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Study of the dynamical behavior of sodium alginate/myoglobin aqueous solutions: A dynamic light scattering study

Caterina Branca *, Ulderico Wanderlingh, Giovanna D'Angelo, Cristina Crupi, Simona Rifici

Dipartimento di Fisica e Scienze della Terra, Università di Messina, Viale Stagno D'Alcontres 31, 98166 Messina, Italy

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ABSTRACT

In the last years, protein/polysaccharide systems have received increasing research interest since they play an essential role in stabilizing food formulations, pharmaceutical preparations and so on. In this study, the diffusive properties of both sodium alginate and sodium alginate/myoglobin water solutions were characterized by dynamic light scattering. By analyzing the effects of concentration, pH and ionic strength on the diffusion coefficient, it emerged that sodium alginate forms aggregates which diffuse as pH sensitive rigid spheres. In the presence of the protein, the polysaccharide is less prone to self-aggregation favoring the formation of more solvent exposed protein/polysaccharide aggregates. These aggregates resulted to be both pH and ionic strength sensitive since their formation arises from a balance between coexisting repulsive and attractive interactions that are strongly influenced by the environmental conditions.
By Fourier-Transform Infrared spectroscopy we evidenced the structural changes that take place in myoglobin during the protein–alginate complex formation. The observed increase in the solvent exposed extended chain conformation supported the hypothesis of a more open conformation adopted by the polysaccharide–protein complex with a consequent reorganization of hydrogen bonding.

1. Introduction

In the last decades much interest has been focused on protein–polysaccharide complexes in response to an increasing demand for novel functional ingredients in pharmaceutical and food industry. Proteins and polysaccharides are for example used together in gels, foams and emulsions [1–3]; they are also used to formulate blood derived products, vaccines and drug delivery systems [4,5]. In the field of pharmaceuticals, one of the greatest challenges is to improve protein stabilization by embedding it in a polysaccharide matrix. Even if this technique is now widely applied, understanding the mechanisms underlying the protein stability is still a complex problem. Previous studies evidenced the presence of aggregative processes mainly driven by electrostatic forces even if hydrogen, hydrophobic or covalent bonds may also contribute significantly [2,6–8]. Consequently, the optimal stability ranges for proteins will depend on a variety of conditions and any study on protein–polysaccharide complexes must take this into account.

One of the most frequently used polysaccharides is sodium alginate, NaAlg, $(NaC_6H_7O_6)_n$, the salt of the long chain carbohydrate alginic acid [9]. It can be found naturally at low concentrations in seawater, where it is secreted by several species of brown algae. Sodium alginate can be characterized as an anionic copolymer comprised of mannuronic acid (M block) and guluronic acid (G block) units arranged along the chain in an irregular pattern of varying proportion of GG, MM and MG blocks [10]. Alginates have COO– and COOH groups along the chain conferring different charge densities depending on pH. The hydrophilic and hydrophobic units along the chain can be altered by the protonation and deprotonation of carboxyl groups in the backbone chain [11]. Alginate is both biopolymer and polyelectrolyte and is considered to be biocompatible, non-toxic, non-immunogenic and biodegradable, which make it an attractive candidate for biomedical applications [12]. Commercially available alginates are used in a variety of technical applications like thickening and gelling agents in the food industry or to immobilize cells in the biotechnological industries [13]. For these reasons, a majority of studies have focused on its gelling properties whereas few studies have investigated the interactions between sodium alginate and surfactants [11,14], or proteins, like BSA [7] and β-lactoglobulin [8]. Starting from these considerations, in this paper we focused our attention on the changes induced by the presence of myoglobin on the diffusive and conformational properties of sodium alginate solutions. Myoglobin has been usually used as a model protein to check binding mechanisms and to reveal structural changes in its native conformation due to its peculiar structure and function. The structure of myoglobin, consisting of a single polypeptide chain of 154 amino acids [15], was first delineated by John Kendrew and colleagues over 40 years ago [16–18]. From these and subsequent works, it emerged that the secondary structure is unusual in that it contains a very high proportion (75%) of α-helical secondary structure. Myoglobin is found mainly in

* Corresponding author.
E-mail address: cbranca@unime.it (C. Branca).

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the muscle tissue where it serves as an intracellular storage site for oxygen [15].

Previous studies showed that globular proteins can interact with anionic polysaccharides to form soluble or insoluble complexes that can be stabilized by electrostatic, ion–dipole or hydrophobic interactions [8]. Several mechanisms of interactions were hypothesized for protein–alginate system, and among them noncovalent electrostatic interactions were mostly studied [19,20], but notwithstanding much effort, they are far from being satisfactorily elucidated. The mechanisms of interactions have obviously a strong influence on the dynamical properties of these systems that up to date were scarcely investigated. Since the mobility, and hence the flexibility of these systems, influences directly the functional activity of the protein, studying the diffusion dynamics of protein–polysaccharide systems under different environmental conditions is of crucial importance. This should lead to the development of novel applications and support the understanding of protein–polysaccharide interactions in general.

In the present study, the dynamical properties of both sodium alginate and sodium alginate/myoglobin water solutions were investigated by dynamic light-scattering (DLS) under different environmental conditions. Moreover, in order to relate the differences in the dynamical properties to structural changes occurring in the systems, Fourier Transform Infrared (FTIR) spectroscopy was also employed. The results were compared with previous investigations on aqueous solutions of sodium alginate tested under similar conditions [21–23]. Such information is of crucial importance for the formulation and optimization of new biocompatible products.

2. Materials and methods

2.1. Materials and solution preparation

Alginic acid sodium salt and horse skeletal muscle myoglobin were purchased from Aldrich (Milwaukee, WI) and Sigma (St Louis, MO), respectively. According to the specifications from the manufacturer, the sodium alginate sample has a weight-average molecular weight of 120,000–190,000 g/mol and a guluronic acid to mannuronic acid (M/G) ratio of 1.56.

As reported in the product information sheet by Sigma, and as also detected by Sheng and Pawliszyn [24], two components can be identified for the myoglobin used in this work; a major component with a pI value of 7.3 and a minor component with a pI of 6.8. Nevertheless, according to recent studies on the same Sigma product [25,26], a value of 7.2 can be considered as a good estimation of the myoglobin pI. For this reason we refer to this value for our following considerations. Like all the globular proteins, at a pH value above the isoelectric point the protein shows a negative net charge. All commercially solvents and reagents used were of analytical grade and no further purifications were made.

The aqueous solutions of sodium alginate were investigated at different concentrations, c (from 0.25 mg/ml up to 5 mg/ml). Each solution was prepared by dissolving a weighed amount of sodium alginate in doubly distilled water; to ensure complete dispersion, the solutions were heated at 30 °C and stirred for 24 h. To free these solutions from dust, they were filtered, through 0.8 μm Millipore filters. All concentrations were prepared at all the desired pH values (3.5, 4.5, 5.5, 6.5, 8, 10.5) using HCl or NaOH. NaCl was added to these solutions in order to have the same starting ionic strength. Then, for each pH value, the ionic strength of the media was varied by adding more NaCl. The contribution to the ionic strength of the sodium counterions from the alginate, about 20 mmol/dm^3 according to the Manning theory [27], was not considered.

A stock solution was prepared by dissolving myoglobin (0.1 mg/ml) in water. The protein/polysaccharide mixtures were prepared by mixing the sodium alginate solution (0.3 mg/ml) at different pHs with the protein stock solution. The starting solutions were then diluted obtaining concentrations of 0.14 mg/ml, 0.2 mg/ml, 0.3 mg/ml, and 0.4 mg/ml at pH values of 5.5, 6.5, 7.5 and 9. The ionic strength of the mixed solution was varied by the addition of NaCl (10 mM–100 mM).

High concentrations of myoglobin (40 mg/ml) and sodium alginate solutions were used to obtain accurate infrared spectra. In more details, we prepared a protein/polysaccharide mixture with a final concentration of 10 mg/ml myoglobin and 3% w/v sodium alginate. This concentration was chosen to maintain roughly the same polysaccharide/protein ratio used for DLS measurements. The pH of each solution was maintained at approximately 7.5. Measurements were performed at 25 °C.

2.2. Dynamic light scattering

DLS experiments were performed by using an Argon laser source ($\lambda = 488$ nm). A standard scattering apparatus with a Brookhaven BI-2030 correlator was employed to analyze the scattered light. Temperature was set at 25 °C and controlled within 0.01 °C by a water circulating apparatus. Every sample was measured several times over the angular range 40°–140°.

In a DLS experiment the measured quantity is the normalized intensity autocorrelation function [28–30]:

$$g_2(Q,t) = \frac{\langle I(Q,0)I(Q,t)\rangle}{\langle I(Q,0)\rangle^2} \tag{1}$$

where $Q = 4\pi n/\lambda \sin(\theta/2)$ is the exchanged wave vector, n being the refraction index of the medium and θ the scattering angle. If the scattered field, $E(Q,t)$, obeys a Gaussian statistic, $g_2(Q, t)$ is related to the normalized field autocorrelation function

$$g_1(Q,t) = \frac{\langle E*(Q,0)E(Q,t)\rangle}{\langle I(Q,0)\rangle} \tag{2}$$

through the Siegert's relation $g_2(Q, t) = 1 + |\alpha \cdot g_1(Q, t)|^2$, α being a constant depending on the experimental setup. For diffusing monodisperse spherical scatterers, the normalized scattered electric field autocorrelation function takes a simple exponential form with decay rate Γ: $g_1(Q, t) = \exp(-\Gamma t)$. Because the investigated solutions were slightly polydisperse, the autocorrelation functions were analyzed in terms of a standard cumulant analysis [28–30]:

$$\ln|g_1(Q,t)| = -\langle\Gamma\rangle t + 1/2!\mu_2 t^2 - 1/3!\mu_3 t^3 + \ldots \tag{3}$$

where μ_n represents the moments of the distribution of the decay rates. If the first moment, $\langle\Gamma\rangle$, is found to be proportional to Q^2, an effective diffusion coefficient can be determined as $D_{eff} = \langle\Gamma\rangle/Q^2$. By fitting $\ln|g_1(Q,t)|$ to a quadratic in t, the mean, $\langle\Gamma\rangle$, and the variance, μ_2, were obtained. The ratio of variance to the square of the mean is a measure of the polydispersity index, PI, of the diffusion coefficient, $PI = \mu_2/\langle\Gamma\rangle^2$.

Before experimentation, the DLS setup was tested using standard polystyrene microbead solutions; precise R_H values have been obtained for samples with particle size between 1 nm and 1 μm with an error of ±2%. For these solutions, it was found that the detector accepts about one coherence area, $\alpha \approx 0.96$. To judge the data quality, only those for which the percent difference between the calculated and measured baseline, reported by the software correlator, was no more than 0.05% were considered. A reduced chi-square parameter less than 0.0001 was used as a measure of goodness-of-fit of the cumulant analysis. Each DLS experiment was repeated in triplicate.

Finally, the Stokes–Einstein relation was used to estimate an average radius of hydration, $\langle R_H\rangle = k_B T/6\pi\eta D_0$, k_B being the Boltzmann's constant, T the absolute temperature and η the solvent viscosity; D_0 was obtained from an extrapolation of D_{eff} to zero concentration.