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ORIGINAL PAPER

Detection of Polymolecular Associations in Hydrophobized Chitosan Derivatives using Fluorescent Probes

Máira R. Rodrigues · Adriana Lima · Lúcia Codognoto · Antonio Balbin Villaverde · Marcos Tadeu Tavares Pacheco · Hueder P. Moisés de Oliveira

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Abstract The microenvironment formed by lauroyl and stearoyl derivatives of chitosan in solution has been studied using two fluorescent probes, pyrene and nabumetone. Existence or not of microdomains formed by polymolecular associations, the inherent hydrophobicity of them in aqueous solution, and the influence of degree of substitution (DS) of derivatives were investigated by emission properties of pyrene and strengthened by the photophysical behavior of nabumetone. Additionally, the ratio between the fluorescence intensities of first (~372 nm) to the third (~384 nm) bands of the emission spectrum of pyrene was used to determine the critical aggregation concentration (CAC). In a previous work, it was already reported the characterization of chitosan derivatives by three spectroscopic techniques (^{13}C-NMR, ^{1}H-NMR and infrared), as well as data on the solubility and swelling-index of them. In addition of that, the new results show that the investigated lauroyl and stearoyl derivatives of chitosan are expected to be potential models for applications in the medical field.

Keywords Chitosan · Hydrophobic derivatives · Drug-delivery systems · Pyrene and nabumetone

M. R. Rodrigues (✉) · A. Lima · L. Codognoto · A. B. Villaverde · M. T. Tavares Pacheco · H. P. Moisés de Oliveira
Instituto de Pesquisa & Desenvolvimento—IP&D, Universidade do Vale do Paraíba—UNIVAP, Av. Shishima Hifumi 2911, Urbanova, CEP: 12244-000 São José dos Campos, SP, Brazil
e-mail: mrr@univap.br

Introduction

Chitosan [1, 2] is derived from chitin polysaccharide by removal of *N*-acetyl groups on the copolymer by deacetylation. Commonly, chitosan dissolves in aqueous acidic medium below pH 6.5. Chitosan and the derivatives of it have been suggested for use as flocculant, food thickener, paper and textile adhesives, membrane and a chelating agent for metals [3–6]. Since chitosan is biodegradable, nontoxic, non immunogenic and biocompatible in animal tissues, research has been directed toward its use in medical applications such as artificial skin, blood anticoagulants and drug delivery [7, 8].

Drugs delivery [9–11] have been produced in the hydrogel form from synthetics [12, 13] and natural polymers [14, 15], as polysaccharides [16, 17], to obtain derivatives capable of forming micelle-like structures in solution and thus to dissolve drugs [18, 19]. Hydrogel structure implies that chemical or physical crosslinks have to be present to avoid dissolution of the hydrophilic polymers chains/segments into the aqueous phase [20, 21]. Physical hydrogels are formed by reversible links when, for example, chitosan is solubilized in an acidic aqueous medium, being that the simplest way to prepare a chitosan hydrogel [1].

In a previous work [22], chitosan was modified by reactions with stearoyl and lauroyl chlorides and product characteristics, such as degree of substitution (DS), solubility and swelling-index, were studied. The aim of work was to generate by synthesis hydrophobic stearoyl (Q-S) and lauroyl (Q-L) derivatives with a possible application as models for drug-delivery. For the application as model for drug-delivery, products must have tendency to form polymolecular associations in an aqueous medium, due to the intermolecular interactions of the hydrophobic groups.

The hydrophobic groups bound to the polysaccharide chain can lead to the formation of large aggregates, which can change solution properties such as viscosity, surface tension and solubility. The aggregates might be similar to surfactant micelles and can be formed above a certain critical aggregation concentration (CAC), which will depend on the degree of substitution. Several polysaccharides have been hydrophobized by the addition of long alkyl chains [23–26] and the CAC values have been determined.

The aim of the present work was to study the aggregation process of hydrophobically modified chitosan and the correlating this process with degrees of substitution. Chitosans modified with acyl chloride were studied using the fluorescent probes pyrene and nabumetone, in order to determine the properties of the aggregates formed by interaction of the polysaccharide chains and CAC. The fluorescent probes nabumetone and pyrene have well-characterized photophysical properties in different environments. The interaction between the solvent molecules and the chromophore affect the energy difference between the ground and excited states, modifying the refractive index and dielectric constant of the solvent [27–29]. This phenomenon have been used with success to investigate the conformational structure of a number of microenvironments [17, 30–36].

Experimental

Chemicals

The photophysical probes, pyrene and nabumetone, were purchased from Fluka Chemie (Basel, Switzerland) and Sigma-Aldrich Co. (St. Louis, MO, EUA), respectively. Chitosan derivatives were prepared and characterized according to previously published procedures [22].

Fluorescence spectroscopy

The addition of fluorophore molecules to hydrogel solutions is being successfully used for monitoring the physic-chemical and structural changes in microenvironments as hydrogels [37–39]; then, pyrene and nabumetone were used to evaluate the local polarity of the microenvironments of derivatives solution. Pyrene (1×10^{-6} mol L^{-1}) and nabumetone (5×10^{-5} mol L^{-1}) were excited at 334 nm and 317 nm, respectively, using a Spex Fluoromax 2 spectrofluorimeter. The absorption spectrum of nabumetone was measured with a Cary 50-Varian spectrophotometer. All measurements were performed at room temperature.

Nabumetone is a drug with emission and absorption spectra sensitive to changes in the molecular microvicinity [34]. Therefore, together with pyrene, it was employed to monitor the solution properties of the derivatives.

Pyrene is a very popular probe due to the well-known fine structure of its fluorescence emission spectrum and the dependence on the polarity and viscosity of the probe neighborhood [27, 29, 30]. The ratio of pyrene fluorescence intensity of the first (~372 nm) to the third (~384 nm) bands reflects the nature of the immediate environment around the probe [27, 40] and it was used to evaluate the polarity of the local environment and to determine the critical aggregation concentration (CAC). This corresponds to a polymer composition or concentration region, at which hydrophobic domains start to be formed similarly to the CMC (critical micellar concentration) of surfactants micelles [27]. Additionally, pyrene produces a well-defined excimer with long lifetime and fluorescence emission band at ~470 nm [27, 40]. These phenomena are suitable to investigate the changes of polarity caused by increasing the DS and by the chain length of substitute in the chitosan derivatives, which in turn induces variations of the probe local concentration.

Results and discussion

In general way, when dealing with polymer (synthetic or natural) aggregations it is not possible to define a micellar concentration or a micelle-like microdomain. Nevertheless, using fluorescent probes can be determined if the microdomains exist and if the probe is placed there. The fluorescence emission spectrum of pyrene in solution and at room temperature presents five principal vibronic bands. The ratio of the intensities of band 1, I_1, to the band 3, I_3, was used to determine the critical aggregation concentration (CAC) and investigated the formation and properties of aggregates of chitosan derivatives. Figure 1 shows a plot of the parameter I_1/I_3 as a function of Q-S3 concentration. The

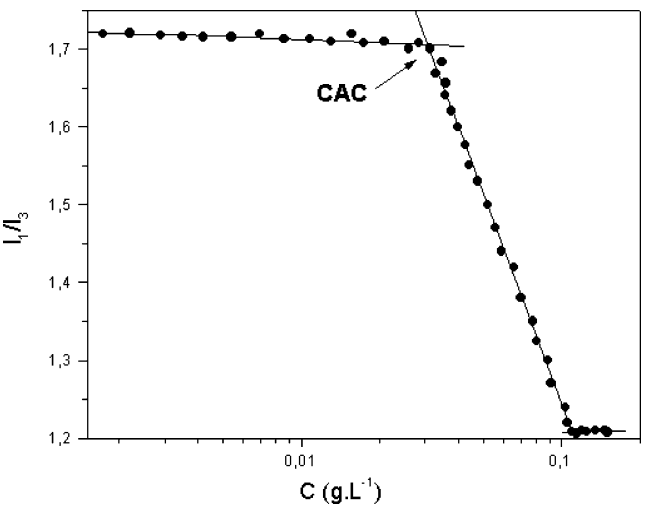

Fig. 1 I_1/I_3 ratio of fluorescence intensities of pyrene (1×10^{-6} mol L^{-1}) as a function of derivative chitosan Q-S3 concentration (C, g L^{-1})

I_1/I_3 values range from 1.87 in water to 0.6 in aliphatic hydrocarbon solvents. Values of 1.1 to 1.2 are typical of aqueous micelles, indicating that pyrene is located in the surface region of the hydrocarbon core [27]. The figure shows that the intensity ratio I_1/I_3 becomes smaller when the pyrene migrates from a rather aqueous environment (I_1/I_3>1.7) to the gradually increasing micelle-like microdomain (I_1/I_3 ~1.2). All derivatives presented the same behavior and CAC values were determined from the threshold concentration, where the intensity ratio of I_1/I_3 started to decrease markedly. Data are presented in Table 1, together with DS values previously calculated from ^{1}H-NMR measurements [22]. The DS was controlled by the acyl chloride or TEA amount. The Q:Ch:TEA molar ratio (where Q:Ch stands for chitosan Q, stearoyl or lauroyl chloride Ch) are shown in Table 1.

Table 1 reports the exponential growing of the DS with the Triethylamine (TEA) concentration (previous results, [22]). Even in the presence of low concentrations of acyl chloride, the DS achieved high values, due to the presence of a favorable amount of TEA. Additionally, the same table shows that the chain length of the substitute seems not to have much influence on aggregation but, for some derivatives (Q-S2 and Q-L2; Q-S3 and Q-L3; Q-S4 and Q-L4) CAC values are attained at lower concentrations for longer hydrocarbon chains for the same DS, similarly to premicelles of surfactants [27, 40]. The relation of the swelling-index of chitosan derivatives, previously described [22], with DS and, consequently, with the aggregation, follows a similar way. The lower the degree of swelling detected (derivative with bigger degree of substitution), the higher the crosslinking density was. Allied to this, the CAC values (range between 0.790×10^{-2} g L^{-1} and 14.0×10^{-2} g L^{-1}) show that a tighter and more compact structure that limits the water uptake must be formed similarly to behavior reported for both synthetic [31, 32, 40] and natural polymers[17, 30, 41, 42].

Figure 2 displays the fluorescence spectra of pyrene as a function of derivative concentration and shows the correspondent changes in the intensity ratio I_1/I_3. It can be observed that the intensity of band 1 is significant enhanced in more polar media while intensity of band 3 is minimally affected. Moreover, the variation of the excimer emission was used to verify the region where pyrene was solubilized: the excimer emission appears when free pyrene molecules are transferred to pre-aggregates of the hydrophobized chitosan and the encounter between two molecules occurs. This emission decreases with the increase of derivative concentration, disappearing when that is bigger than the CAC, which is caused by the redistribution of pyrene molecules among the polymer aggregates. We believe that this region corresponds to a concentration at which long alkyl chains start to interact in a mutually cooperative way, the critical aggregation concentration, CAC.

All this data were strengthened by the photophysical behavior of nabumetone. This fluorescent probe can exist in two preferred conformations [34]. Nabumetone is a naphthalene derivative with fluorescent properties, which contains a butanone substituent in the 2-position. This can interact with the aromatic moiety causing a decrease of probe fluorescence when the drug is in aqueous medium. Another conformation, in which the interaction does not occur, appears when nabumetone is in an apolar solvent and its fluorescence is intensified. These properties of nabumetone have promoted its use as a reporter of microenvironment for chitosan derivatives. Figure 3 shows the emission spectra of nabumetone at several concentrations of chitosan derivative Q-L3. With the increase of derivative concentration, the probe is transferred to a more hydrophobic microenvironment where it is protected from water quenching and the intensity of emission of the band centered on 350 nm increases. In addition, when the derivative concentration achieves a value near that of CAC (obtained values with pyrene) the emission spectrum remain practi-

Table 1 Degree of substitution (DS) from previous work [22], calculated critical aggregation concentration (CAC) and molar ratios (Q:Ch:TEA) for the chitosan derivatives with chitosan (Q), acyl chloride (Ch) and TEA amounts

Stearoyl derivatives	Molar ratio Q:Ch:TEA	DS	CAC $\times10^{-2}$ (g L^{-1})	Lauroyl derivatives	Molar ratio Q:Ch:TEA	DS	CAC 10^{-2} (g L^{-1})
TQ-S1	1.0:1.0:1.7	0.0033	14.0				
TQ-S2	1.0:1.0:4.2	0.024	9.50				
TQ-S3	1.0:1.0:5.0	0.032	8.00				
Q-S1	1.0:1.0:6.7	0.10	0.810	Q-L1	1.0:1.0:6.7	0.11	0.790
Q-S2	1.0:0.70:6.7	0.085	1.40	Q-L2	1.0:0.70:6.7	0.10	1.80
Q-S3	1.0:0.33:6.7	0.079	3.00	Q-L3	1.0:0.33:6.7	0.075	3.10
Q-S4	1.0:0.15:6.7	0.071	3.80	Q-L4	1.0:0.15:6.7	0.056	5.70
Q-S5	1.0:0.10:6.7	0.030	8.70	Q-L5	1.0:0.10:6.7	0.043	8.50

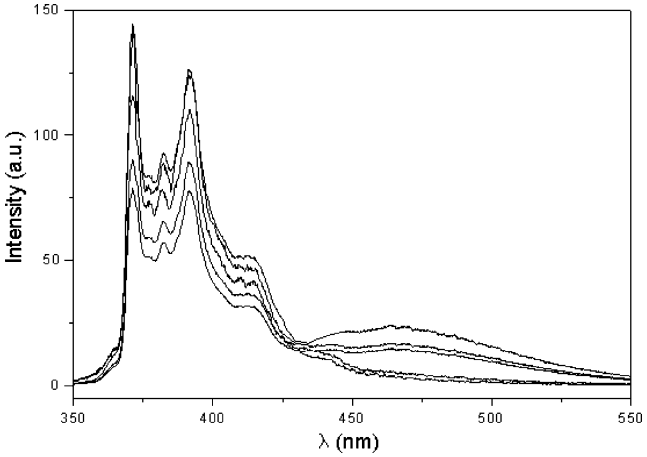

Fig. 2 Fluorescence spectra of pyrene (1×10^{-6} mol L^{-1}) as a function of Q-S3 concentration (10^{-2} g L^{-1}): water; 3.8; 2.8; 2.5; 1.0 (*from the base to the top*)

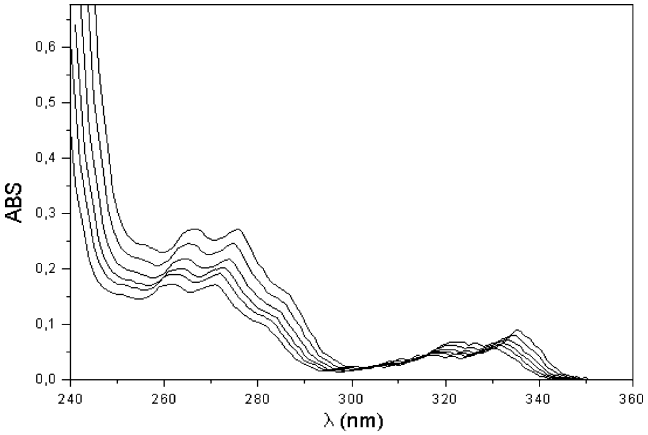

Fig. 4 Absorption spectra of nabumetone (5×10^{-5} mol L^{-1}) as a function of Q-L3 concentration (10^{-2} g L^{-1}): water; 1.0; 1.5; 2.5; 3.0; 3.5 (*from the base to the top*)

cally constant showing that the nabumetone probe was incorporated into the aggregates. Nevertheless, the absorption spectra of nabumetone (Fig. 4) are shifted to longer wavelengths when the chitosan derivative concentration increases, indicating that nabumetone molecules are transferred from water to a less polar environment inside the aggregates of chitosan derivative. All spectra are characteristics of naphthalene 2-substituted compounds [43] where the longer wavelength band is the most sensitive to changes in the solvent polarity [34] and the maximum absorption can be related to the relative permeability of the drug environment [44]. All derivatives presented the same behavior.

With the use of pyrene and nabumetone probes, the results in this article provide insight into the nature of the hydrophobic environment created by the stearoyl and lauroyl chitosan derivatives in aqueous solutions: above a certain concentration of chitosan derivative, CAC, intermolecular hydrophobic interactions lead to the formation of polymolecular associations where hydrophobic molecules such as probes and drugs can be better solubilized. This association process seems to be influenced by the increase of DS and sometimes, by the chain length of the substitute.

The aggregates are formed at concentrations in the range between 0.79×10^{-2} g L^{-1} and 14.0×10^{-2} g L^{-1}, independent of the composition of the derivatives. Therefore, these products will be able to exhibit thickening properties equivalent to those observed for higher molecular weight homopolymers and play important role as viscosity modifiers in a variety of new technologies, including paints, inks, cosmetics and drug deliveries.

As a drug delivery, chitosan can be prepared in various forms such as hydrogels, particles, and membranes. Hydrogels have been more used since drugs can be easily dispersed in the matrix and their release is achieved through the selection of various polymer networks.

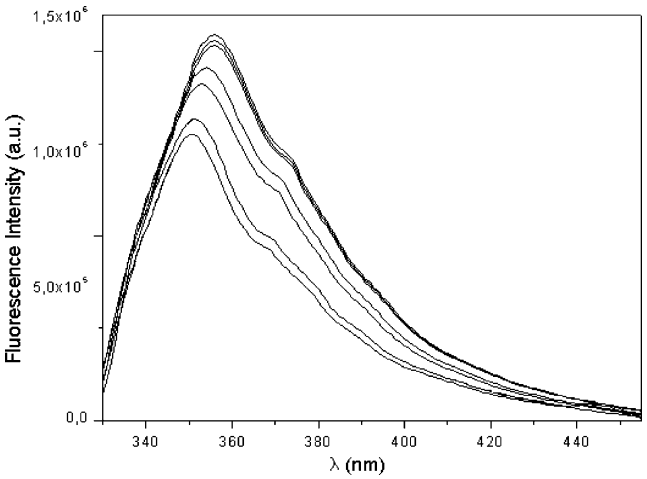

Fig. 3 Fluorescence spectra of nabumetone (5×10^{-5} mol L^{-1}) as a function of Q-L3 concentration (10^{-2} g L^{-1}): water; 0.7; 1.0; 1.8; 2.9; 3.0; 3.2 (*from the base to the top*)

Conclusion

The results in this article clearly illustrate the potential of new chitosan derivatives and their ability to form a moderately hydrophobic environment in aqueous solution. By selective substitute groups, it should be possible to generate derivatives that display a variety of properties in aqueous media. The good performance of chitosan derivatives accompanied by the more thorough understanding of the physical and chemistry properties of chitosan-based gels, leads to expect that chitosan gels will have an expanding range of applications in the near future.

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