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Aggregation of some water-soluble derivatives of chitin in aqueous solutions: Role of the degree of acetylation and effect of hydrogen bond breaker

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ABSTRACT

By dynamic light scattering in combination with fluorescence spectroscopy and TEM it was shown that aggregation in aqueous solutions is inherent not only to chitosan, but also to two other water-soluble derivatives of chitin: O-carboxymethylchitin and di-N,N-carboxymethylchitosan. Aggregation is observed even for the samples without N-acetyl-D-glucosamine units, which remain upon incomplete chemical modification of chitin, indicating that specific interactions between residual chitin repeat units cannot be the main reason for the aggregation. At the same time, 7 M urea weakens the aggregation, thus testifying that hydrogen bonding and/or hydrophobic interactions are partially responsible for this phenomenon. The incomplete disruption of aggregates in 7 M urea may arise from crystallization of junction zones between different macromolecules, which makes some hydrogen bonds inaccessible for urea or too stable for breaking by this agent.

1. Introduction

Chitin is the second most abundant polysaccharide on the earth after cellulose. It is extracted from the outer shell of crustaceans, shrimps and crabs (Peter, 1995). Chitin is composed of (1 → 4)-linked N-acetyl-D-glucosamine and D-glucosamine units with high degree of N-acetylation (DA). It is not soluble in most available solvents, which essentially restricts the area of its application. But chitin can be converted into water-soluble forms by chemical modification with incorporation of charged groups. Among water-soluble derivatives of chitin, the most common are positively charged chitosan obtained by partial deacetylation and negatively charged carboxymethylated chitin and chitosan. These polymers are nontoxic, biocompatible and biodegradable. Due to these properties, they are used extensively in such fields as food industry, pharmacy, cosmetics, and medicine (Lang & Clausen, 1989; Peter, 1995). Most of these applications deal with aqueous solutions. Therefore, it is important to understand the properties of these polymers in aqueous media.

Among chitin derivatives, the most studied is chitosan. It is soluble in water only at pH < 6, when most of the amino groups are protonated. But even under these conditions the solutions can contain some intermolecular aggregates along with molecularly dispersed polymer chains. The presence of aggregates was demonstrated by static and dynamic light scattering (DLS) techniques (Anthonsen, Vårum, Hermansson, Smidsrød, & Brant, 1994; Korchagina & Philippova, 2010; Schatz, Pichot, Delair, Viton, & Domard, 2003; Wu, Zhou, & Wang, 1995). Often the aggregation is accompanied by the formation of hydrophobic domains which was evidenced by fluorescence spectroscopy with pyrene as a probe (Amiji, 1995; Philippova et al., 2001). This allows one to suggest that the hydrophobic domains may link different macromolecules with each other in the aggregates. The presence of hydrophobic surfaces enriched in CH-groups that may be responsible for such aggregation was recently demonstrated by molecular modeling of chitosan chains (Mazeau & Rinaudo, 2004). The important role of hydrophobic interactions in the aggregation of chitosan macromolecules is also manifested in the LCST behavior of their aqueous solutions (Mazeau & Rinaudo, 2004). Along with hydrophobic interactions, hydrogen bonding is also suggested to contribute to the aggregation of chitosan (Amiji, 1995; Argüelles-Monal, Goycoolea, Lizardi, Peniche, & Higuera-Ciapara, 2003; Nyström, Kjøniksen, & Iversen, 1999; Philippova et al., 2001). It should be noted that junction zones formed by multiple hydrogen bonds between different macromolecules can be also sensed by pyrene as hydrophobic domains (Frank, Hemker, & Oyama, 1991). Indeed, each repeat unit in chitosan contains both hydrophilic and hydrophobic groups, but only hydrophilic groups are involved in hydrogen bonding (Okuyama, Noguchi, Miyazawa, Yui, & Ogawa, 1997). As a result of such bonding the interacting hydrophilic groups become "screened" from

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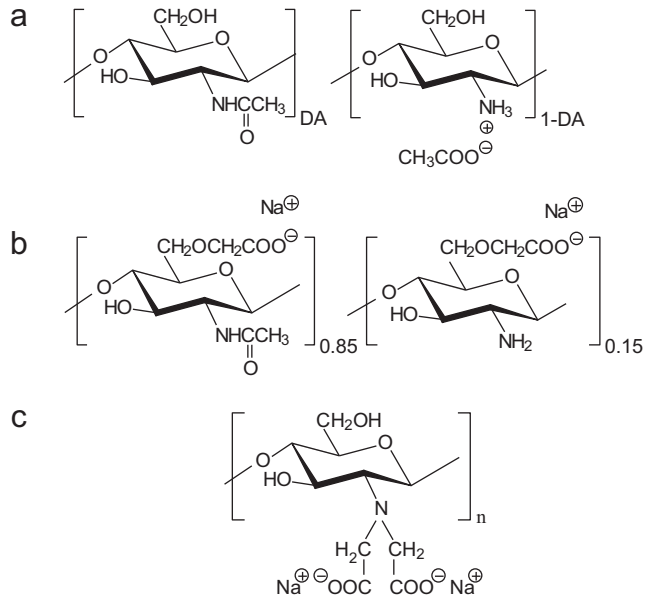

Fig. 1. Chemical structures of chitosan (a), O-carboxymethylchitin sodium salt (b) and di-N,N-carboxymethylchitosan sodium salt (c) studied in this paper.

solvent, which effectively increases the hydrophobicity of the polymer (Yu, Tanaka, Tanaka, & Tanaka, 1992).

Although most of the studies of aggregation behavior were performed with chitosan solutions, recently the formation of aggregates was demonstrated also for O- and N-carboxymethylchitosans (Chen, Du, Tian, & Sun, 2005; De Freitas, Drenski, Alb, & Reed, 2010; Felicio et al., 2008; Zhu, Chan-Park, Dai, & Li, 2005; Zhu, Dai, Li, & Zhao, 2006). At the same time, it still remains unclear, whether such aggregation is typical for other water-soluble derivatives of chitin.

In this paper we report the use of DLS, fluorescence spectroscopy with pyrene as a probe and TEM to check whether aggregation can be observed in two water-soluble derivatives of chitin: O-carboxymethylchitin and di-N,N-carboxymethylchitosan. Particular attention is paid to determination of the factors that affect the aggregation. Such information is essential for the development of a molecular explanation of the aggregation behavior. In particular, these studies provide a critical test for the hypothesis that the main role in this phenomenon is played by uncharged N-acetyl-D-glucosamine units, which remain upon incomplete chemical modification of chitin (Aiba, 1991).

2. Materials and methods

2.1. Materials

Acetic acid (99.5%) and ethanol (96%) from Fluka were used as received. A fluorescence probe pyrene obtained from Aldrich was repeatedly recrystallized from absolute ethanol to water. Water was purified with a Milli-Q system (Millipore).

Chemical formulae of polymers under study are presented in Fig. 1. Chitosan with DA of 0.56 (lot A32E03) was supplied by Aber Technologies (France). It is completely soluble in 0.3 M acetic acid. The samples of chitosan with smaller DA were obtained by progressive deacetylation following the technique reported previously (Rinaudo & Domard, 1989). By ^{1}H NMR spectroscopy it was shown that in these samples the distribution of N-acetyl-D-glucosamine units along the backbone is random. The values of molecular weight and of DA of chitosans under study are summarized in Table 1. The samples have the polydispersity ratio $M_w/M_n = 1.3$, where M_w is the weight average molecular weight and M_n is the number average molecular weight.

Table 1
The characteristics of chitosan samples under study.

Chitosan samples	Degree of acetylation DA	M_w[a] (kg/mol)
1	Traces	110
2	0.12	135
3	0.24	127
4	0.40	101
5	0.56	115

[a] Determined by SEC-MALLS method after filtration through 0.22 μm pore size membrane (eluent: 0.3 M acetic acid/0.2 M sodium acetate; concentration of chitosan solution: 0.5 g/L).

Sodium salt of O-carboxymethylchitin with molecular weight of 160 kg/mol, DA = 0.85 and degree of substitution of 0.9 obtained from chitosan from crab shells was kindly provided by Dr. G.A. Vikhoreva (A.N. Kosygin Moscow State Textile University).

Sodium salt of di-N,N-carboxymethylchitosan has molecular weight equal to 160 kg/mol, DA about zero, and degree of substitution of nearly 2 (Rinaudo, Le Dung, Gey, & Milas, 1992; Rinaudo, Le Dung, & Milas, 1992).

The overlap concentration C^* for chitosan (sample 2) solution was estimated from the departure of the dependence of specific viscosity on polymer concentration from Huggins behavior. It equals to ca. 6×10^{-3} monomol/L. The C^* values for O-carboxymethylchitin and di-N,N-carboxymethylchitosan are expected to be of the same order of magnitude because these polymers have close values of the degree of polymerization.

2.2. Solution preparation

Polymer aqueous solutions were prepared by weighing the components and stirring for at least 24 h. Solutions of chitosan were made in 0.3 M CH_3COOH, where the amino groups of polymers are fully protonated (Rinaudo, Pavlov, & Desbrières, 1999). Solutions of sodium salts of O-carboxymethylchitin and di-N,N-carboxymethylchitosan were prepared in pure water; pH of the resulting solutions was slightly alkaline. Under these conditions aminogroups of O-carboxymethylchitin are not protonated.

Solutions for fluorescence measurements were made by pipetting a small quantity of pyrene stock solution in ethanol (3×10^{-4} mol/L) 24 h before the measurements. The concentration of pyrene in all samples was equal to 8×10^{-7} mol/L.

2.3. Fluorescence spectroscopy

Fluorescence measurements were performed on a Hitachi MPF-4 fluorescence spectrophotometer in a thermostated cuvette holder at 25 °C. The pyrene spectra were obtained by exciting the solutions at 338 nm and recording the emission over the range 350–550 nm at the scan rate of 15 nm/min. The slit width was set at 5 nm for the excitation and 1.5 nm for the emission. To increase the precision of the determination of the values of intensities of different vibronic peaks, the averaged (during at least 2 min) fluorescence intensities were recorded at the maximum of each peak.

2.4. Dynamic light scattering (DLS)

DLS measurements were carried out with ALV/DLS/SLS-5022F compact goniometer system equipped with an ALV-6010/EPP multiple tau digital time correlator (ALV Laser Vertriebs GmbH, Langen, Germany) and Uniphase He/Ne laser (model 1145P) operating at a power of 22 mW at $\lambda = 632.8$ nm. The intensity autocorrelation function $g_2(t)$ was measured at a scattering angle of 90° relative to

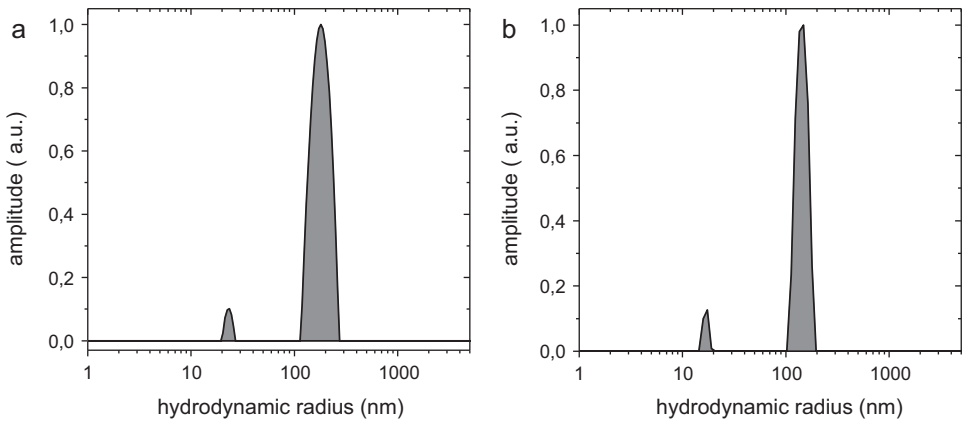

Fig. 2. The size distribution functions obtained by CONTIN analysis of intensity autocorrelation function measured at 90° scattering angle for (a) 1.2×10^{-3} monomol/L solution of sodium salt of O-carboxymethylchitin in water in the presence of 0.05 M NaCl and (b) 1.2×10^{-3} monomol/L solution of chitosan with DA = 0.12 (sample 2) in 0.3 M CH_3COOH in the presence of 0.05 M CH_3COONa.

the primary beam. A nonlinear regularized inverse Laplace transformation technique (CONTIN) was employed to obtain the decay time distribution and to extract the translational diffusion coefficients. With Stokes–Einstein equation the diffusion coefficients were converted to the hydrodynamic radii.

All polymer solutions under study were filtered with a 0.45 μm cellulose acetate membrane filter directly into a measuring cell with 10 mm outer diameter. The cells were immersed in a thermostated index matching bath with toluene. Samples were measured for at least 3 min, and measurements were repeated three times to verify the reproducibility of the results.

2.5. Transmission electron microscopy (TEM)

Electron micrographs were obtained with a LEO912 AB OMEGA transmission electron microscope at the accelerating voltage of 100 kV. The samples for TEM observations were prepared as follows. First, 2.5 μL of 0.05% (w/v) polymer solution in 0.3 M acetic acid (for chitosan) or in water (for di-N,N-carboxymethylchitosan) was deposited on a 140 mesh Formvar-coated copper grid and dried for 1 min. The excess of solution was blotted off. Then 2.5 μL of 1 wt.% staining uranyl acetate solution in water at pH = 4.5 was added to the top of the sample, blotted off and dried in air.

3. Results and discussion

3.1. Aggregation of different derivatives of chitin

In some recent works the appearance of intermolecular aggregates in aqueous solutions of chitosan (Amiji, 1995; Anthonsen et al., 1994; Korchagina & Philippova, 2010; Lang & Clausen, 1989; Peter, 1995; Philippova et al., 2001; Schatz, Viton, Delair, Pichot, & Domard, 2003; Wu et al., 1995) and O- and N-carboxymethylchitosan (Chen et al., 2005; De Freitas et al., 2010; Felicio et al., 2008; Zhu et al., 2005, 2006) was observed. To determine whether such aggregation behavior is inherent to other derivatives of chitin as well, we used O-carboxymethylchitin and di-N,N-carboxymethylchitosan.

3.1.1. O-carboxymethylchitin

Fig. 2a shows the DLS data for aqueous salt solutions of O-carboxymethylchitin. For comparison the data for chitosan are presented as well (Fig. 2b). It is seen that dilute aqueous solutions of chitosan contain species of two different sizes: 18 and 150 nm. According to estimations by the formula (Grosberg & Khokhlov, 1994):

$$R_h \sim \left(\frac{3\pi}{128}\right)^{1/2} (2Ll_p)^{1/2} \quad (1)$$

using persistence length l_p value of 6 nm (Berth, Cölfen, & Dautzenberg, 2002) and contour length L value of 420 nm, the hydrodynamic radius for single chitosan macromolecules with molecular weight of 135 kg/mol should be of 19 nm. Therefore, the smaller species are single chitosan macromolecules (unimers). The larger species are intermolecular aggregates, as was evidenced in our previous paper (Korchagina & Philippova, 2010).

From Fig. 2 it is seen that the size distribution function for O-carboxymethylchitin shows two populations of particles, as in the case of chitosan. Thus, intermolecular aggregates together with single chains are also observed in solutions of the anionic chitin derivative O-carboxymethylchitin.

Simultaneously, fluorescence spectroscopy with pyrene as a probe was used to detect the aggregation. The ratio of intensities of the first (371.5 nm) to third (383 nm) vibronic peaks I_1/I_3 in the fluorescence spectra of pyrene is known to be quite sensitive to the polarity of microenvironment of the probe (Kalyanasundaram & Thomas, 1977). The value of the ratio I_1/I_3 ("polarity parameter") is higher in more polar media (e.g., in water (polar solvent) $I_1/I_3 = 2.0$, while in hexane (nonpolar solvent) $I_1/I_3 = 0.6$) (Kalyanasundaram & Thomas, 1977). When in polar medium (water) the hydrophobic domains are formed, and pyrene being quite hydrophobic is solubilized in their nonpolar interior, which leads to the decrease of the polarity parameter. Due to this property, pyrene is widely used to detect the formation of hydrophobic domains in aqueous media (Blagodatskikh et al., 2002; Kalyanasundaram & Thomas, 1977; Philippova & Starodoubtzev, 1993; Philippova, Hourdet, Audebert, & Khokhlov, 1997).

Fig. 3 shows the results of measurements of polarity parameter of pyrene in aqueous solutions of O-carboxymethylchitin and chitosan. For proper comparison of the experimental data for polymers with different structure of repeat units the concentration of polymer is expressed in monomol/L, i.e., in moles of repeat units/1 L of solution. From Fig. 3 it is seen that anionic derivative of chitin, O-carboxymethylchitin, forms hydrophobic domains in the same region of polymer concentration as the cationic derivative, chitosan.

The fluorescence curve starts to decrease at a polymer concentration of 7×10^{-4} monomol/L indicating the onset of aggregation, which is in agreement with DLS data (Figs. 2 and 3). At the same time, it should be noted that in such dilute solutions DLS is more

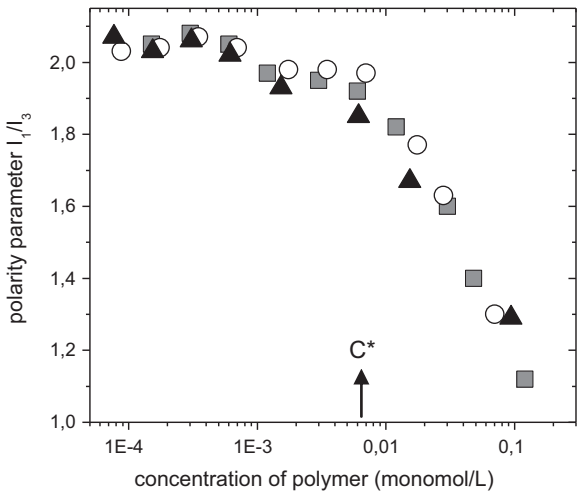

Fig. 3. Polarity parameter I_1/I_3 of pyrene as a function of polymer concentration in solutions of chitosan with DA = 0.12 (sample 2) (squares) in 0.3 M CH_3COOH and of sodium salts of O-carboxymethylchitin (circles) and di-N,N-carboxymethylchitosan (triangles) in water. C* is the coil-overlap concentration for chitosan (sample 2).

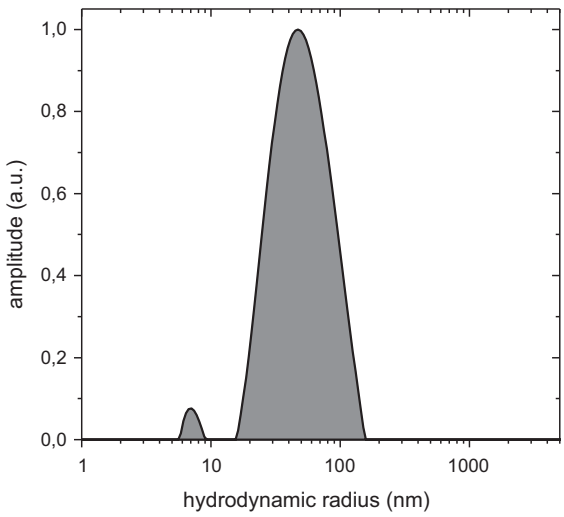

Fig. 4. The size distribution functions obtained by CONTIN analysis of intensity autocorrelation function measured at 90° scattering angle for 1.2×10^{-3} monomol/L solution of sodium salt of di-N,N-carboxymethylchitosan in water in the presence of 0.05 M NaCl.

sensitive to the presence of aggregates, because light scattering intensity increases strongly with particle size; as a result a very small population of aggregates can yield a large scattering intensity. For example, in the case of chitosan dissolved in 0.3 M CH_3COOH/0.05 M CH_3COONa, where DLS data are dominated by the aggregates (Fig. 2b), the fraction of aggregated macromolecules was shown to be less than 0.1 (Korchagina & Philippova, 2010). Most probably, at such low content of aggregates the total amount of hydrophobic domains inside them is so small that most of pyrene molecules still reside in water, and therefore the drop of polarity parameter of pyrene is rather small. The amount of hydrophobic domains increases tremendously only in the semidilute regime when the intermolecular interactions become much more pronounced. This observation is consistent with the results recently obtained for N-carboxymethylchitosan (De Freitas et al., 2010; Felicio et al., 2008) as in its aqueous solutions DLS detects the aggregates at polymer concentration of 0.1 g/L, whereas fluorescence probe method reveals the hydrophobic domains generated by aggregates only at much higher concentrations (1–10 g/L). By contrast, in hydrophobically modified chitosan fluorescence spectroscopy finds the hydrophobic domains already in dilute regime (Philippova et al., 2001), because in this case the amount of these domains is much higher. Thus, one can note that for the unmodified samples the DLS is more suitable to detect aggregation in the dilute regime, whereas the fluorescence probe method is preferable in the semidilute regime, where the DLS data are more difficult to interpret.

So, a combination of DLS and fluorescence spectroscopy evidences the formation of aggregates of O-carboxymethylchitin in aqueous medium.

3.1.2. Di-N,N-carboxymethylchitosan

Fig. 4 shows that di-N,N-carboxymethylchitosan, like O-carboxymethylchitin and chitosan, gives two peaks in the size distribution function obtained by DLS, and Fig. 3 demonstrates the drop in polarity parameter of pyrene indicative of aggregation. To the best of our knowledge, this is the first observation of aggregation in aqueous solutions of di-N,N-carboxymethylchitosan.

The aggregates of di-N,N-carboxymethylchitosan were visualized by TEM. The results are shown in Fig. 5. It is seen that the aggregates are of spherical shape. The average radius of the aggregates on TEM images is lower than the $R_{h\,agg}$ value obtained by DLS, which is expected taking into account the fact that TEM visualizes the dried aggregates on the surface, whereas DLS deals with swollen aggregates in the solvent. Similar pictures were recently obtained for aggregates of chitosan (Korchagina & Philippova, 2010) and N-carboxymethylchitosan (Felicio et al., 2008). The analysis of experimental data performed previously (De Freitas et al., 2010; Korchagina & Philippova, 2010) has shown that most probably the aggregates are spherical microgel particles.

Thus, the data obtained evidence that the aggregation is inherent to various water-soluble derivatives of chitin including both positively charged chitosan and negatively charged O-carboxymethylchitin and di-N,N-carboxymethylchitosan.

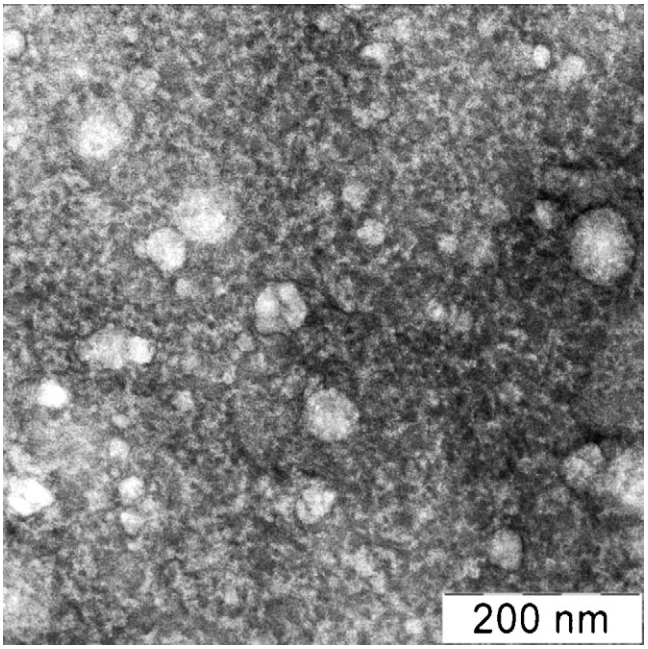

Fig. 5. TEM micrographs of multichain aggregates of di-N,N-carboxymethylchitosan negatively stained with uranyl acetate.

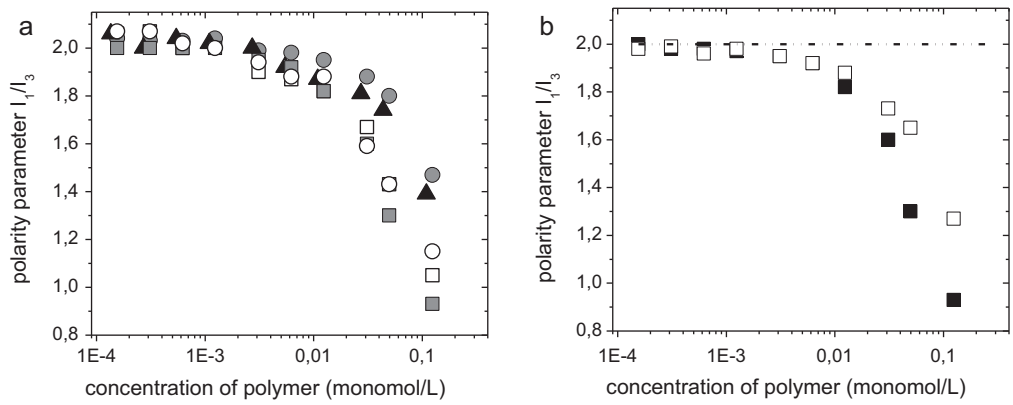

Fig. 6. Polarity parameter I_1/I_3 of pyrene as a function of polymer concentration in (a) solutions of chitosan with different DA: DA = 0 (sample 1) (open squares), DA = 0.12 (sample 2) (solid squares), DA = 0.24 (sample 3) (open circles), DA = 0.4 (sample 4) (solid circles), DA = 0.56 (sample 5) (triangles) in 0.3 M CH_3COOH; (b) solutions of chitosan with DA = 0.12 (sample 2) in 0.3 M CH_3COOH (solid squares) and in 0.3 M CH_3COOH/7 M urea (open squares). Dotted line shows the value of the polarity parameter for the solvents without polymer.

3.2. Role of the degree of acetylation

As aggregates are observed in many derivatives of chitin, it is reasonable to suggest that they may be due to interaction between N-acetyl-D-glucosamine repeat units that remain upon incomplete chemical modification of this polysaccharide. Indeed, in chitin itself the intermolecular interaction between these units is so strong that the polymer is insoluble in most common solvents and prefers to keep its semicrystalline structure stabilized by multiple hydrogen bonds. The importance of the impact of interaction between N-acetyl-D-glucosamine repeat units on the aggregation behavior of chitosan was recognized by many researchers especially when these units form rather long sequences along polymer chains (Aiba, 1991; Kurita, Sannan, & Iwakura, 1977; Matsumoto, Kawai, & Masuda, 1991; Vårum, Anthonsen, Grasdalen, & Smidsrød, 1991). It is suggested that these groups interact with each other via hydrogen bonding (Aiba, 1991); also they represent hydrophobic sites on chitosan chains and therefore may contribute to hydrophobic interactions (Schatz, Pichot, Delair, et al., 2003; Schatz, Viton, Delair, et al., 2003).

As regards chitosan with random distribution of N-acetyl-D-glucosamine repeat units, the available data about the effect of DA on its aggregation behavior are rather contradictory. Thus, Anthonsen et al. (Anthonsen et al., 1994; Ottøy, Vårum, Christensen, Anthonsen, & Smidsrød, 1996) showed that the aggregation is more pronounced for high DA values (0.52 (Ottøy et al., 1996) or 0.60 (Anthonsen et al., 1994), which was suggested to be due to the intermolecular hydrophobic interactions between the acetyl groups. By contrast, Kubota and Eguchi (1997) demonstrated that chitosan with DA about 0.50 shows the highest water-solubility among chitosan samples with other DA values. Similar data were obtained by Sogias, Khutoryanskiy, and Williams (2010). This behavior was explained by lower degree of crystallinity of such polymer (Qin et al., 2006; Taghizadeh & Davari, 2006) indicating weaker intermolecular interactions because of steric hindrances imposed by bulky acetyl substituents.

Analysis of literature data shows that the second type of behavior (better solubility of chitosan at DA around 0.50) is observed mainly for the samples obtained by homogeneous reacetylation of completely deacetylated chitin. On the other hand, the first type of behavior (greater aggregation at high DA) is often found for the samples prepared by partial deacetylation of chitin. In this case one can expect that some crystalline zones may remain as a result of incomplete dissolution of the polymer.

To clarify further the situation, in the present paper aggregation in chitosan samples with different DA (i.e., with different content of N-acetyl-D-glucosamine groups) was studied by three complementary techniques: the fluorescence probe method, DLS and TEM. The

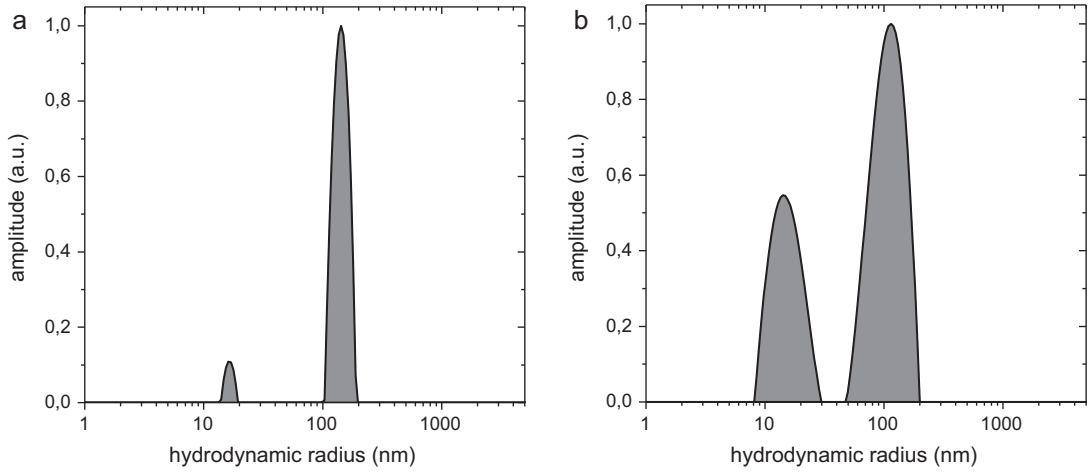

Fig. 7. The size distribution functions obtained by CONTIN analysis of intensity autocorrelation function measured at 90° scattering angle for 1.2×10^{-3} monomol/L solution of chitosan with DA = 0 (sample 1) (a) and with DA = 0.56 (sample 5) (b) in 0.3 M CH_3COOH in the presence of 0.05 M CH_3COONa.

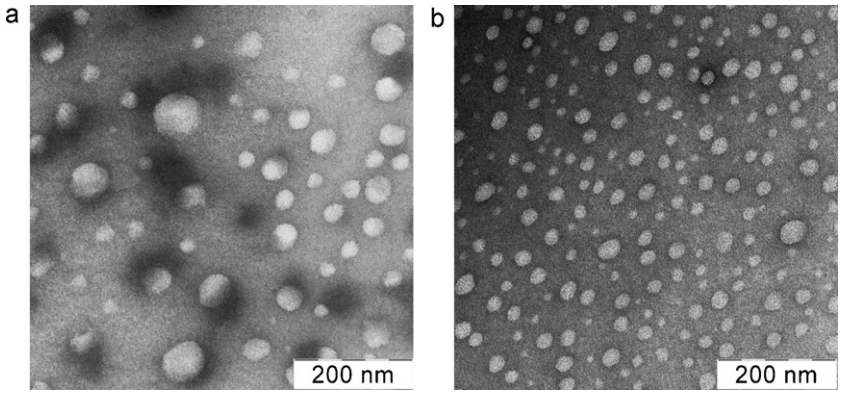

Fig. 8. TEM micrographs of multichain aggregates of chitosan with DA = 0.12 (sample 2) (a) and DA = 0.56 (sample 5) (b) negatively stained with uranyl acetate.

samples were prepared by deacetylation of the same initial polymer and they have very close values of molecular weight, random distribution of N-acetyl-D-glucosamine groups along the backbone, and values of DA varying in a wide range from 0 to 0.56 (Table 1). If the aggregation is indeed due to the interaction between N-acetyl-D-glucosamine units, it should be more pronounced at higher content of these units.

Fig. 6a shows the dependence of the polarity parameter of pyrene on the concentration of chitosan samples with different DA values in aqueous solutions of acetic acid. It is clearly seen that all the samples are able to aggregate in acidic medium independently of the content of N-acetyl-D-glucosamine groups. Especially it should be noted that the aggregation is observed even for the sample of chitosan that does not contain N-acetyl-D-glucosamine groups at all (DA = 0) (Fig. 6a).

The data obtained by the fluorescence probe method were confirmed by DLS results. Fig. 7 shows that the chitosan sample with DA = 0 forms intermolecular aggregates with hydrodynamic radius of 145 nm (for comparison R_h of single chitosan macromolecules with molecular weight of 110 kg/mol at θ-conditions should be ca. 17 nm according to estimation with Eq. (1)). These observations unambiguously demonstrate that the aggregation in acidic aqueous solutions of chitosan is not mainly due to the interactions between N-acetyl-D-glucosamine groups. Therefore, the results obtained disprove the commonly accepted hypothesis that the cause of aggregation is the interactions between N-acetyl-D-glucosamine monomer units.

The aggregation of chitosan samples without N-acetyl-D-glucosamine groups may be explained by their high ability to crystallize (Belamie, Domard, Chanzy, & Giraud-Guille, 1999; Ogawa, Hirano, Miyanishi, Yui, & Watanabe, 1984; Sogias et al., 2010), because of the more regular structure (all the repeat units are identical) and the absence of bulky side groups hampering the close packing of macromolecules. Such crystallites are known to be strengthened by multiple intra- and intermolecular hydrogen bonds (Okuyama et al., 1997).

WAXD data show that the ability to crystallize is significantly suppressed in half-acetylated chitosan samples, which may be due to steric hindrances imposed by acetyl substituents (Qin et al., 2006; Taghizadeh & Davari, 2006). The fluorescence and DLS results obtained in the present paper demonstrate that the samples with high DA (DA = 0.4 and 0.56) are indeed somewhat less prone to aggregation than their less acetylated counterparts. For instance, for the sample with DA = 0.56 the drop of polarity parameter of pyrene is shifted to higher polymer concentration (Fig. 6a) and the fraction of the intensity of scattered light provided by single chains is much higher (Fig. 7b) than for the fully deacetylated sample (Fig. 7a). Thus, the samples of chitosan prepared by deacetylation of chitin demonstrate lower tendency toward aggregation at DA of ca. 0.5 similar to the reacetylated samples (Kubota & Eguchi, 1997; Sogias et al., 2010).

To visualize the aggregates of chitosan with different DA values TEM was used. Typical pictures obtained are presented in Fig. 8. It is seen that the aggregates are of nearly spherical shape. Also, for the half-acetylated chitosan they are somewhat smaller than for the samples with lower DA, which is in accordance with DLS data.

As chitosan in a solid state contains crystallites (Brunel et al., 2009; Rinaudo, 2006), the question arises whether the crystal domains, which seem to be responsible for the aggregation behavior, arise from incomplete dissolution of crystallites or are formed already in the solution. To answer this question, the chitosan solutions were filtered through 0.1 μm cellulose acetate filter, which has pores of smaller diameter than the size of the aggregates, so that preformed aggregates should remain on the filter. In our experiments it was observed that there is almost no polymer loss on the filter. As to the aggregates, DLS data show that, having been destroyed by filtering, they are re-formed in solution, indicating that they are in dynamic equilibrium with single macromolecules (Korchagina & Philippova, 2010). Thus, preformed crystallites remaining upon incomplete dissolution of polymer are not responsible for the aggregation. The aggregates are mainly formed as a result of intermolecular interaction between dissolved macromolecules.

So, the data obtained show that the aggregation proceeds even for fully deacetylated chitosan, indicating that the N-acetyl-D-glucosamine units are not responsible for this phenomenon. Moreover, at rather high content of these units (DA = 0.4 and 0.56) the tendency to aggregation is reduced.

When considering other derivatives of chitin, let us note that the aggregation of di-N,N-carboxymethylchitosan, which does not contain N-acetyl groups at all, clearly evidences that these groups are not necessary for the aggregation.

Thus, it is obvious that the aggregation of chitin derivatives in aqueous medium can occur even in the absence of N-acetyl-D-glucosamine units remaining upon incomplete modification of chitin.

3.3. *Effect of urea*

In chitin derivatives each repeat unit possesses both proton donor and proton acceptor groups able to interact with each other via hydrogen bonding. Moreover, each unit contains not only hydrophilic, but also hydrophobic groups. Therefore, the aggregation of the ionic derivatives of chitin under study may be caused by a combination of hydrogen bonding and hydrophobic interactions between any repeat units (not only N-acetyl-D-glucosamine ones).

To check the role of hydrogen bonding we studied the effect of 7 M urea on the aggregation phenomenon. Urea is known to break hydrogen bonds (Hua, Zhou, Thirumalai, & Berne, 2008; Pelmont, 1989) and also to weaken hydrophobic interactions (Mukerjee & Ray, 1963) in water systems. Indeed, the driving force for hydrophobic association in aqueous media is partially attributed to the need for the hydrophobic moieties to minimize the surface area of their contact with water and consequently to minimize the amount of water that must be "structured" in order to solubilize hydrophobic parts. The addition of urea to aqueous solutions disrupts the structuring ability of water, thereby weakening the hydrophobic interactions in the solution (Mukerjee & Ray, 1963). Thus, urea can produce a double effect: it can break down the hydrogen bonds and weaken the hydrophobic interactions. Therefore, if hydrogen bonding and/or hydrophobic interactions are responsible for the aggregation, the aggregation should be suppressed by the addition of 7 M urea.

Fig. 6b shows the effect of 7 M urea on the polarity parameter of pyrene I_1/I_3 in aqueous solutions of chitosan. It is seen that urea hinders the formation of aggregates, which allows one to suggest that hydrogen bonding and/or hydrophobic interactions contribute to the aggregation. Unfortunately, we were unable to check this suggestion by DLS since it was impossible to get reliable DLS data in so concentrated a solution of urea because of the strong scattering from the solvent itself.

Thus, both hydrogen bonding and hydrophobic interactions seem to be among the factors responsible for the aggregation of chitosan. Nevertheless, the fluorescence data show that although urea hinders the formation of the aggregates, it does not prevent the aggregation completely. This may indicate that not all hydrogen bonds are disrupted by 7 M urea. Incomplete breaking of hydrogen bonds by urea has been reported for several polysaccharides including amylose (Erlander, Purvinas, & Griffin, 1968) and starch (Erlander & Tobin, 1968). Such behavior was explained (Erlander & Tobin, 1968) by the bulkiness of urea molecules, which cannot come in close proximity of interacting OH groups to destroy the hydrogen bond between them. One can speculate that similar behavior can be observed in chitosan, when, for example, (i) long sequences of cooperative hydrogen bonds or (ii) some microcrystallites are formed in the junction zones connecting different macromolecules inside the aggregate. In these cases the simultaneous disruption of multiple hydrogen bonds is required in order to destroy the junctions. But the literature data show that even highly cooperative hydrogen bonds between complementary macromolecules are effectively broken by urea (Hao et al., 2010). On the other hand, polysaccharides keeping most of the hydrogen bonds upon addition of urea (amylose, starch) are mainly stereoregular and prone to crystallization. Therefore, it seems likely that the low effect of urea on the aggregation of chitosan may result from the formation of microcrystallites, which make the hydrogen bonds inaccessible to urea and/or more stable.

Indeed, chitosan is known to be able to crystallize and its unit cell is stabilized by N2···O6 hydrogen bonds (Okuyama et al., 1997). If in such crystallites the hydrophilic groups participating in hydrogen bonds are screened from the solvent, this means that the surface of crystallites should be enriched in hydrophobic groups, which may induce the hydrophobic aggregation between them sensed by pyrene. It should be noted that for reacetylated chitosan samples with DA of ca.0.5, for which the crystallization is essentially suppressed, the effect of urea is much more pronounced (Sogias et al., 2010).

Thus, some suppression of the aggregation of chitosan by 7 M urea indicates that both H-bonding and hydrophobic interactions may contribute to the association. On the other hand, urea cannot destroy the aggregates completely, which is suggested to be due to partial crystallization of junction zones inside the aggregates.

4. Conclusions

The aggregation in aqueous solutions of anionic derivatives of chitin (O-carboxymethylchitin, di-N,N-carboxymethylchitosan) was observed. It was found to occur at the same region of polymer concentrations at which the aggregation in solutions of chitosan of the similar molecular weight was detected. It was demonstrated that the aggregation takes place even in the absence of residual N-acetyl-D-glucosamine repeat units, which remain upon incomplete chemical modification of chitin. Although the exact mechanism of the formation of aggregates is not yet well understood, it is assumed that the junction zones connecting different macromolecules inside the aggregates may contain crystalline domains. These findings are quite important for the design of new systems based on water-soluble chitin derivatives.

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