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Antifungal activity of oligochitosans (short chain chitosans) against some *Candida* species and clinical isolates of *Candida albicans*: Molecular weight–activity relationship

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ABSTRACT

A series of oligochitosans (short chain chitosans) prepared by acidic hydrolysis of chitosan and characterized by their molecular weight, polydispersity and degree of deacetylation were used to determine their anticandidal activities. This study has demonstrated that oligochitosans show a high fungistatic activity (MIC 8–512 μg/ml) against *Candida* species and clinical isolates of *Candida albicans*, which are resistant to a series of classic antibiotics. Flow cytometry analysis showed that oligochitosan possessed a high fungicidal activity as well. For the first time it was shown that even sub-MIC oligochitosan concentration suppressed the formation of *C. albicans* hyphal structures, cause severe cell wall alterations, and altered internal cell structure. These results indicate that oligochitosan should be considered as a possible alternative/additive to known anti-yeast agents in pharmaceutical compositions.

1. Introduction

The ascomycete yeast *Candida* is the most common cause of opportunistic mycosis worldwide.

Among *Candida* species, *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida kruisei*, *Candida scotti*, and *Candida guilliermondii* are the most widespread ones. *Candida* species can be found in the oral cavity, throat, gastrointestinal tract, vagina, nails, and skin, and they are responsible for most (>91%) yeast infections with *C. albicans* being their major cause [1]. Over the last decades, the predominant pathogenic *Candida* species have changed, and infections caused by non-albicans *Candida* species have grown increasingly [2,3]. Conversely, the prominence of non-albicans, such as *C. kruisei*, *C. parapsilosis* and *C. tropicalis*, has increased their antibiotic resistance bringing challenges for effective antifungal therapy [4,5].

Moreover, misuse of antibiotics and the growing number of diseases caused by fungi not susceptible to antibiotics have increased problems with human allergy and have become a big problem globally [6], especially taking into account that there are a limited number of antifungal drugs [3]. Therefore, there is a need for new non-toxic fungicides which would be active against invasive and noninvasive human pathogens and could, at least, reduce the level of administration and side effects of classic antibiotics [7].

Chitin, poly[β-(1 → 4)-2-acetamido-2-deoxy-D-glucose], is the most abundant among the natural amino polysaccharides and represents a constituent of the cell walls of most fungi, algae, insects and crustaceans. Term "chitosan" belongs to a group of polysaccharides consisting of glucosamine and *N*-acetylglucosamine or glucosamine only, which are derived from chitin and are soluble in an acidic aqueous media. Conditionally, chitosans can be distinguished by their molecular weight (MW): high molecular weight chitosan (HMW), low molecular weight (LMW) chitosan,

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and oligochitosan (short chain chitosan). Although the boundaries between these groups are fluid, the term "oligochitosan" can used for chitosan molecules with fewer than 100 glucosamine units, i.e. MW ≈ 16 kDa [8]. These types of chitosan have found several applications in food and pharmaceutical industries, mainly due to its high biodegradability and antimicrobial properties [9–12].

Many reviews of antimicrobial activities of chitosan and oligochitosan against bacteria, fungi and viruses have been published [9,10,12–19]. As a result of these investigations, chitosan and oligochitosan were shown to be non-toxic to higher organisms and to be biocompatible [8–12]. In addition, they provide synergistic/additive effects in combination with antibiotics, indicating that chitosan and oligochitosan can be used to enhance the antimicrobial activity of pharmaceuticals [22–27].

As a result of numerous investigations, it was shown that variation in molecular weight leads to two different mechanisms of chitosan and target microorganism interaction: the first – adsorption of chitosan onto cell walls leading to cell walls covering, membrane weakening, disruption and cell leakage – is mainly connected with a high molecular weight (HMW) chitosan; the second – penetration of chitosan into living cells leading to the inhibition of various enzymes and disruption of proteins synthesis, interfacing with the synthesis of mRNA – is mainly connected with a low molecular weight (LMW) chitosan [13,15,16,28–36].

Yeast-like fungi, including *Candida* species, are also susceptible to the impact of different types of chitosan. Unfortunately, these results have concerned the anti-yeast activity of HMW and LMW chitosans having MW > 32 kDa so far [24,26,37–39].

Although from the practical point of view, oligochitosans (short chain chitosans) have several advantages over HMW and LMW chitosans; nevertheless, the interaction of *Candida* species, including *C. albicans* clinical isolates, with oligochitosan has been described [40,41].

In this paper, we describe the activity of well-characterized oligochitosan samples varying in MW and having a narrow polydispersity against *Candida* species, including clinical isolates of *C. albicans*, mainly focussing on the MW–activity relationship, hyphae formation and morphological changes of *C. albicans* cells.

2. Results

2.1. Oligochitosan samples preparation and characterization

There are several chemical methods for deep depolymerization of chitosan including acidic and enzymatic depolymerization as well as that by means of nitrous acid (sodium nitrite), periodic acid (sodium periodate), and hydrogen peroxide. To prepare a set of oligochitosan samples differing in molecular weights we used the most suitable method for depolymerization of LMW chitosan, namely the depolymerization of chitosan by hydrochloric acid. This method was chosen since a) it does not require the usage of either an expensive purified form of chitosanolytic enzyme or a cheaper complex of chitosanolytic enzymes whose usage in turn requires separation of oligochitosan from the enzymatic dirt and lead to enzyme type-dependent antimicrobial activity of oligochitosan [8,15,42,43]; b) its impact on the chemical structure of chitosan is minimal in comparison with the method that uses hydrogen peroxide [44,45]; c) the product does not have reactive aldehyde end-groups capable of producing Schiff-bases and other by-products which are formed during depolymerization of chitosan by means of nitrous acid (sodium nitrite) or periodic acid (sodium periodate) [46–48]. The method of depolymerization of chitosan in hydrochloric acid solution is highly specific towards the cleavage of glycosidic bonds between two conjunct *N*-acetylglucosamine units and between *N*-acetylglucosamine–glucosamine ones and doesn't modify chitosan chemically [49,50]. After the hydrolysis, oligochitosan hydrochlorides were analysed by HP–HPLC and ^{1}H NMR methods to determine their M_w, M_n and DD. Partial deacetylation was also observed and, therefore, the degrees of deacetylation of oligochitosans obtained after the acidic hydrolysis (Table 1) were higher (DD 93–99 mol.%) than that of the parent chitosan (DD 80%) in accordance with the previously published data [51]. As a result, eight oligochitosan samples having MWs in the range of 0.73–19.99 kDa and low polydispersity were obtained. The physicochemical characteristics of oligochitosan samples used in this investigation are shown in Table 1.

2.2. Susceptibility of Candida species towards oligochitosans

Candida species obtained from the ATCC and RCM collections were used to examine their susceptibilities toward oligochitosans. It was shown that MICs of oligochitosans were significantly dependent on oligochitosan MWs (Table 2). As the MW increased in the range 0.73–19.99 kDa, MICs decreased 8–70 folds rounding up, but increased in the case of LMW and HMW chitosans. *C. albicans* ATCC 90028 was found to be the least sensitive to MW of oligochitosan. Its MIC reduced twice from one oligochitosan sample to another, and the reduction was about 8 folds in this MW interval so that the MIC 256 μg/ml was found in the range of 15.06–19.99 kDa.

Compared to *C. albicans*, the other *Candida* species were more sensitive to MW of oligochitosan. Their MICs decreased with increasing MW from 0.73 to 5.98 kDa and were minimal in the range between 8.30 and 19.99 kDa. As a result, oligochitosan showed a high antimicrobial activity against *Candida* species used in these investigations, and cell growth was completely inhibited by 0.0016–0.025% (16–256 ppm) concentrations (rounding down) of oligochitosan having MW in the range of 6–20 kDa. On the other hand, oligochitosans with MW 0.73–2.09 kDa were ineffective against all *Candida* species with the exception of *C. scotti*. In general, their effects were found to be species-dependent with *C. albicans* being the strain with lowest sensitivity to the oligochitosans tested.

2.3. Micrographic analysis

The morphological changes induced by oligochitosan were examined using Transmission Electron Microscopy (TEM). *C. albicans* ATCC 90028 cells treated with ¼ MIC oligochitosan (M_w 9.69 kDa) were fixed, and TEM micrographs were recorded (Fig. 1). The micrographs of treated cells showed disturbance in *C. albicans* cell walls after the treatment with oligochitosan even at its sub-MIC concentration. TEM observations provided clear evidence of morphological and structural alterations of fungal cells. The micrographs showed that untreated oval cells (Fig. 1a) had a normal smooth surface, whereas after treatment with oligochitosan (Fig. 1b) severe cell wall alterations appeared: outer cell wall layers were puffier and inner cell wall layers were thinner than intact ones. Besides, oligochitosan caused changes in the internal cell

Table 1
Physicochemical characteristics of oligochitosan samples.

Sample number	M_w ±0.05 kDa	M_w/M_n	Average DP[a]	DD ±1 mol.%
1	0.73	1.41	4	95
2	1.52	1.39	8	93
3	2.09	1.40	12	97
4	5.95	1.22	34	96
5	8.39	1.61	48	95
6	9.69	1.44	56	97
7	15.06	1.61	87	94
8	19.99	1.66	116	98

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

Table 2
MICs of oligochitosans and chitosans towards *Candida* species.

Candida species	Oligochitosan, M_w (kDa)										Control, cells/ml × 10^{-5}[b]
	0.73	1.52	2.09	5.98	8.39	9.69	15.06	19.99	70[a]	600[a]	
C. albicans	≥2048	≥2048	≥2048	1024	512	512	256	256	1024	≥2048	5.0 ± 0.5
C. kruisei	≥2048	≥2048	1024	256	64	64	64	32	64	1024	5.5 ± 0.5
C. parapsilosis	≥2048	≥2048	1024	128	32	32	32	32	128	1024	5.0 ± 0.5
C. tropicalis	≥2048	≥2048	512	128	32	32	32	32	64	512	4.0 ± 0.5
C. scotti	256	256	64	32	16	16	16	16	16	128	5.0 ± 0.5
C. glabrata	≥2048	≥2048	512	62	32	32	32	32	64	512	5.0 ± 0.5

[a] M_w.
[b] Control without oligochitosan (chitosan).

structure – the amount of vacuoles increased and the nucleus was hardly distinguished. After prolonged exposure to lethal oligochitosan concentrations, cells disruption, lyses and release of intercellular components also occurred.

2.4. Membrane permeability and cells viability

The changes in the integrity of the *C. albicans* ATCC 90028 cytoplasmic membranes and cells viability after treatment with oligochitosan were characterized by flow cytometric analysis using PI as a DNA binding dye that cannot permeate undamaged cell membranes and FDA as a cell binding dye. When the cell membranes were damaged, PI permeated into the cell cytoplasm and bound to DNA increasing fluorescence of cells by 20–30 times [52].

Flow cytometry showed that after the incubation of *C. albicans* cells with oligochitosan (M_w 9.69 kDa) used in the amount of 512 μg/ml, the number of living cells was reduced from 99,6% (in control) to 7.9% (Fig. 2, top). About 40% of survived cells had damaged cytoplasmic membranes, and the membranes of 87.7% dead cells were found damaged as well. The perturbation of cytoplasmic membrane permeability was observed as a result of cell membrane damage caused by oligochitosan, the corresponding permeation of PI into the cell cytoplasm, PI binding to DNA and corresponding 17-fold increase of the cells fluorescence (Fig. 2, bottom). The proportions between intact and damaged initial cells (control) and living, dead and damaged cells (oligochitosan) are shown in Table 3. The results testified that oligochitosan caused cell membrane disintegration as well as cytoplasmic components leakage as it was shown for *Saccharomyces cerevisiae* membrane perturbation by a LMW chitosan [53].

2.5. Antihyphal activity of oligochitosan

The outstanding attribute of *C. albicans* is its ability to produce a diversity of morphological forms, ranging from unicellular budding yeast (blastospores) to hyphae structures (pseudo-hyphae and true hyphae) [54]. The conversion of *C. albicans* to the filamentous stage (pathogenic) starts with the production of germ tubes that results in the formation of hyphal structures [55]. This morphological transformation ability has been suggested as an important factor for *C. albicans* virulence [56,57].

The influence of oligochitosan on the morphology of *C. albicans* ATCC 90028 cells was investigated by fluorescent microscopy. It was found that in the liquid SGB medium the strain formed pseudo mycelium. Fig. 3a,b shows typical examples of hyphae and unicellular structures in control experiments (a) and the structure of agglomerated yeast-cells in the presence of ¼ MIC concentration of MW 9.69 kDa oligochitosan where hyphal structures are absent (b). The suppression of hyphae structures formation by oligochitosans varied in MW depending on oligochitosan MIC values was also investigated (Table 4). It was found that the degree of hyphal structure formation depended on MW and concentration of oligochitosan. Actually, the samples with MW from 0.73 to 2.09 kDa showed inhibition degrees of 35–55% even at 1/2 MIC concentrations. The complete suppression of hyphal structure formation occurred when samples having MW in the range of 8.30–19.99 kDa were used in concentrations of 1/2–1/8 MIC. A significant

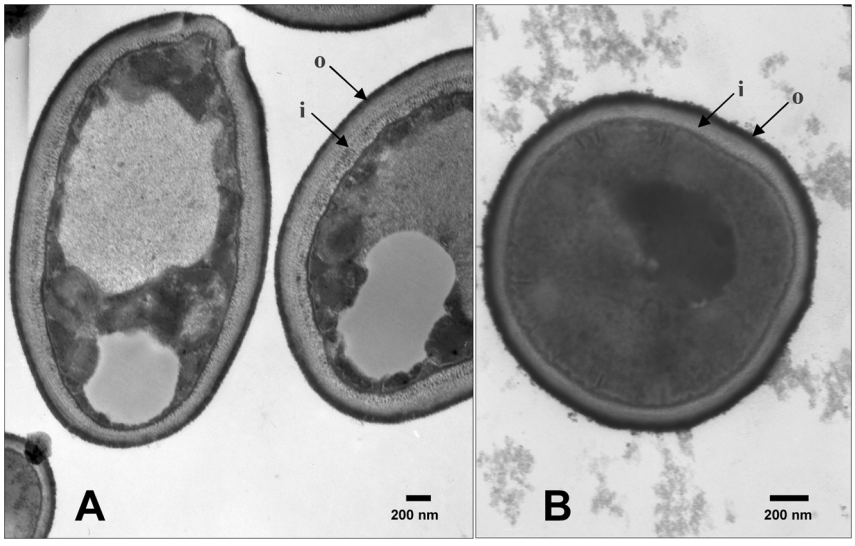

Fig. 1. TEM micrographs of *C. albicans* ATCC 90028 cells: A – intact cells; B – cell treated with ¼ MIC 9.69 kDa oligochitosan. Arrows show: o – outer layer, i – inner layer.

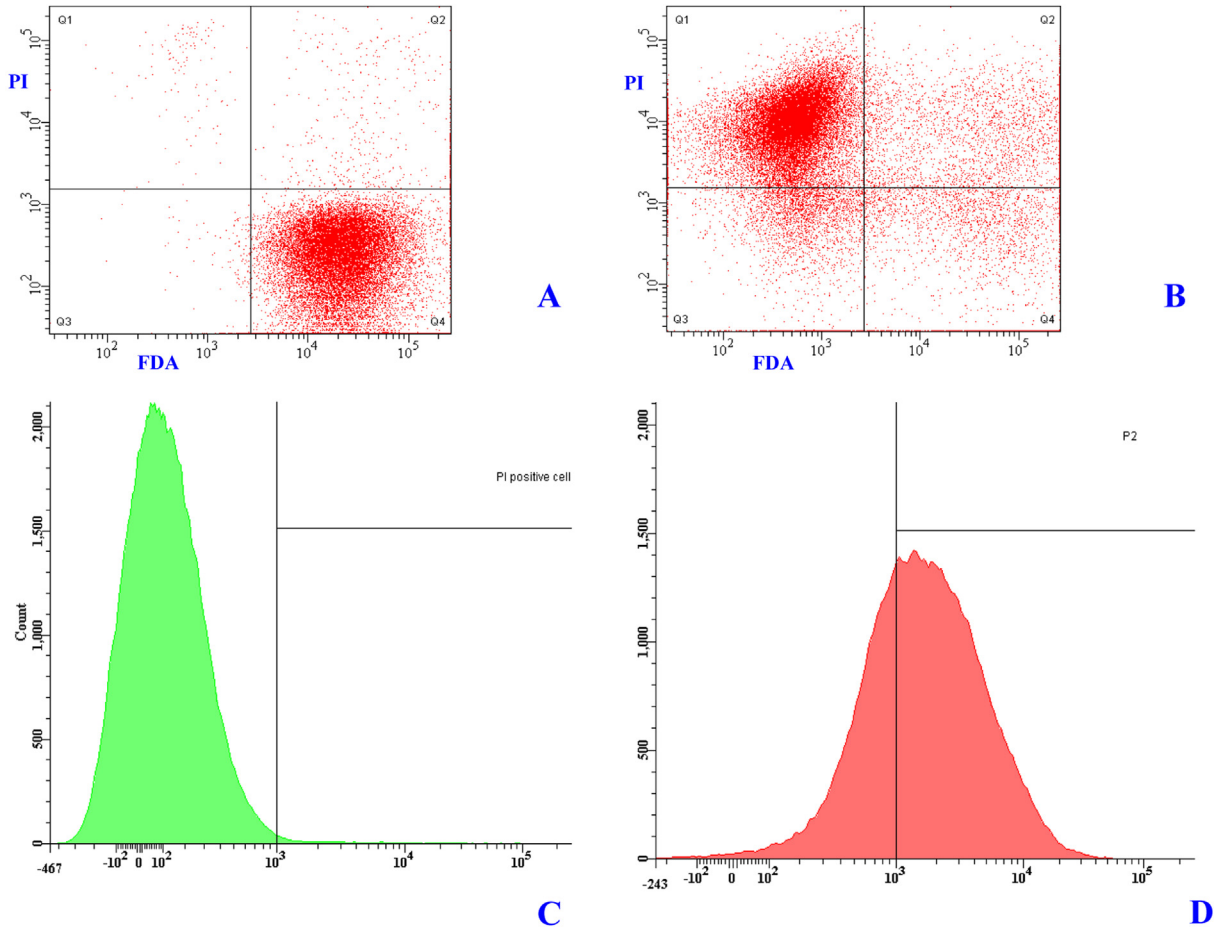

Fig. 2. Flow cytometric analysis of *C. albicans* cells viability. A and B – cells stained with PI and FDA; C and D – cells stained with PI. A and C – control, B and D – cells treated with (9.69 kDa, 512 g/ml) oligochitosan. A, B: *X*-axis – FDA fluorescence intensity; C, D: *X*-axis – PI fluorescence intensity; A, B: *Y*-axis – PI fluorescence intensity; C, D: *Y*-axis – count cells.

Table 3
Compositions of intact and treated cells.

Cell status	Intact cells, %	Treated cells, %
Living cells	98.6	4.8
Membrane-damaged living cells	1.0	3.1
Membrane-damaged dead cells	0.4	80.9
Membrane-intact dead cells	0.0	11.2

difference was also found between HMW chitosan (Mw 600 kDa) and oligochitosans (Table). Oligochitosans whose MW were in the range of 8.30–19.9 kDa showed much higher antihyphal activity in the suppression of hyphal formation at 1/8–1/64 MIC concentration in comparison with LMW chitosan and HMW chitosan (MW 70 and 600 kDa, accordingly), while the lowest oligochitosans were practically ineffective. Also, the data showed that oligochitosans at lower concentrations (1/2–1/32 MIC) significantly inhibited hyphal structure formation if their MW $>$ 5 kDa.

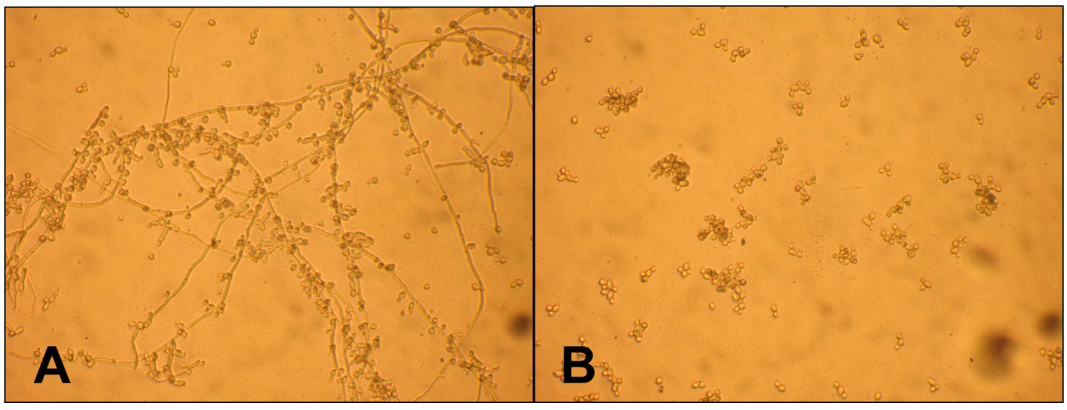

Fig. 3. Micrographs of *C. albicans* ATCC 90028 untreated (a) and treated (b) with ¼ MIC oligochitosan (M_w 9.69 kDa).

Table 4
Molecular weight and concentration dependences of antihyphal activity of oligochitosans (corresponding MIC values are shown in Table 2).

Sub-MIC	Number of hyphal structures in the presence of oligochitosan (M_w, kDa)									
	0.73	1.52	2.09	5.98	8.39	9.69	15.06	19.99	70[a]	600[a]
1/2	38 ± 7	35 ± 8	24 ± 5	0	0	0	0	0	0	0
1/4	72 ± 6	71 ± 8	49 ± 7	7 ± 5	0	0	0	0	0	17 ± 6
1/8	74 ± 6	70 ± 8	70 ± 7	16 ± 6	0	0	0	0	15 ± 7	22 ± 6
1/16	72 ± 8	72 ± 10	70 ± 8	34 ± 8	17 ± 6	9 ± 3	4 ± 3	4 ± 3	20 ± 7	55 ± 7
1/32	75 ± 8	72 ± 10	73 ± 7	54 ± 6	31 ± 5	28 ± 6	24 ± 5	24 ± 5	59 ± 7	71 ± 7
1/64	72 ± 6	72 ± 8	70 ± 6	68 ± 5	55 ± 7	56 ± 7	56 ± 8	57 ± 5	65 ± 8	72 ± 7
1/128	70 ± 7	72 ± 12	72 ± 10	77 ± 12	76 ± 13	70 ± 4	74 ± 5	73 ± 8	67 ± 7	72 ± 6
1/256	72 ± 10	73 ± 9	76 ± 5	75 ± 10	70 ± 7	70 ± 7	71 ± 7	70 ± 7	67 ± 9	73 ± 5
Control	70 ± 12	75 ± 12	75 ± 11	73 ± 6	73 ± 11	70 ± 9	70 ± 8	75 ± 9	70 ± 5	75 ± 8

[a] M_w.

2.6. Susceptibility of clinical C. albicans isolates

Thirty-six clinical *C. albicans* isolates were separated from different parts of the human body, including oral cavity (10), genital tract (10), skin (4), nails (9), and auricle (3). These isolates associated with *C. albicans* infections were preliminary tested towards a series of classic antibiotics, and the series was divided into three groups in accordance with their resistances: highly resistant strains (marked "R"), moderately resistant (marked "I"), and sensitive (marked "S"). Afterwards, susceptibilities of the isolates towards three oligochitosan samples differing in MW were determined. As a result, it was found that these isolates were more or less susceptible to oligochitosan application. Most of these isolates, including the highly resistant ones, were inhibited within the concentration range from 8 to 64 μg/ml when 9.69 kDa and 19.99 kDa oligochitosans were applied (Table 5). Although oligochitosan of lower M_w

Table 5
Oligochitosan MICs and fungicide susceptibilities of *C. albicans* clinical isolates: highly resistant strains – R, moderately resistant – I, and sensitive – S.

Body side	Isolate number	NYS[a]	KET[b]	FLU[c]	TER[d]	INT[e]	CLO[f]	PIM[g]	Oligochitosan M_w and MIC, μg/ml		
									2.09 kDa	9.69 kDa	19.99 kDa
Oral cavity	2882	I	I	I	R	I	I	I	256	32	32
	2425	I	I	S	S	S	S	S	512	64	32
	2899	S	I	R	R	R	S	S	256	32	32
	2429	S	I	I	S	I	I	I	64	8	8
	2568	S	I	S	S	S	S	S	128	16	16
	2422	S	I	S	S	S	S	S	128	16	16
	2597	S	R	S	S	I	S	I	128	8	8
	2243	I	S	I	S	I	S	I	512	64	64
	2515	S	I	I	I	S	I	I	512	256	128
	2236	I	R	I	S	I	I	I	256	128	64
Genital tract	2306	S	R	I	I	S	R	S	256	32	32
	2320	S	I	S	I	S	I	I	512	32	32
	2326	S	I	S	S	S	S	S	521	256	256
	2368	S	I	S	I	S	S	S	128	16	16
	2559	I	I	I	I	I	I	I	64	8	8
	2453	R	R	R	R	S	I	I	64	8	8
	2350	I	I	I	I	I	I	I	512	32	32
	2296	I	I	S	I	I	I	I	128	16	16
	2450	S	R	R	R	R	R	I	256	64	64
	2519	I	I	I	I	I	I	I	256	64	64
Auricle	2226	S	S	S	S	S	S	S	16	8	8
	2315	S	I	S	S	S	S	S	128	32	32
	2324	S	S	S	S	S	S	I	32	8	8
Skin	2859	S	S	S	S	S	S	S	64	8	8
	2845	S	S	S	S	S	S	S	256	64	32
	2844	S	I	S	S	S	S	S	512	512	256
	2834	S	S	S	S	S	S	S	64	8	8
Nails	2554	S	S	S	S	S	S	S	32	8	8
	2638	S	I	S	S	S	S	S	64	8	8
	2624	S	R	I	I	I	I	S	64	8	8
	2888	S	S	S	S	S	S	S	64	8	8
	2467	S	S	S	S	S	S	S	64	8	8
	2401	S	I	S	S	S	S	S	64	8	8
	2472	S	S	S	S	S	S	S	64	8	8
	2404	S	I	I	S	S	S	S	64	16	8
	2613	S	S	S	S	S	S	S	128	16	16

[a] Nystatin.
[b] Ketoconazole.
[c] Fluconazole.
[d] Terbinafine.
[e] Itraconazole.
[f] Clotrimazole.
[g] Pimaricin.

(2.09 kDa) were also active against all clinical strains, their MICs were found to be higher (64–512 μg/ml).

3. Discussion

3.1. Uncertainty in the chitosan MW–activity relationship

From the practical point of view, oligochitosan has several advantages over HMW and LMW chitosans: a) oligochitosan has better absorption profiles, oligochitosan can be considered as safe, non-toxic candidate for pharmaceutical and biomedical applications, b) oligochitosan is much more soluble in aqueous media and it forms solutions of much lower viscosity; c) oligochitosan is more compatible with surfactants, stabilizers and emulsifiers, sugars, 60% ethanol, salt, glycerin, organic acids and colorants, and therefore has no undesirable impact on the physicochemical properties of consumer goods; d) application of oligochitosan in pharmaceutical, cosmetic and food products does not interfere with current industrial technologies [8,10,12,17,19–21,27].

As a result of several investigations it is commonly accepted that antimicrobial activity of various types of chitosan depends on its MW and DA, target microorganism, and experimental conditions (pH, concentration, incubation medium, method of chitosan sample preparation, etc.). Regarding DA, the activity increases with increasing DD. In the case of MW–activity relationship, contradictory data regarding a correlation between antifungal activity and chitosan molecular weight has been discussed [58,59]. Thus, it was shown in some studies that the increase in chitosan molecular weight led to the decrease in biocidal activity of chitosan [31,60–63]. In the others, an increased activity of HMW chitosans in comparison with low molecular weight chitosans was found [43,64–68]. Only ones a bell-like dependence of fungistatic activity versus MW was found [58], where oligochitosans having MW in the range 5–10 kDa possessed maximal activity in comparison with lower and higher oligochitosans and LMW chitosans.

In our opinion, the contradictory results found in the activity–MW relationship has been observed mainly because of the reasons described above and because most investigators have used chitosan samples of differing in MW, DA, polydispersity (PI), and preparation methods [31,60–68]. In other words, the differences in biological effects reported for antimicrobial action of different chitosan samples can also be caused by the presence of different types and quantities of lowest and highest chitooligosaccharides in the samples. The contradiction may also be caused by the presence of by-products and variation in the chemical structure of terminal groups of polysaccharide chains and acetyl-group distribution along oligochitosan chains due to the differences in methods used for hydrolysis of chitosan. Consequently, there is good reason to believe that molecular heterogeneity is the main reason for an ambiguity in the experimental data on correlation between biocidal activities and physicochemical characteristics of different types of chitosan. This means that before investigating every chitosan sample must be characterized at least by its molecular weight, degree of acetylation, and polydispersity [58,59].

The published data concerning the biocidal activity of oligochitosan against *Candida* species, including *C. albicans*, contain results on the relationship between MW and activity of chitosan that are as contradictious as the data for other fungi and bacteria. Moreover, LMW chitosan (MW 70 kDa, DD >75%) at concentration 5000 mg/ml showed an important inhibitory effect against *C. albicans* ATCC 64550. LMW chitosan was found active against other *Candida* species, including clinical isolates, and had MICs in the range of 2–2500 mg/ml [26]. On the other hand, two LMW chitosans (MW 32 and 38 kDa, DD 94 and 88%, respectively) and two HMW chitosans (MW 138 and 184 kDa, DD 84 and 86%, respectively) showed low activities (MIC ~1.2–3.2 mg/ml) towards *C. albicans* ATCC 10231 [37]. The concentrations of HMW chitosan (DD 85%, MW unknown) and LMW chitosan (DD 84.5%, MW unknown) providing 80% inhibition of the growth of *C. albicans* DSM 11225, *C. kruisei* ATCC 6258 and *C. glabrata* DSM 11226, were in the range of 1.86–6.97 mg/ml [22]. Negligible differences were found between HMW and LMW chitosans regarding their activities towards *C. kruisei* and *C. glabrata*, while HWM chitosan was found 4-fold more active towards *C. albicans* in comparison with LMW chitosan. Chitosan oligosaccharides (SIGMA–ALDRICH, MW < 5 kDa) tested in this work had a very low activity [38].

Fraction of oligochitosans (MW 1 and 3 kDa, DD and PI unknown) showed low activity (MIC 1.56 mg/ml) against *C. albicans* (strain unknown), while oligochitosan (MW 5 and 10 kDa, DD and PI unknown) showed a high activity (MIC <0.04 mg/ml) against this yeast strain [40]. The absence of statistical difference in activities between three enzymatically produced oligochitosans (DD 85%) with MW 5, 12 and 27 kDa (MIC 100 μg/ml) towards *C. albicans* ATCC 885653 was found, while other samples with MW 6 kDa and 15.7 kDa had MICs twice lower (MIC 50 μg/ml) or sixth times higher (MIC 600 μg/ml), respectively. On the other hand, all these samples were surprisingly inactive against *C. kruisei* RCM Y-2594 [41].

A series of well-characterized chitosan and oligochitosan samples with different MW prepared by depolymerization with hemicellulose was used to determine the activity towards *C. albicans*, and it was shown that two oligochitosans (M_w 1.4 kDa, PI 1.52 and M_w 2.8 kDa, PI 1.61) and HMW chitosan (M_w 400 kDa, PI 4.12) were not active against a hospital strain of *C. albicans*. On the other hand, the activity of LMW chitosans (PI 2.91–3.69, DD 85–86%) taken in the concentration of 300 μg/ml increased in the order 48–78 > 17 > 130 kDa, and they could completely inhibit the growth of *C. albicans* at the concentration of 1400 μg/ml [68].

As reviewed above, in most cases, chitosan samples were not characterized by PI and DD (DA) values, and only few oligochitosan samples were used in many earlier studies. Therefore, there was a lack of data on biocidal activity of a wide range of oligochitosan samples differing in chain lengths against *C. albicans*, especially the clinical isolates. The basic question which should be clarified first is what molecular weight oligochitosan must have in order to possess the highest activity against *Candida* species?

3.2. Plausible reasons in the differences in oligochitosan MW–anticandidal activity relationship

In the present study we have used a series of well-characterized oligochitosan samples and demonstrated that oligochitosan possesses both fungistatic and fungicidal activities. Suppression of hyphal structure formation by oligochitosan (as well as chitosan) was reported for the first time, although the mode of action is still unclear. Actually, cell adhesion and conversion of *C. albicans* from unicellular form to the filamentous stage, which are the most important determinants of pathogenesis, and many regulatory aspects of the adhesion and conversion to the filamentous stage remain unclear so far. Nevertheless, it should be noted that several adhesins responsible for biofilms formation have been identified for *Candida* species so far. Thus, *C. albicans* cells surfaces can express the adhesin Als1p, which binds specifically to fucose- and glucosamine-containing glycans and plays an important role in the adhesion to host cells and in the development of candidiasis [69,70]. Also, proteins known as septins have been found in *C. albicans* during the development of hyphal structures from yeast mother cells [71]. In addition, some mannoproteins form part of the extracellular matrix in *C. albicans* biofilms and play a role in the adhesiveness of biofilms to surfaces [72]. Presently, we hypothesize that oligochitosan may suppress the expression of extracellular

adhesin(s), interfere with a regulatory mechanism responsible for germ tube development and hyphal growth initiation, and may represent a possible binding site for *C. albicans* extracellular adhesin(s).

In this investigation we tested the susceptibility of a series of clinical isolates of *C. albicans* towards the members of three types of antifungals belonging to azoles (4), polyenes (2) and allylamines classes (1) of systemic drugs currently used in clinical practice. These antifungals affect biosynthesis of ergosterol, the major sterol in fungal cell membranes but not present in animals, nucleic acid and cell wall biosynthesis [3]. Since the resistance of *C. albicans* to these antifungals spreads and increases, there is a need for new non-toxic substances targeting on cells rigidity, permeability, and proliferation, cells adhesion to animal organs, germ tube development, hyphal growth initiation, and suppression of fungal adhesin expression. In this study, oligochitosan with MW 9.69 and 19.99 kDa showed high activities (MIC < 100 μg/ml) against most of *C. albicans* isolates cross-resistant to many antifungals. Some strains were more resistant towards the action of oligochitosans with MW 9.69 and 19.99 kDa (>100 μg/ml) and among them three strains had MIC >250 μg/ml. Plausible reasons in differences in anti-candidal activity of the samples used in this investigation may be caused by differences in sample cell wall adsorption and cell membrane penetration abilities as well as by differences in sample interactions with outer and inner cell components. Also, cellular surface charge can influence chitosan interaction with cell walls of target organisms: the larger the negative cell surface charge is the higher its binding ability to positively charged chitosan macromolecules is [73,74]. This was confirmed for a series of *C. albicans* mutant cells, where the degree of phosphomannan loss and concomitant reduction in surface negative charge was correlated with the reduced level of peptide binding to the cells and with the corresponding reduction of fungicidal action of the cationic antimicrobial peptide – dermaseptin [75].

4. Materials and methods

4.1. Samples preparation and characterization

LMW chitosan (M_w 70 kDa, DD 80%) and HMW chitosan (M_w 600 kDa, DD 75%) were purchased from ALDRICH.

Oligochitosan samples were prepared following the protocol described in Ref. [59]. Briefly: LMW chitosan was dissolved in 1 M hydrochloric acid, and the solution heated at 70 °C for 1÷6 h. Oligochitosan hydrochloride was precipitated with ethanol (1:3 v/v) and dried in vacuum over solid sodium hydroxide. The yield of oligochitosan hydrochloride was in the range of 20–90% depending on MW of the final product. Oligochitosan stock solutions were filtrated through a 0.22 μm pore-size syringe filter (Millipore, Swinnex) and stored at 4 °C until usage.

Weight-average (M_w) and number-average (M_n) molecular weight characteristics were determined by high performance size exclusion chromatography (HP-SEC) using procedure published in Ref. [76]. Agilent 1200 Series Chromatography system equipped with an isocratic pump, refractive index detector, and PL-OH mixed column was applied at 25 °C, buffer 0.2 M CH_3COOH/0.15 M CH_3COONH_4 was used as an eluent. A series of monodispersed pullulans (Fluka) with M_p from 1.08 to 710 kDa and D-glucosamine were used as calibration standards.

Degree of deacetylation (DD, mol.%): ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) was used to determine degrees of acetylation (DA, mol.%) of LMW chitosan and oligochitosans in accordance with the method described in Ref. [77].

DD was calculated as follows: DD (%) = 100% – DA%.

Table 6
Antibiotic susceptibility groups.

Antibiotic	Inhibition zone diameter, mm		
	R	I	S
Nystatin	≤13	13÷17	≥18
Ketoconazole	≤19	20÷25	≥26
Fluconazole	≤19	20÷28	≥29
Terbinafine	≤13	14÷18	≥19
Itraconazole	≤13	14÷18	≥19
Clotrimazole	≤7	7÷11	≥12
Pimaricin	≤13	14÷18	≥19

4.2. Candida species and clinical isolates

Candida species: *C. albicans* ATCC 90028, *C. kruisei* RCM 251, *C. parapsilosis* RCM Y-1345, *C. tropicalis* RCM Y-61, *C. glabrata* ATCC 90030, and *C. scotti* RCM 0736 were obtained from the American Type Culture Collection (ATCC) and Russian Collection of Microorganisms (RCM). Clinical *C. albicans* isolates were obtained from the Mycological Laboratory of Kazan Scientific Research Institute of Epidemiology and Microbiology. All isolations were identified biochemically by carbohydrate assimilation tests (Candifast, International Microbe, France), and the identification was completed by micromorphological study on chromogenic medium (CHROMagar Candida, France) [78]. The isolated yeasts were grown and stored on Sabouraud Dextrose Agar (SDA, Merck, Darmstadt, Germany) at 4 °C. Inocula for the assays were prepared by dilution of the harvested cell suspension in 0.85% NaCl solution, and cell counting was done using Haemocytometer. Cell concentration was then adjusted to $(4.0 \pm 0.5) \times 10^4$ cells/ml using the above-mentioned saline solution.

4.3. Flow cytometry: membrane permeability and cell survival assays

C. albicans ATCC 90028 cells at the mid-log phase were centrifuged at 1000 rpm for 5 min and then resuspended in 0.02 M MES–Na buffer (pH 7.4) containing 0.5% NaCl. The final concentration: 4×10^5 cell/ml. Cells were treated with 512 μg/ml oligochitosan (9.69 kDa) for 2 h at 30 °C. After the treatment, cells were stained with 10 μg/ml propidium iodide (PI) (Sigma) for 10 min and 10 μg/ml fluorescein diacetate (FDA) (Sigma) for 5 min at room temperature prior to cytometry analysis. Flow cytometry was performed with a FACSCanto II Flow Cytometer (Becton Dickinson, USA).

4.4. Antibiotics susceptibility of clinical C. albicans strains

The agar-based assay for the susceptibility of isolates of *C. albicans* strains to various antifungals was carried out in accordance with the method described in Ref. [79]. Commercially available filter (9 mm in diameter; Scientific Research Centre of Pharmacotherapy, Russia) were preloaded with nystatin (80 U/disk), ketoconazole (20 μg/disk), fluconazole (40 μg/disk), terbinafine (100 μg/disk), itraconazole (10 μg/disk), clotrimazole (10 μg/disk), pimaricin (100 μg/disk). *C. albicans* isolates were cultured on SGA at 35 °C for 24 h. Then, yeast cells were suspended in 5 mL of sterile physiological serum and thoroughly vortexes to achieve a smooth suspension with optical density (OD) from 0.08 to 0.1 at the wavelength 600 nm (0.5 McFarland's standard turbidity). Cell suspension was placed on a sterile cotton swab (90 mm in diameter) containing 0.5 μg/ml Methylene blue. The discs containing antibiotics were placed on the swabs and incubated at 35 °C, and zones of inhibition around the disks were measured after 24 h (slowly growing isolates were additionally incubated for 24 h) [80].

The isolates were conditionally divided into three groups (Table 6) depending on their antibiotic susceptibility: S – sensitive, I – intermediate, R – resistant (Recommendations of the Scientific Research Centre of Pharmacotherapy, Russia).

4.5. Minimal inhibitory concentration (MIC)

MICs of oligochitosan samples were determined by a 2-fold microdilution assay in accordance with the modified method described in Ref. [81]. A stock oligochitosan solution (8.192 mg/ml) in distilled water was sterilized by means of filtration through a 0.22 μm pore-size syringe filter (Millipore, Swinnex) and stored at 4 °C until usage. Briefly: a set of 2-fold serially diluted oligochitosan solutions in Sabouraud glucose broth (SGB) adjusted with 0.05 M Ac–Na buffers (pH 5.7) to a final volume of 200 μl were prepared in a sterile 96-well polystyrene microtiter plate. An inoculation loop with the yeast cell material was mixed with SGB and shaken for 24 h with 200 rpm. The probes (100 μl) containing $(3.0 \pm 0.1) \times 10^3$ cfu/ml were applied to each well of the plate. The plate with *C. albicans* was incubated at 35 °C at shaking (200 rpm) and monitored at 600 nm after 48 h incubation by an ELISA reader (Biolog, Hayward, CA). The corresponding control experiments were carried out without oligochitosan application. All experiments were carried out in triplicates. MIC was defined as the lowest concentration required to suppress cells multiplication.

4.6. Hyphal structures formation

Suspensions of *C. albicans* cells were incubated with and without oligochitosan at 30 °C for 48 h, and then 20 μl suspensions were placed on a glass plate and dried. Unicells and hyphal structures were observed and counted by means of a light microscope.

4.7. Light microscopy

Micrographs of *C. albicans* ATCC 90028 treated with oligochitosan (M_w 9.69 kDa) as well as the untreated cells were captured using a MICMED-6 light microscope (Lomo, Saint Petersburg, Russia) 48 h after the treatment.

4.8. Transmission electron microscopy (TEM)

C. albicans ATCC 90028 cells treated with ¼ MIC oligochitosan concentration as well as the untreated control cells were collected, and TEM studies were carried out as described in Ref. [34]. Micrographs were captured using a JEm-100 CXII transmission electron microscope (SPSS, Chicago, IL).

4.9. Confocal fluorescent microscopy

β-Glucan and chitin layers of the cell walls were visualized using Calcofluor White (SIGMA) as described in Ref. [82] and captured using a JEOL 1200EX microscope at 80 kV using UV light DAPI filter.

5. Conclusion

In our investigations a series of oligochitosan samples appropriately characterized by their molecular weight, polydispersity and degree of deacetylation have been prepared and used to determine their anticandidal activities. This study has demonstrated that oligochitosans showed a high fungistatic activity (MIC 8–512 μg/ml) against *Candida* species and clinical isolates of *C. albicans*, which are resistant to a series of standard antibiotics. Flow cytometry analysis showed that oligochitosan also possessed a high fungicidal activity. Oligochitosans with MW between 10 and 20 kDa displayed maximal activity in suppressing yeast cells multiplication and caused severe cell wall alteration. Plausible reasons in differences in anticandidal activity of the samples used in this investigation may be caused by differences in sample cell wall adsorption and cell membrane penetration abilities as well as by differences in sample interactions with outer and inner cell components.

For the first time it was shown that even sub-MIC oligochitosan concentrations suppress *C. albicans* hyphal structure formation. Although the mode of antimicrobial action of chitosan and its derivatives has been discussed in many cited papers, we speculate that the mechanism of oligochitosan activity in the suppression of *C. albicans* ability to form hyphal structures may significantly differ from that of oligochitosan bactericidal activity. We suggest that the mechanisms responsible for antihyphal activity of oligochitosan are connected with the differences in the cell surface charge and level of adhesin(s) and septin(s) expression between treated and intact *Candida* cells, but this suggestion must be confirmed experimentally. Taking into account that the antimicrobial action of chitosan is aimed at cell walls weakening and penetration, interfering with DNA transcription, mRNA function, and protein synthesis, oligochitosan in combination with antifungals may facilitate cell walls permeability by standard antibiotics and play an important role in suppression of fungal pathogenicity. In addition, the discovered effect of suppression of hyphal structure development by oligochitosan can be used for the creation of pharmaceutical compositions capable of reducing the pathogenic potential of *C. albicans* and also other *Candida* species. In our opinion, in order to appropriately recognize the mode of oligochitosan action on the suppression of *C. albicans* hyphae structures development, multiple factors, such as the impact of oligochitosan on the suppression of germ tube development, hyphal initiation and expression and bonding of fungal adhesin(s), must be considered, i.e. further work is necessary to explain the molecular mechanism(s) underlying the sensitivity of *C. albicans* and other *Candida* strains to oligochitosan and to evaluate the role of oligochitosan as a putative anticandidal agent.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.12.017.

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