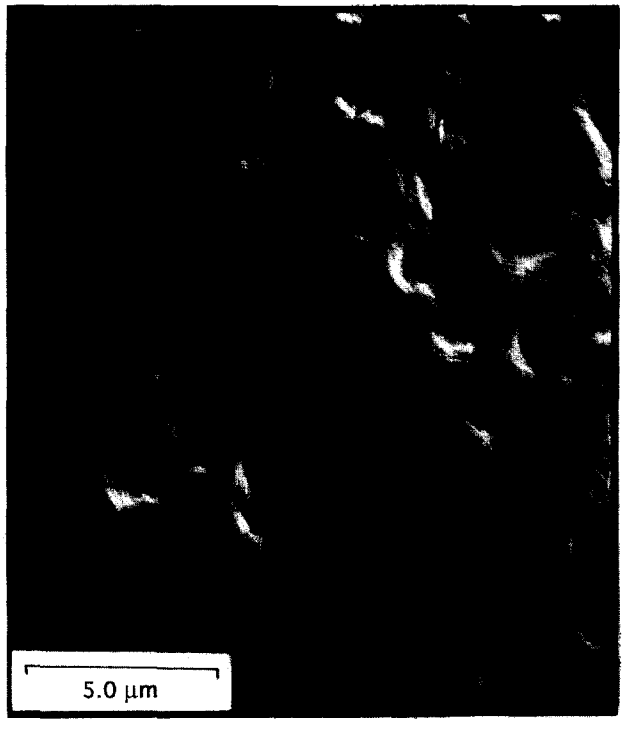

Fig. 6. SEM micrograph of the external, shiny side of the chitosan/undecylenic acid asymmetrical membrane.

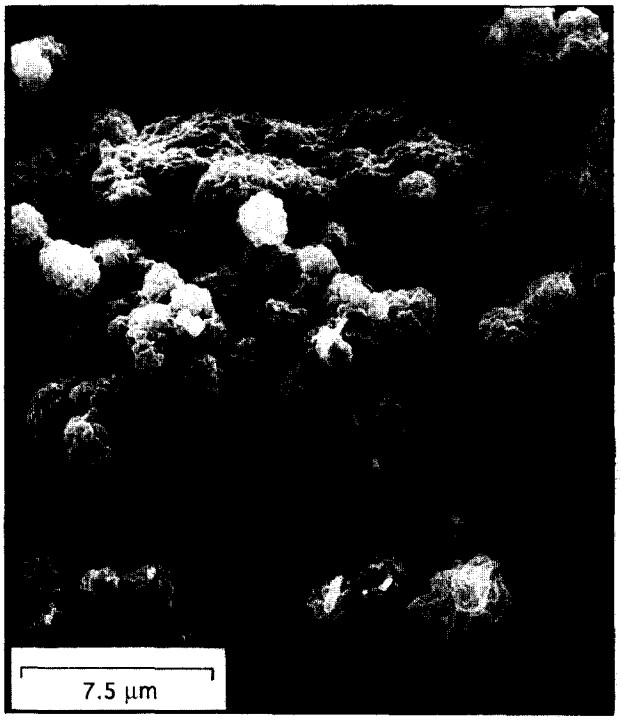

Fig. 7. SEM micrograph of the internal, dull side of the chitosan/undecylenic acid asymmetrical membrane.

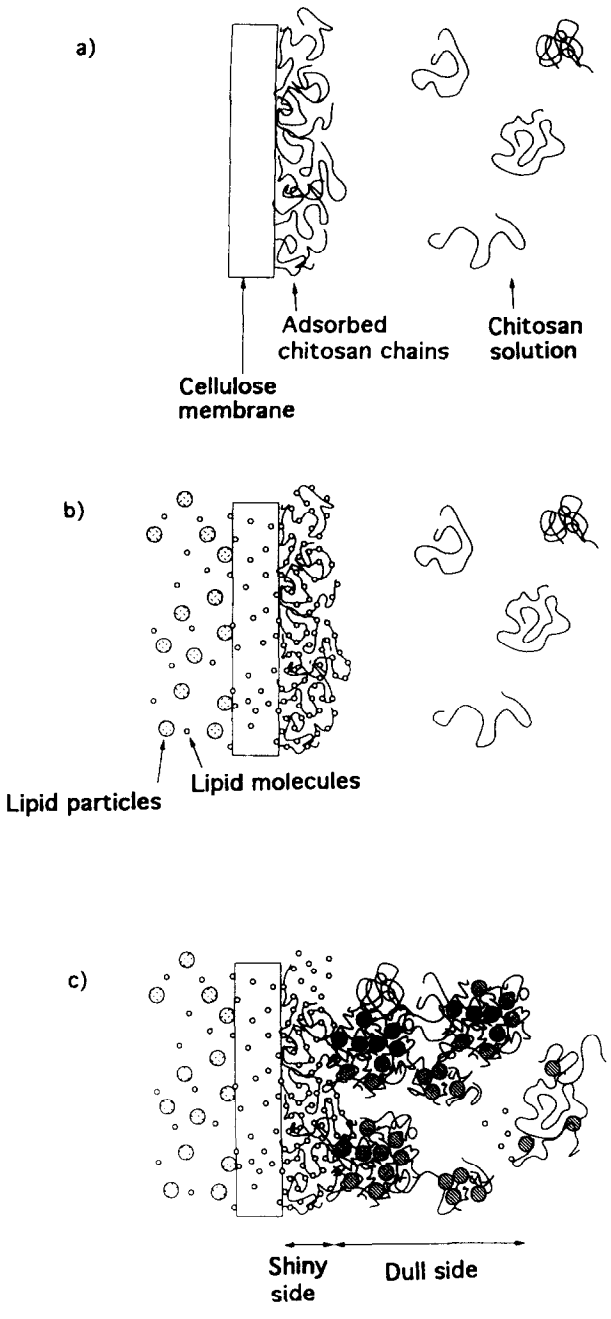

Fig. 8. Schematic representation of the proposed process for the formation of the chitosan/undecylenic acid asymmetrical membrane.

which, in a final stage, will be completely desorbed from the dialysis bag. The permeability of the asymmetrical membrane to lipid molecules is demonstrated by the continuous decrease of the external concentration of lipid (Fig. 4), even after its formation is markedly visible by eye (after ≈24 h).

CONCLUSIONS

This work allowed us to identify the morphologies of the two states induced during the addition of chitosan to a dispersion of undecylenic acid, i.e. the flocs and the redispersed particles. They both correspond to fractal structures and as such, they could find interesting applications in the biomedical field, in particular as drug delivery systems. The asymmetrical membrane prepared in some circumstances allows the use of a relatively stable system which associates both hydrophilic and hydrophobic behaviours. These properties, in addition to the biological properties of the two components, should lead to interesting applications of these membranes in the field of biomaterials.

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