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Molecular weight and pH aspects of the efficacy of oligochitosan against methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Oligochitosan samples varying in molecular weight (M_w) and having narrow polydispersities were prepared by means of depolymerization of chitosan in hydrochloric acid, and their antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) was measured at pH values 5.5–8.0. The antibacterial testing of oligochitosans obtained showed that oligochitosans having M_w in the range of 0.73–20.0 kDa could be used both at slightly acidic and neutral pH values, and that the activity against MRSA remained moderate for oligochitosan samples having M_w about 3–5 kDa even at slightly basic pH values. The self-assembling behavior of oligochitosan macromolecules in the dilute solution at various pH values as a function of chain length was investigated. At first it was shown that oligochitosans formed supramolecular aggregates in dilute solutions below the critical pH value 6.5. Despite the aggregation phenomenon, the formation of nano-sized aggregates did not prevent oligochitosan from demonstrating the bactiostatic activity.

1. Introduction

Among Gram-positive bacteria the mostly collected in hospitals, public buildings, and aircraft cabins is Gram-positive *Staphylococcus aureus* that can cause a wide variety of diseases in humans and animals. *S. aureus* is becoming more and more resistant to many commonly used antibiotics including penicillin, amoxicillin, tetracycline, erythromycin, linezolid, vancomycin, and methicillin (Gandara, 2006). Increased problems with human allergy also have been observed in the patients receiving antibiotic agents for treatment. As a result, benefits and safety of many biocides are the subjects of debates among regulators specializing in medicine, food, cosmetics, environmental sciences, and toxicology (Donadio, Maffioli, Monciardini, Sosio, & Jabes, 2010). Therefore, there is a need for new non-toxic biocides that could be active against broad spectrum of invasive and noninvasive human pathogens and could reduce the level of administration of classic antibiotics.

Chitosan produced by a partial or complete deacetylation of chitin represents a collective name for a group of polysaccharides consisting of glucosamine and *N*-acetylglucosamine or glucosamine only. Chitosan and chitooligosaccharides have attracted considerable interest due to their different biological activities (Xia, Liu, Zhang, & Chen, 2011). Numerous investigations of antimicrobial activity of chitosan, its derivatives and analogues named oligochitosan and chitooligosaccharides against many bacteria, including *S. aureus* (Muzzarelli et al., 1990), filamentous fungi and yeasts have been published so far, and nowadays it is commonly accepted that the activity depends on molecular weight (M_w), degree of deacetylation (DD), target microorganism, and experimental conditions. As to DD, the higher DD is, the higher activity occurs. On the other side, the controversial evidences for a correlation between biocidal activity and M_w of chitosan have been found so far. It was shown in some studies that the increase in chitosan molecular weight led to the decrease in biocidal activity of chitosan (Hernández-Lauzardo et al., 2008; Jung, Chung, & Lee, 2002; Tikhonov et al., 2006; Xu, Zhao, Han, & Du, 2007; Yun, Kim, & Lee, 1999; Zheng & Zhu, 2003). In the others an increased activity of high molecular weight chitosans in comparison with low molecular weight chitosans was found (Hirano & Nagao, 1989; Kim, Thomas, Lee, & Park, 2003; Li, Feng, Yang, Wang, & Su, 2008; Lin, Lin, & Chen, 2009; Liu, Guan, Yang, Li, & Yao, 2001; Qin et al., 2006; Shahidi, Arachchi, & Jeon, 1999; Zhang, Tan, Yuan, & Rui, 2003). It was only ones that the bell-like dependence of fungistatic activity versus molecular weight was found (Tikhonov et al., 2011). The M_w-activity relationship is also found dependent on the

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target microorganism: the lower molecular weight of chitosan is, the stronger antimicrobial effect against Gram-negative bacteria is. In the case of Gram-positive bacteria, the effect of higher molecular weight of chitosan is stronger than that of lower molecular weight chitosan (Eaton, Fernandes, Pereira, Pintado, & Malcata, 2008; Fernandes et al., 2008).

In our opinion, the controversial results concerning biocidal activity and its correlation with M_w of chitosan have been found mainly because so far most investigators either have used only few chitosan samples, or molecular weight distribution/polydispersity have not been taken into account. That is, some of the biological effects reported for chitosan may be caused by the presence of lower molecular weight chitosan macromolecules and chitooligosaccharides. This means that before investigating each sample also must be characterized by its polydispersity. The controversy may also be caused by presence of by-products and variation in the chemical structure of end-units and acetyl-group distribution along polysaccharide chains due to the differences in the methods used for depolymerization of HMW chitosan.

In this paper, we describe a preparation of well-characterized oligochitosan samples varying in M_w and having a narrow polydispersity as well as their bactericidal activities against methicillin-resistant (MRSA) *S. aureus* mainly focusing on the M_w–pH-activity relationship. Also, we describe our first results of the self-assembling behavior of oligochitosan macromolecules in the dilute solution at various pH values as a function of chain length.

2. Materials and methods

2.1. Preparation of oligochitosan

Low molecular weight (LMW) chitosan (M_w 70 kDa, DD 80 mol%) used for preparation of oligochitosan samples was purchased from ALDRICH. LMW chitosan was hydrolyzed in hydrochloric acid at 70 °C. Oligochitosan hydrochloride was precipitated with ethanol and dried in vacuum over sodium hydroxide. The yield of oligochitosan hydrochloride was in the range of 20–90% depending on M_w of the final product.

2.2. Molecular weight and polydispersity

The weight-average (M_w), number-average (M_n) molecular weight, and polydispersity indexes (PI = M_w/M_n) of LMW chitosan and oligochitosan samples were determined by HP SEC method described in Lopatin, Derbeneva, Kulikov, Varlamov, & Shpigun (2009).

2.3. Degree of deacetylation

Degree of deacetylation (DD, mol%) was determined by ^{1}H NMR method (Hirai, Odani, & Nakajima, 1991).

2.4. Effective pK_a values

Effective pK_a values of oligochitosans were determined by potentiometric titration of oligochitosan hydrochlorides in accordance with the modified method published in Qin et al. (2006). Briefly, 50 ml of oligochitosan hydrochloride was dissolved (1 mg/ml) in distilled water and pH was adjusted by HCl to pH 3.00–3.05. The solution was titrated with 0.5 M NaOH while monitoring the solution pH. The equivalent inflexion points of titration curves were taken as pK_a of oligochitosan samples. All experiments were carried out in triplicate, and average values are shown in Table 1.

2.5. Dynamic light scattering (DLS)

DLS measurements were performed using PhotoCor Complex spectrometer (PhotoCor Instruments, Russia) equipped with pseudo cross-correlation system of photon counting and He–Ne laser as a light source (λ = 633 nm). The real-time correlator was employed in the logarithmic configuration. Measurements were performed in dilute solutions at 25 °C within the range of scattering angles of 30–140°. Distributions over decay time and hydrodynamic radius were obtained by means of CONTIN program. Apparent self-diffusion coefficients D were determined for each diffusive mode from angular dependence of the reciprocal relaxation time τ in accordance with the relation $D = 1/\tau q^2$, where $q = (4\pi n/\lambda)\sin(\theta/2)$ is wave vector. The corresponding hydrodynamic radii R_h were calculated from Stokes–Einstein relation:

$$R_h = \frac{kT}{6\pi\eta D}$$

where k is Boltzmann's constant, η is the solvent viscosity.

Sample solutions for DLS studies were prepared as follows: oligochitosan hydrochloride samples were dissolved in 0.1 M acetic acid (c = 3 mg/ml for sample 10, and 10 mg/ml for samples 3, 6 and 7), then the solution of 3 M tris(hydroxymethyl)aminomethane (TRIS) was added dropwise to oligochitosan solutions in order to adjust a desirable pH. Solutions were filtered through 0.22 μm pore-size Spartan nitrocellulose membranes. At critical pH value, 0.8 μm Millipore mixed cellulose ether membranes were used. No aging effect was observed in solutions except for the solutions corresponding to the critical points.

2.6. Transmission electron microscopy (TEM)

Transmission electron microscopy was performed with a LEO912 AB OMEGA electron microscope. The samples were prepared using 0.5–1% solution of oligochitosan hydrochlorides in 0.1 M acetic acid–TRIS buffer. A droplet of sample solution was deposited on Formvar-coated copper grid and dried for 1 min, then the excess of the solution was blotted off. The staining solution (1%

Table 1
Characteristics of oligochitosan samples.

Sample number	$M_w \pm 0.03$ (kDa)	PI ± 0.08	DP[a]	DD ± 1 (mol%)	p$K_a \pm 0.1$
1	0.73	1.41	4	95	7.1
2	1.52	1.39	8	93	6.7
3	2.09	1.40	12	97	6.6
4	3.58	1.71	20	95	6.5
5	4.22	1.38	24	97	6.5
6	6.40	1.56	39	97	6.5
7	9.69	1.44	56	97	6.4
8	12.80	1.39	74	95	6.4
9	15.10	1.61	87	94	6.4
10	20.00	1.66	116	98	6.4

[a]Average degree of polymerization was calculated in accordance with DD values.