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Polyelectrolyte complex formation between iota-carrageenan and poly(L-lysine) in dilute aqueous solutions: a spectroscopic and conformational study

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Abstract

The polyelectrolyte complex formation between iota- (ι-)carrageenan and poly(L-lysine) (PLL) has been investigated in HEPES 5 mM, NaCl 150 mM pH 6.5 at 25 °C, in dilute conditions. After building of the ternary phase diagram of the system, the formation of soluble polyelectrolyte complexes in transparent systems has been evidenced by means of spectroscopic techniques. Absorption spectrum of methylene blue (MB^+) interacting with ι-carrageenan chains, in presence of increasing amounts of the polypeptide, as well as fluorescence polarization of a FITC-labeled PLL and circular dichroism spectrum of PLL, in presence of increasing amounts of the polysaccharide, have been studied. In all cases, the endpoint occurred for a peptide to polysaccharide weight ratio of about 0.82, indicating an approximate 1/1 charge stoichiometry of the polyelectrolyte complex formed. Furthermore, the polyelectrolyte complex formation process was proved to be conformation-directing as PLL adopts a right-handed α-helical conformation when mixed with ι-carrageenan.

Keywords: Iota-carrageenan; Poly(L-lysine); Polyelectrolyte complex; Stoichiometry; Fluorescence; Conformation

1. Introduction

The formation of complexes though the binding of oppositely-charged polyelectrolytes is well known. Recently, polyelectrolyte complexes based on natural polysaccharides have attracted considerable attention in the pharmaceutical domain due to their potential applications in drug delivery systems. Microencapsulation technologies using polyelectrolyte complexes have emerged, allowing the formation of coacervate-type nano or microparticles with promising results in terms of stability and controlled drug release (Dumitriu & Chornet, 1998; Whateley, 1992). The well-known association of alginate with poly(L-Lysine) (PLL) to form a capsule core material of calcium alginate gel–sol enclosed by a polyanion–polycation complex membrane is a good example of what can be obtained by using polyelectrolyte complexes to design drug delivery systems (Thu, Bruheim, Espevik, Smidsrod, Soon-Shiong, & Skjak-Braek, 1996). Layer-by-layer assembly of oppositely charged polyelectrolytes can also be performed, leading to polyelectrolyte multilayers allowing the formation of semi-permeable microcapsules of special interest for controlled drug release (Qiu, Leporatti, Donath, & Möhwald, 2001).

Among the polysaccharides particularly suitable for theses applications, due to their biocompatibility and non-cytotoxicity, are carrageenans. Carrageenans (Yalpani, 1988) are polydisperse linear sulfated galactans extracted from different species of marine red algae of the class Rhodophyceae. Their main chain consists of alternating copolymers of 1,4-α- and 1,3-β-D-galactopyranose and 3,6-anhydro-D-galactopyranose. They come in three major

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types designated by means of Greek letters as κ, ι and λ, where the main structural difference among them is in the sulphated group degree of substitution. They are well known for their gel forming properties and are used extensively in food and pharmaceutical industry as gelling or thickening agents. They are also used increasingly in the pharmaceutical field to prepare microspheres (Garcia & Ghaly, 1996; Sipahigil & Dortunc, 2001) or microcapsules in association with other polyelectrolytes (Bartkowiak, 1999; Patil & Speaker, 2000; Prokop, 1998; Suzuki & Lim, 1994; Tomida, Nakamura, & Kiryu, 1994), as their gelation process (Te Nijenhuis, 1997) allows beads preparation by the ionotropic gelation method. ι-carrageenan, which bears two sulphated charges by monomer unit, is the most commonly used in these formulations. Recently, ι-carrageenan prepared in HEPES buffer or sodium saline was found to be the most suitable for the formation of capsules (Bartkowiak & Hunkeler, 2001).

By analogy with the alginate-PLL association, it seems to us that the association of ι-carrageenan with PLL could be of interest. Preliminary experiments performed in our laboratory to further design a drug delivery system constituted with these two polyelectrolytes showed us that a combination of HEPES 5 mM and NaCl 150 mM, pH 6.5, leaded to the formation of mechanically stable capsules. As a consequence, we first focused on the study of the formation of ι-carrageenan and PLL complexes in these ionic conditions.

A number of studies have demonstrated that the formation of protein or peptide–polysaccharide complexes and coacervates is sensitive to chemical, physical and structural parameters (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Most of them considered the effect of parameters such as pH, ionic strength, biopolymers charge density or molecular weight on the extent of the phase separation (Nairn, 1995; Schmitt, Sanchez, Thomas, & Hardy, 1999). Phase separated-systems are quite difficult to study: phase diagrams based on turbidity of the solutions, and chemical analysis of the coacervate and equilibrium fluid phases are the most currently applied techniques. Moreover, the information gained from these techniques is far from satisfying, as it does not give information on the microstructure of the mixed system. To our knowledge, fewer studies have been performed on the interaction between a peptide and a polysaccharide in the dilute regime, mostly because of a lack of techniques available or sensitive enough to be applied in the concentrations domain where the complexes formed are soluble. In this context, we decided to develop various methodologies based on spectroscopic techniques to study the formation of complexes between PLL and ι-carrageenan. Among the structural parameters known to influence the formation and stability of complexes, we focused on the polyelectrolytes weight ratio and charge ratio, which both affect the extent of the electrostatic interaction.

The aim of the present work is to study the interaction between PLL and ι-carrageenan in dilute conditions. We have studied the behavior of the PLL-ι-carrageenan mixtures in terms of stoichiometry of the resulting macromolecular complex and conformation of PLL upon interaction with the polysaccharide.

2. Materials and methods

2.1. Reagents

The solvent used during the experiments was a solution of HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethansulfonic acid], Sigma, USA) 5 mM, NaCl (RP Normapur, Prolabo, France) 150 mM, in ultrapure water (Milli-Q, 18 mΩ obtained with a Millipore water purification system). It was filtered through a 0.1 μm filter (Millex HV, Millipore, USA), degassed and pH was adjusted to 6.5 with NaOH (RP Normapur, Prolabo, France) 1N.

ι-carrageenan was supplied by Sigma (USA) and certified by the supplier as obtained from a single species to produce essentially pure ι-carrageenan. The sample was transformed into its sodium salt form following a general procedure which has already proved to be safe and reliable for avoiding undesired aggregation phenomena, for both ι- and κ-carrageenan (Bongaerts, Paoletti, Denef, Vanneste, Cuppo, & Reynaers, 2000; Bongaerts, Reynaers, Zanetti, & Paoletti, 1999). We have characterized the polysaccharide in HEPES 5 mM, NaCl 150 mM pH 6.5 by size exclusion chromatography (SEC) coupled to a multi-angle laser light scattering detector (MALLS Dawn, Wyatt, USA) and a refractometer (RI ERC, Japan) according to previously described conditions (Girod, Baldet-Dupy, Maillols, & Devoisselle, 2002). Briefly, SEC was performed on Shodex OH-Pak columns B-806/HQ, B-805/HQ and B-804/HQ in series, placed in a oven set at 60 °C, to preclude any aggregate formation. The analysis of the sample resulted in a weight-average molar mass M_w of 790 700 g/mol, a polydispersity index M_w/M_n of 1.2, and an average radius of gyration r_g of 65 nm.

Poly(L-lysine) hydrobromide 15–30 kD and poly(L-lysine) hydrobromide 15–30 kD labeled with fluorescein isothiocyanate (FITC) (degree of substitution: 0.005 mole FITC per mole lysine monomer) were purchased from Sigma (USA). The amino groups of the PLL side chains present a p*K*a of approximately 9. We performed a SEC-MALLS-RI analysis of PLL and PLL-FITC in the solvent, which resulted in Mw of 18 150 and 17 800 g/mol, respectively.

Methylene Blue, 3,7-bis(dimethylamino)-phenazothionium chloride, was a Sigma product used without further purification.

2.2. Preparation of PLL and/or ι-carrageenan dilute solutions

PLL and PLL-FITC were dispersed in a cold solvent. ι-Carrageenan was dispersed in a cold solvent and then dissolved under stirring at 80 °C for 30 min.

ι-carrageenan/PLL systems were prepared by mixing and stirring at 80 °C for 10 min. In the case of samples containing methylene blue, the desired quantity of methylene blue stock solution was added to the samples which were stirred at 80 °C for 10 min more. The samples were then cooled to 25 °C and allowed to equilibrate at this temperature in complete darkness for 6 h.

2.3. Ternary phase diagram of PLL/ι-carrageenan mixed aqueous dispersions

Ternary phase diagram of PLL/ι-carrageenan mixed aqueous dispersions was established at pH 6.5 and at 25 ± 1 °C. The macroscopic bulk phase separation points (called 'turbid systems') were determined by the appearance of turbidity on the basis of macroscopic observations. The so-called 'transparent systems' were those for which no macroscopic phase separation was observed. The amounts of PLL and ι-carrageenan used for the preparation of the dispersions were determined by weight (±0.0001 g) using a Mettler AE163 weighing device (Germany). The weight% polymer concentrations could then be calculated. The resulting solvent, PLL, ι-carrageenan values (in wt%) were compiled to build up a ternary phase diagram represented by an equilateral triangle using the SigmaPlot 7.0 software (SPSS Inc., USA).

2.4. UV-visible absorption spectra of methylene blue (MB^+)

UV-visible absorption spectra of methylene blue (MB^+) in presence of ι-carrageenan and increasing amounts of PLL (PLL/ι-carrageenan weight ratio ranging from 0.22 to 1.1) were recorded at 25 °C with a Uvikon 922 spectrophotometer using 1-cm quartz cuvettes. The spectrophotometer, set at a spectral resolution of 1 nm, was used in the 400–750 nm range.

A MB^+ stock solution was prepared by stirring at room temperature for 1 h. The MB^+ concentration in the samples was adjusted so that the MB^+ alone spectrum showed a maximum at 664 nm (corresponding to the monomer form of the dye) with an optical density of about 1, the optical density where a linear response is obtained with the spectrophotometer. The corresponding MB^+ concentration was 9.35×10^{-3} g/l.

2.5. Fluorescence polarization experiments

The fluorescence measurements were performed on a RF 5302 Shimadzu spectrofluorometer (Japan) equipped with a Xenon light source (UXL-150S, Ushio, Japan). All measurements were carried out at 25 °C in 1 × 1 cm-path length quartz cuvettes (Hellma, Germany) under gentle magnetic stirring (Cuv-o-stir 633, Hellma, Germany).

Samples with PLL/ι-carrageenan weight ratio ranging from 6.46 to 0.21 (with PLL-FITC at a constant concentration of 0.02 g/l) were prepared. The samples were illuminated with vertically polarized light and the components of the emitted intensity, respectively, polarized vertically ($I_{\parallel}$) and horizontally ($I_{\perp}$), with reference to the polarization direction of the excitation light, were measured successively for each sample. The excitation and emission wavelengths were set to 490 and 515 nm, respectively, with 1.5 and 3 nm bandwidths. The steady-state fluorescence polarization factor p was determined according to the following equation:

$$P = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + GI_{\perp}}$$

where G is the compensating factor for the anisotropic sensitivity of the instrument (0.672 in our experimental case). The contribution of scattered light to the fluorescence emission ($I_{\parallel}$ and $I_{\perp}$) was determined independently for an unlabeled reference solution of the same composition. At the maximum carrageenan concentration, the contribution of scattered light to the total fluorescence emission was less than 5%. All measurements were carried out in triplicate.

2.6. Chiroptical spectroscopy

Circular dichroism (CD) spectra were recorded using a CD6 Jobin–Yvon (France) spectropolarimeter with molar ellipticity [θ] (degree cm^2 $dmol^{-1}$) data based on the mean residue molecular weight of poly(L-lysine) hydrobromide. Spectra of PLL alone (at a concentration of 0.2 g/l) or in presence of ι-carrageenan (PLL/ι-carrageenan weight ratio ranging from 3.7 to 0.2) were recorded at 25 °C in a 0.1-cm quartz cell in the region of the characteristic absorption bands of the amide groups, between 200 and 270 nm.

3. Results and discussion

3.1. Ternary phase diagram of PLL/ι-carrageenan mixtures

First a ternary phase diagram of PLL/ι-carrageenan mixtures in non-gelling conditions has been established in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C (Fig. 1). The phase diagram is typical of systems involving attractive interactions leading to the formation of complexes between oppositely charged macromolecules at low ionic strength, with a two-phase region located in the water-rich corner (Piculell, Bergfeldt, & Nilsson, 1995). This region in the two-phase corner typically contains the equilibrium between a rich-solvent phase and a rich-polymer phase. Similar

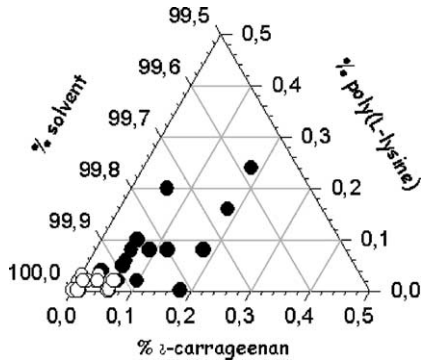

Fig. 1. Ternary phase diagram of poly(L-lysine) and ι-carrageenan in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C (●: turbid systems; ○: transparent systems).

behavior has already been reported for a number of polymeric systems (Michon, Vigouroux, Boulenguer, Cuvelier, & Launay, 2000; Thalberg, Lindman, & Karlström, 1990; Xia & Dublin, 1994). It is usually explained in terms of entropic and enthalpic effects between the polymers and the solvent molecules (Tolstoguzov, 1997). In fact, the polymer association decreases the entropy of mixing of the system by lowering the number of possible macromolecule conformations, but this effect is counter-balanced by a strong enthalpic contribution, arising from the liberation of water molecules and counter-ions. The balance between the two effects contributes to the stabilization of the system.

To study the system under non-gelling conditions, we have worked preferentially in the region located in the solvent-rich corner. Two types of behavior have been observed as a function of the polyelectrolyte concentrations (Fig. 1). When PLL and ι-carrageenan concentrations are, respectively, higher than 0.05 and 0.1 wt%, a macroscopic phase separation is observed. When the concentrations of polymers are lower, no macroscopic bulk phase separation is observed and the systems are transparent. Even if interactions still exist, complex coacervation is suppressed by dilution. In the following experiments, we have worked with mixtures located in the concentration domain corresponding to transparent systems, permitting to analyze further the PLL/ι-carrageenan interactions to be investigated by spectroscopic techniques.

3.2. Molecular evidence of the polyelectrolyte complex formation in transparent systems

We have carried out a set of experiments aimed at gathering evidence for the formation of polyelectrolyte complexes in PLL/ι-carrageenan transparent systems. These experiments were based on the ability of methylene blue (MB^+), a cationic phenotiazinium dye, to form clusters in the presence of an anionic polyelectrolyte (Tuite & Kelly, 1993).

Fig. 2, dashed line, displays the typical spectrum of a MB^+ aqueous solution at 9.35×10^{-3} g/l, with an absorption band centered at 664 nm (α-band) together

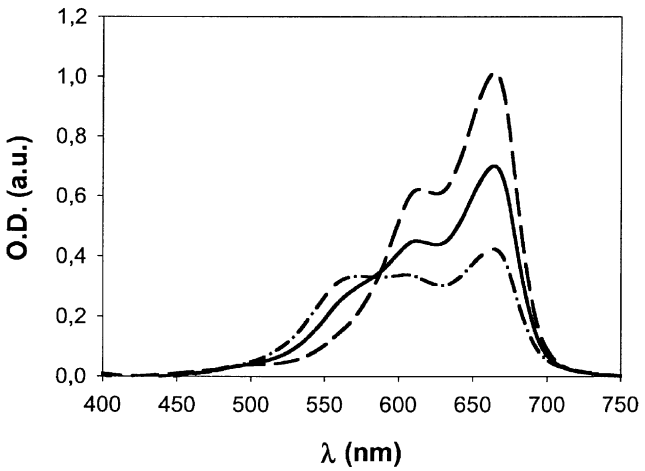

Fig. 2. Absorption spectra of MB^+: free in solution (– – –); in presence of ι-carrageenan (– • –); in presence of ι-carrageenan and PLL for a PLL/ι-carrageenan weight ratio of 0.44 (—), in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C (MB^+ concentration: 9.35×10^{-3} g/l; ι-carrageenan concentration: 1.81×10^{-2} g/l).

with a shoulder at 610 nm (β-band). The former is assigned to the monomeric dye, while the latter to a face-to-face dimeric form (Tuite & Kelly, 1993). An increase of the dye concentration favors the formation of dimers with a consequent increase of the β-band. Multimeric stacks of dye molecules will be formed at higher concentration with the development of a large absorption band around 570 nm.

The presence of a negatively charged polyelectrolyte favors the formation of multimeric aggregates even in dilute solution (Schoenberg & Moore, 1964). In Fig. 2, dash-dot line, the absorption in the visible range of a MB^+/ι-carrageenan mixture, with an unchanged MB^+ concentration and a ι-carrageenan concentration of 1.81×10^{-2} g/l, is reported. It differs from the MB^+ spectrum in solution at the same concentration: a decrease both of the α- and β-bands with a corresponding increase of a new band at 570 nm is observed, in agreement with the spectral behavior already reported in the literature for MB^+/carrageenan systems (Snoeren, 1976; Soedjak, 1994). This was explained by an ionic interaction occurring between the planar cationic dye molecules and the anionic macromolecules having regular spaced polar groups (Schoenberg & Moore, 1964). In this case, long-range forces involving water and methylene blue molecules can take place and maintain the planar cationic molecules relatively close to each other. Then they can form metachromatic complexes in which methylene blue molecules in parallel absorb at 570 nm. If another cationic molecule able to associate with ι-carrageenan complicates the system, a competition for the sulphated groups of the polyanion takes place between the planar cationic MB^+ and this molecule. Some MB^+ molecules are removed from the sulphated groups sites

and then released in solution. As a consequence, the peak at 664 nm increases and the shoulder at 570 nm decreases. This method has already been used to demonstrate the associative nature of the interaction between heparin and poly(alkylenimines) (Casu, Torri, Legramandi, & Righetti, 1982), pectate and PLL (Paradossi, Chiessi, & Malovikova, 1999) or ι-carrageenan and gelatin (Michon et al., 2000). As interactions between PLL and ι-carrageenan have never been studied before, and as the structural characteristics of the polyanion are known to greatly influence the formation of polyelectrolyte complexes (Paradossi et al., 1999), the aim of our work was to show if such attractive interaction could occur in dilute transparent solution between PLL and ι-carrageenan.

First an 'optimal' concentration in ι-carrageenan has been determined. To perform this, the evolution of the optical density measured at 664 nm (called $OD_{664\ nm}$) as a function of ι-carrageenan has been studied (data not shown). The $OD_{664\ nm}$ decreases as ι-carrageenan concentration increases up to a concentration limit of 1.81×10^{-2} g/l above which the $OD_{664\ nm}$ remains constant. Below this concentration, the $OD_{664\ nm}$ decrease corresponds to the formation of new MB^+/ι-carrageenan metachromatic complexes. At the plateau, all MB^+ molecules are interacting with the sulphated groups and addition of carrageenan does not change the number of 'fixed' MB^+ molecules: it can be assumed that all the MB^+ molecules are fixed and that all the sulphated groups are saturated. The ι-carrageenan concentration limit of 1.81×10^{-2} g/l was chosen for the study of the interaction with PLL. At this ι-carrageenan concentration, the maximum difference compared with MB^+ alone spectrum is obtained. Moreover, all sulphated groups are saturated by MB^+ molecules. As a consequence, if PLL interacts associatively with carrageenan, some of the MB^+ fixed will necessary be released in the medium, and this will lead to an increase of $OD_{664\ nm}$.

The absorption spectra of MB^+/ι-carrageenan/PLL mixtures, with PLL/ι-carrageenan weight ratio ranging from 0.22 to 1.1, have been measured. As PLL concentration in solution increases, a decrease of the shoulder at 570 nm (decrease of the concentration of MB^+/ι-carrageenan complexes) and an increase of the peak at 664 nm (release of free MB^+ molecules) can be observed. As an example, the MB^+ spectrum for a PLL/ι-carrageenan weight ratio of 0.44 is given on Fig. 2, solid line. To better visualize the MB^+ spectrum change as a function of PLL/ι-carrageenan weight ratio, $OD_{664\ nm}$ evolution has been represented as a function of this ratio (Fig. 3). In the absence of PLL, $OD_{664\ nm}$ of MB^+ in presence of ι-carrageenan represents the lower limit of the curve (Fig. 3, lower dashed gray line). Upon addition of PLL in solution, $OD_{664\ nm}$ increases regularly since the PLL/ι-carrageenan weight ratio of 0.22. Between the ratios of 0.77 and 1.1, $OD_{664\ nm}$ reaches a plateau corresponding to the $OD_{664\ nm}$ obtained for free dye in solution (Fig. 3, upper dashed gray line).

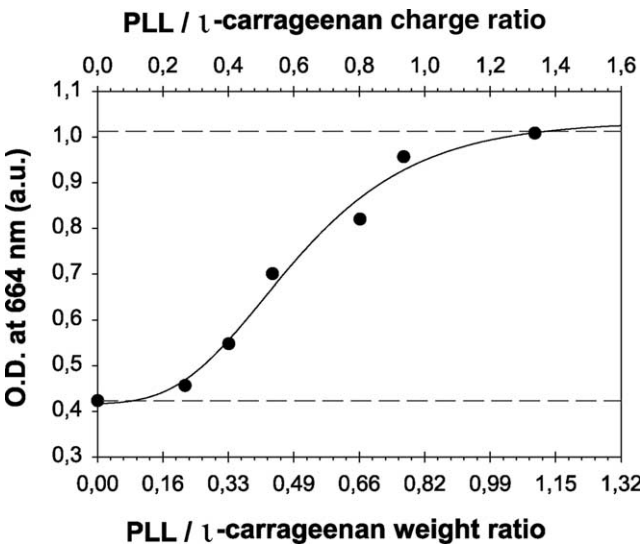

Fig. 3. $OD_{664\ nm}$ of MB^+ in presence of ι-carrageenan and increasing amounts of PLL, expressed in terms of PLL/ι-carrageenan weight ratio (bottom *x*-axis) or lysine/disaccharide unit charge ratio (top *x*-axis), in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C. The charge ratio calculation is based on the idealized formulas of the repeating units of both polyelectrolytes. Dashed gray lines represent the $OD_{664\ nm}$ lower and upper limits, corresponding to $OD_{664\ nm}$ values when MB^+ is in presence of ι-carrageenan or free in solution, respectively, (MB^+ concentration: 9.35×10^{-3} g/l; ι-carrageenan concentration: 1.81×10^{-2} g/l).

These results clearly show that PLL associates with ι-carrageenan and displaces the interaction between MB^+ and ι-carrageenan. The progressive recover of the free MB^+ spectrum demonstrates that PLL efficiently binds to ι-carrageenan until the complete displacement of MB^+ from ι-carrageenan sulphate groups. It seems that the association of PLL and ι-carrageenan is complete for a PLL/ι-carrageenan weight ratio around 0.82.

3.3. Stoichiometry of the polyelectrolyte complex formation evaluated by the motion of a fluorescent probe linked to poly(L-lysine)

To get more insights on the association between PLL and ι-carrageenan, fluorescence polarization has been used. Fluorescent molecules have been widely used to study polymer conformation behavior (Ebdon, Lucas, Soutar, & Swanson, 1994) or to probe the microenvironment of organized host media such as polyelectrolyte complexes (Caruso, Donath, Möhwald, & Georgieva, 1998; Heyward & Ghiggino, 1989) or polysaccharide/membrane interaction (Girod, Cara, Maillols, Salles, & Devoisselle, 2001). Among them, the fluorescein amine-reactive derivatives, like FITC, are the most common fluorescent derivatization reagents used for covalently labeling peptides and proteins. Indeed, when covalently attached to the polymer backbone, their motion abilities are directly dependent of the polymer chain motion. This makes them useful for gaining information about complexation of polymers (Nikiforov &

Jeong, 1999) by measuring their steady-state fluorescent polarization. If PLL is labeled with a fluorophore such as FITC, then the PLL-mediated binding of the probe to the ι-carrageenan molecules should lead to a significant change in the fluorescence polarization of the fluorophore.

The fluorescence polarization of a FITC-labeled PLL at a constant concentration has been measured in presence of various amounts of ι-carrageenan so that PLL/ι-carrageenan weight ratio varied from 6.46 to 0.21. First, excitation and emission spectra of FITC linked to PLL, alone or in the presence of ι-carrageenan, have been studied. The spectra exhibit well separated absorption and emission bands, with an excitation maximum at 490 nm and an emission maximum at 515 nm (data not shown). No change is observed in the wavelengths of the emission and excitation maxima of FITC whatever the ι-carrageenan concentration range used in our experiments, allowing us to use this probe to study its motion in the presence of ι-carrageenan.

The polarization of the probe as a function of PLL/ι-carrageenan weight ratio has been determined (Fig. 4). Above a PLL/ι-carrageenan weight ratio of 2.22, the steady state fluorescence polarization of FITC-PLL remains constant; no significant change in fluorescence polarization can be observed in comparison with the fluorescence polarization measured when FITC-PLL is alone in solution at the same concentration. This means that, at the considered PLL/ι-carrageenan weight ratios, the micro-environment of the fluorescent probe remains the same: the major part of the polypeptide has not entered into complexation with ι-carrageenan. Then the polarization exhibits an abrupt increase on increasing ι-carrageenan concentration, between PLL/ι-carrageenan weight ratios of 1.66 and 0.59. Below the weight ratio of 0.59, the polarization reaches a plateau corresponding to a value of polarization significantly higher than the one measured when PLL-FITC is alone in solution. The increase of FITC polarization indicates a decrease in the probe molecular motion, directly connected with a change of its micro-environment. This change can be attributed to ionic interaction between PLL and ι-carrageenan which stiffens the polycation backbone and/or to a change of the polypeptide conformation for a less flexible one. As the increases of FITC polarization value and of $OD_{664\ nm}$ in MB^+ spectrum occur for the same range of weight ratios, we conclude that both phenomenon are induced by the formation of complexes between ι-carrageenan and PLL chains, the maximum complex-forming interaction being reached for a PLL/ι-carrageenan weight ratio of about 0.82. However, the loss of PLL chain flexibility upon the polyelectrolytes association could also be accompanied by a change of the polypeptide chain conformation.

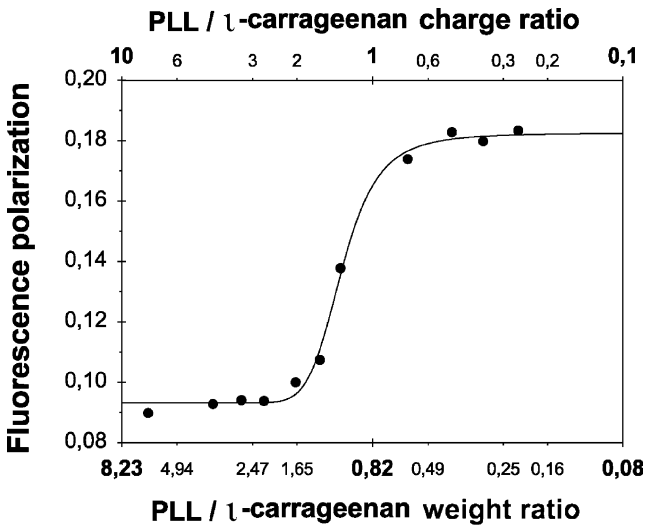

Fig. 4. Fluorescence polarization of FITC-labeled PLL 0.02 g/l in presence of increasing amounts of ι-carrageenan, expressed in terms of PLL/ι-carrageenan weight ratio (bottom *x*-axis) or lysine/disaccharide unit charge ratio (top *x*-axis), in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C. The charge ratio calculation is based on the idealized formulas of the repeating units of both polyelectrolytes.

3.4. Study of the conformation of poly(L-lysine) in the presence of ι-carrageenan

In order to elucidate this point, the conformation of PLL has been studied in dilute solution. Various amounts of ι-carrageenan have been added to PLL at a constant concentration so that PLL/ι-carrageenan weight ratio varied from 3.7 to 0.2. If the polypeptide conformation changes upon interaction with the polysaccharide, this will be reflected in its CD spectra. It is well known that the amide groups of poly(amino acid)s show characteristic $\pi\pi^*$ and $n\pi^*$ transitions between 200 and 270 nm that are sensitive to their secondary structures (Fasman, 1996). Moreover, in this region, carrageenan chains do not absorb (data not shown).

The CD curve of PLL alone at neutral pH in aqueous solution (Fig. 5, dotted line) is characterized by a strong negative band at ≈200 nm ($\pi\pi^*$ transition) and a small

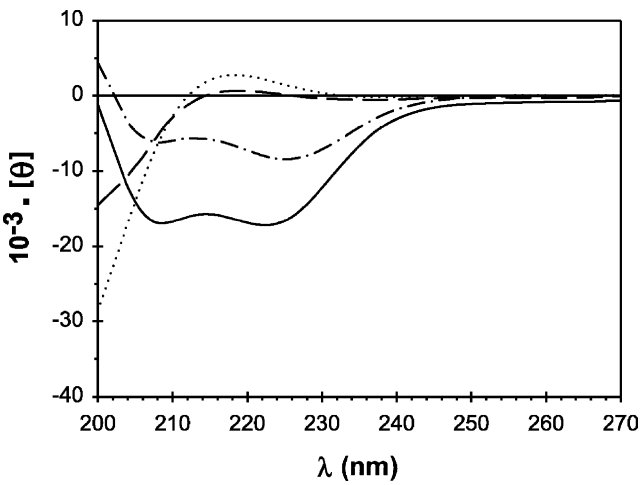

Fig. 5. CD spectra of PLL 0.2 g/l: alone (•••); in presence of increasing amounts of ι-carrageenan [corresponding to PLL/ι-carrageenan weight ratio of 2.63 (– – –); 1.18 (– • –); 0.59 (—)] in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C.

positive band in the range 215–220 nm ($n\pi^*$ transition). Its shape is identical to published spectra which have been interpreted to be representative of a dynamic state involving the extended left-handed P_{II} conformation of the polypeptide (Drake, Siligardi, & Gibbons, 1988), and is characteristic of a 'charged coil' structure (Greenfield & Fasman, 1969). Upon addition of ι-carrageenan, the PLL spectrum changes progressively (Fig. 5, dashed, dash-dot and solid lines), indicating a conformational change of PLL from a random coil to a regular arrangement. At the ratio of 2.63 (Fig. 5, dashed line), a maximum is still present at about 220 nm ($n\pi^*$ transition) but the $[\theta]$ values are nearly zero at higher wavelengths. These characteristics are in agreement with the interpretation that the PLL chains are still predominantly in a random coil conformation. By contrast, for PLL/ι-carrageenan weight ratios between 2.63 and 0.59 (see for example Fig. 5, dash-dot line, for a PLL/ι-carrageenan weight ratio of 1.18), the curves present a different shape. They show negative $[\theta]$ values above 205 nm, with two weak minima around 208 and 222 nm. The intensity of these respective minima increases on increasing ι-carrageenan concentration (i.e. on lowering the PLL/ι-carrageenan weight ratio). This means that significant amounts of higher ordered structures are present, in mixture with random coil structures. When the PLL/ι-carrageenan weight ratio is 0.59 (Fig. 5, solid line), the PLL spectrum is more distinct and characterized by a positive band at 200 nm ($\pi\pi^*_{//}$ transition), and two negative bands with minima at about 208 nm ($\pi\pi^*_{\perp}$ transition) and 222 nm ($n\pi^*$ transition). This spectral shape is typical of a right-handed α-helical conformation (Greenfield & Fasman, 1969). A contribution of β-sheet structures to the spectra was not found.

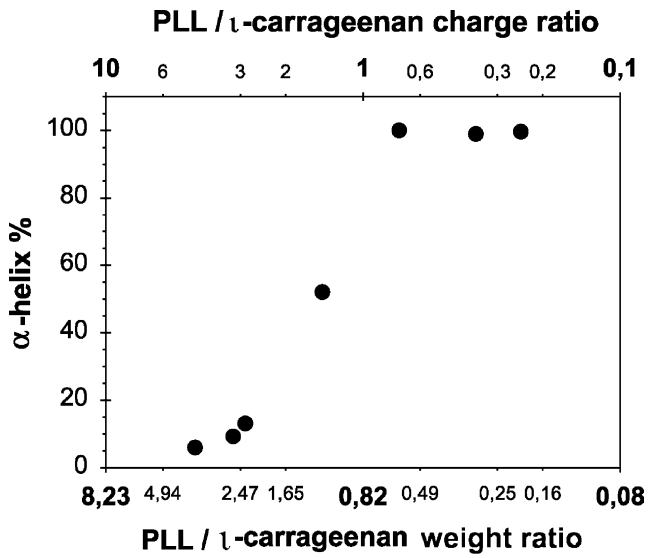

Fig. 6. α-helix content (%) of PLL 0.2 g/l in presence of increasing amounts of ι-carrageenan, expressed in terms of PLL/ι-carrageenan weight ratio (bottom *x*-axis) or lysine/disaccharide unit charge ratio (top *x*-axis), in HEPES 5 mM, NaCl 150 mM, pH 6.5 at 25 °C. The charge ratio calculation is based on the idealized formulas of the repeating units of both polyelectrolytes.

According to Greenfield and Fasman (1969), the content of α-helices in the samples has been estimated from the mean molar ellipticity at the wavelength of 222 nm. The α-helix content is plotted as a function of PLL/ι-carrageenan weight ratio in Fig. 6. Figure shows that the α-helix content grows from the PLL/ι-carrageenan weight ratio of 3.70 to the ratio of 0.59, with an abrupt increase between 2.36 and 0.59. Below this limiting ratio, the α-helix content of PLL/ι-carrageenan mixtures has nearly constant values of about 100%, indicating that the highest degree of ordered structure has been obtained. When comparing these results with those obtained by studying the MB^+ visible spectra and fluorescence polarization of a FITC-labeled PLL, it is clear that the change of the PLL conformation occurs at ratios for which the $OD_{664\ nm}$ of MB^+ and the polarization of FITC-PLL reach a plateau. These results reveal a very intensive conformational change of polypeptide connected with the maximum complex formation, occurring for weight ratios around the value of 0.82. Such polysaccharide-induced coil-helix transition of PLL has already been reported in literature (Bystricky, Malovikova, & Sticzay, 1991; Gelman, Rippon, & Blackwell, 1973; Mulloy, Crane, Drake, & Davies, 1996). This has been inferred to strong electrostatics interactions between the polyelectrolytes and to the energetically favorable properties of an α-helix conformation for PLL, which both contribute to the stability of the complex (Mulloy et al., 1996). It is probable that a higher ordered conformation allows better interaction of PLL with ι-carrageenan.

However, the results obtained on different model polypeptides–polysaccharide systems show that the association processes can induce different conformational behavior for the polypeptidic chain (Paradossi et al., 1999). As far as carrageenans are concerned, in literature, only kappa-carrageenan (which bears one sulphate charge per monomer unit) sodium salt and PLL hydrobromide interactions have already been studied (Domard & Rinaudo, 1981). Whatever the concentration ratio, no modification of the CD spectrum of PLL was observed, although conductimetric and potentiometric studies showed an uncharged complex formation with a 1/1 stoichiometry. So it seems that ι-carrageenan interacts differently than κ-carrageenan with PLL. This could be inferred to their difference in charge density or to their conformation, which affects the intercharge distances (Paoletti, Smidsrod, & Grasalen, 1984). But Hugerth, Caram-Lelham, and Sundelof (1997) have shown that these parameters are in fact of little importance. They suggest that the decisive factor in polyelectrolyte complex formation with ι- or κ-carrageenan is the presence of aggregated helical elements in solution, which permit polyelectrolyte complexes to be obtained with a surplus of negative charge.

In an attempt to go further in the knowledge of the stoichiometry of the complex formation, we have expressed

the PLL/ι-carrageenan ratio in term of charge ratio. To achieve this, we have considered the idealized structures of the monomer units of both polyelectrolytes, considering that PLL bears one charge per monomer unit, and ι-carrageenan two. This calculation must be taken as an approximation, as it does not take in account the presence of 'defects' in the carrageenan structure, related to the cultivation and harvesting of red algae as well as to the further processing of carrageenan (Therkelsen, 1982). However, the ι-carrageenan sample used in this study was certified by the supplier as obtained from a single species to produce essentially pure ι-carrageenan and a more precise determination of the 'exact' stoichiometry of the complex lies outside the scope of this paper. Nevertheless, the lysine/disaccharide unit charge ratios were calculated and reported as top *x*-axis on Figs. 3, 4 and 6. All the curves show that when an increasing amount of one of the polymer was added all of it was incorporated into the complex until a stoichiometric charge ratio around unity was obtained. The equicharge pairing of oppositely charged groups deduced from Figs. 3,4 and 6 indicates the formation of electroneutral complexes.

So it seems that the lysine/disaccharide unit charge ratio is insufficient to explain the conformation directing interaction of ι-carrageenan with PLL. Bystricky, Malovikova, and Sticzay (1990, 1991) have shown that complex formation is not only governed by a stoichiometric ratio of the charged groups, but also by stoichiometric compatibility of the charge densities, i.e. by the ability of polysaccharide to take up the suitable orientation for the complementary saturation of charges. This could explain why PLL conformation changes in the presence of ι-carrageenan, and not in presence of kappa-carrageenan.

4. Conclusion

The polyelectrolyte complex formation between ι-carrageenan and poly(L-lysine) in dilute conditions has been studied by various spectroscopic techniques. They all show the association of the two polyelectrolytes, leading to a change of the PLL conformation. A weight ratio PLL/ι-carrageenan of 0.82 has been determined as the optimal ratio of interaction of PLL with ι-carrageenan. The calculation of the corresponding charge ratio, on the basis of the idealized structures of the monomer units of both polyelectrolytes, supports the hypothesis of a 1:1 stoichiometry complex formation.

However, some questions arise from this study, especially considering the effect of type of carrageenan on the formation and structure of complexes. This point necessitates further investigation concerning the polysaccharide conformation upon interaction with PLL and the consecutive stability of the complexes formed. The study of the influence of ionic strength and temperature could lead to a better understanding of the key parameters implied in PLL/carrageenan chains association. These points are currently under investigation in our laboratory.

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