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Antimicrobial activity of piperazine derivatives of chitosan

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ABSTRACT

Well characterized methylpiperazine, mono-quaternary dimethylpiperazine and di-quaternary trimethylpiperazine derivatives of chitosan, with different degrees of substitution, were investigated for antibacterial activity against five strains of Gram-positive and Gram-negative bacteria. Some of the methylpiperazine and the dimethyl piperazine derivatives of chitosan, with low degree of substitution, were active against bacteria at pH 5.5 with minimum inhibitory concentration (MIC) $\geqslant$64 μg/ml. Chitosan derivatives with the di-quaternary substituents were active against bacteria with MIC as low as 8 μg/ml and in general more active at pH 7.2 than at pH 5.5. The minimum lethal concentrations (MLC) were the same as the MIC within 1–2 dilutions. The most active compound induced gradual decrease in the count of viable bacteria over 8 h at 8× MIC. The results are consistent with the interpretation that methylpiperazine and mono-quaternary dimethylpiperazine substituents do not contribute to activity against bacteria, whereas di-quaternary trimethylpiperazine moiety, will contribute to antibacterial activity.

1. Introduction

Chitosan, a cationic polysaccharide composed of β-1 → 4 linked D-glucosamine and *N*-acetyl-D-glucosamine units, is active against Gram-positive and Gram-negative bacteria (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitosan can therefore be used as film-forming agent and additive to improve the shelf-life of food products (Devlieghere, Vermeulen, & Debevere, 2004). Antibacterial properties can also be important when chitosan is considered as a pharmaceutical excipient (Illum, 1998), as a permeation enhancer (Schipper et al., 1997) or as a hemostatic agent in wound dressing (Burkatovskaya et al., 2006). It is generally accepted that the polycationic chitosan can adsorb to negatively charged cell membranes, which will then lead to decrease in the osmotic stability of the cell and leakage of intracellular constituents (Rabea et al., 2003; Devlieghere et al., 2004). However, the exact mechanism of the antimicrobial action is still unknown and several other mechanisms that may contribute to the antimicrobial action have also been suggested, such as the formation of an impermeable coat on the bacterial surface (Zheng & Zhu, 2003), uptake of low molecular weight chitosans that will interact with electronegative substances in the cell (Zheng & Zhu, 2003), and inhibition of bacterial growth through chelation of trace metals (Rabea et al., 2003). The mechanism for the interaction may be different for Gram-positive and Gram-negative bacteria. For example, it has been reported that there is a positive correlation between the molecular weight (M_w) of chitosan and activity against the Gram-positive bacteria, *Staphylococcus aureus*, whereas the correlation is negative for Gram-negative bacteria (Zheng & Zhu, 2003). Other studies have, however, found that M_w of the material has little effect on activity against these organisms (No, Park, Lee, & Meyers, 2002). Helander and colleagues (Helander, Nurmiaho-Lassila, Ahvenainen, Rhoades, & Roller, 2001) have reported that chitosan disrupts the barrier function of the outer membrane of Gram-negative bacteria. Chitosan may therefore sensitize Gram-negative bacteria to bacteriolytic agents, although there is no direct bactericidal effect at low concentrations.

The pK_a for native chitosan is around 6.5 and the ionization of the polymer therefore only occurs under acidic conditions. Antibacterial activity of chitosan has been linked to the cationic charge. For example, it has been shown that native chitosan is significantly more active against Gram-negative and Gram-positive bacteria at pH 4.5 than at pH 5.9 (Rabea et al., 2003). Many studies have been conducted on the antibacterial activity of native chitosan, but concentrations reported to be required for activity against the same specific target organism can vary up to 500-fold (Rabea et al., 2003). This concentration may depend on the degree of polymerization and deacetylation but assay conditions such as pH of the growth medium, and presence or absence of substances such as trace metal ions, lipids and criteria used to define activity, will also have considerable effect.

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The main limitation of chitosan for biomedical applications is its low activity and solubility at neutral or alkaline conditions. Various derivatives of chitosan have therefore been investigated as potential substitutes for chitosan with enhanced properties. Derivatives with fixed cationic charge, such as *N*,*N*,*N*-trialkyl (Avadi et al., 2004; Jia, Shen, & Xu, 2001), *N*-[(2-hydroxy-3-trimethylammonium)propyl] (Kim, Lee, Lee, & Park, 2003; Lim & Hudson, 2004a, 2004b; Qin et al., 2004), dialkylaminoethyl (Lee, Lim, & Kim, 2002) have been reported and investigated for antimicrobial activity and other cytotoxic activity. Neutral and zwitter-ionic derivatives have also been investigated for the antibacterial effect (Kurita, Shimada, Nishiyama, Shimojoh, & Nishimura, 1998; Muzzarelli et al., 1990; Peng, Han, Liu, & Xu, 2005; Tikhonov et al., 2006). In general it has been reported that these derivatives with high density of quaternary amino groups are effective against bacteria, whereas some neutral derivatives such as hydroxypropyl chitosan have been found to be inactive (Peng et al., 2005). Antibacterial activity has therefore been linked to cationic charge density on the polymer (Jia et al., 2001; Peng et al., 2005). However, it is difficult to compare activities reported in these studies as the assay conditions have not been identical. Different species of bacteria have been used and the strains within each species may vary considerably. Thus it is not clear what type of modification will give most active material and how activity can be further improved. Recently we have reported on the activity chitosan-*N*-betainates (Holappa et al., 2004, 2006a). These were investigated for activity against *S. aureus* and *Escherichia coli*, strains from the ATCC (American Type Culture Collection), representing clinically important species recommended by the NCCLS (now CLSI) as quality control strains for susceptibility testing. Minimum Inhibitory and Minimum Lethal concentrations (MIC and MLC) were determined according to NCCLS standard assays (NCCLS, 2003). Our investigation showed that introducing the cationic betainoyl group (*N*-1-carboxymethyl-2-trimethylammonium) on the 2-amino group of chitosan is negatively correlated with activity. Using the same assay conditions, we have shown that activity of *N*,*N*,*N*-trimethylation of chitosan is positively correlated with activity at pH 7.2, whereas the correlation is negative at pH 5.5 (Rúnarsson et al., 2007). Thus it is apparent that the activity of quaternary amino derivatives of chitosan is not only dependent on the charge but also on the chemical structure.

In the current investigation we have studied the antibacterial activity of piperazine derivatives of chitosan (*N*-1-carboxymethyl-2-(piperazinyl)chitosan). The synthesis procedure for piperazine derivatives with mono-, di- or tri-methylation of amino groups of the piperazinyl moiety has previously been reported (Holappa et al., 2005, 2006b). Antibacterial assays were conducted as before according to the CLSI (formerly NCCLS) standard methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (CLSI, 2006), using Gram-negative and Gram-positive bacteria, both sensitive and resistant strains representing clinically important species. Killing curves for the most active material are also reported.

2. Experimental section

2.1. Materials

Synthesis and detailed NMR characterization of *N*-1-carboxymethyl-2-(4-methylpiperazinyl) chitosan, *N*-[1-carboxymethyl-2-(1,4-dimethylpiperazinium)] chitosan chloride, *N*-[1-carboxymethyl-2-(4,4-dimethylpiperazinium)] chitosan chloride and *N*-[1-carboxymethyl-2-(1,4,4-trimethylpiperazi-1,4-dium)] chitosan dichloride, with different degrees of substitution, have previously been reported (Holappa et al., 2005, 2006b).

2.2. Antibacterial testings

The CLSI standard method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically was used (CLSI, 2006) for MIC testing and counting of viable cells.

2.3. Organisms

The organisms used were *S. aureus* ATCC 29213 and ATCC 43300, *E. coli* ATCC 25922, *Streptococcus pneumoniae* ATCC 4961, ATCC 6306 and ATCC 6303, *Enterococcus faecalis* ATCC 29212 and *Pseudomonas aeruginosa* ATCC 27853. The strains were kept frozen in tryptose-glycerol freezing media (Difco Laboratories, Detroit, MI) at −80 °C, cultured and then subcultured once on 5% horse blood agar (Oxioid, Hampshire, UK) prior to the testing.

2.4. Culture medium

Mueller–Hinton broth (Oxoid) at pH 7.2 with divalent cations Ca^{2+} and Mg^{2+} was adjusted by the manufacturer. The Mueller–Hinton broth was also adjusted with HCl (5 N) to pH 5.5 for measurements at the lower pH. The pH was measured with a PHM220 Lab pH meter MederLab (Radiometer, Copenhagen, Denmark). For testing of *S. pneumoniae* the broth was supplemented with 5% defibrinated sheep blood. For plating, bacterial count and minimal lethal concentration measurements, blood agar with 5% horse blood was used.

2.5. Microdilution

The chitosan derivatives were weighed and dissolved in Mueller–Hinton broth at pH 7.2 and pH 5.5 to obtain a concentration of 8192 μg/ml. Then 50 μl of this solution was added to the first two wells on a microtiter plate and 2-fold dilutions were done in 50 μl Mueller–Hinton broth from well two.

The final range (after addition of the inoculum) was from 4096 to 2 μg/ml, giving the possibility of reporting ⩾8192 μg/ml as the highest result.

2.6. Inoculum

The inoculum was made by direct suspension of colonies in Mueller–Hinton broth. It was adjusted to the turbidity of MacFarland standard 0.5, or approximately $1–2 \times 10^8$ CFU/ml. The suspension was then diluted 1:100 ($1–2 \times 10^6$) with the aim to achieve 5×10^5 CFU/ml in the test, as the diluted suspension is further diluted 1:2 when 50 μl of the diluted suspension was added to 50 μl chitosan derivatives, creating a 2-fold dilution in the microtiter wells.

2.7. Incubation

The microtiter plates were incubated at 35 °C in a sealed plastic bag in ambient air for 16–20 h.

2.8. MIC determination

MIC was determined according to the standard, as the lowest concentration [of an antimicrobial agent] that prevents visible growth of a microorganism in a broth dilution susceptibility test.

2.9. MLC determination

MLC was determined after reading the results for the MIC. Blood agar was plated with two times 10 μl of all the dilutions that showed no growth, and incubated at 35 °C in ambient air for 16–

20 h. MLC was determined as the lowest concentration that achieved a 99.9% decrease of viable bacteria.

2.10. Quality control

Growth control was 50 μl of Mueller–Hinton broth without chitosan derivatives, but inoculated as the tests. Sterility control was 100 μl of Mueller–Hinton broth. Performance control was ceftriaxone 32 μg/ml diluted 2-fold as the other substrates in the test. Viable cell count was done by making 10-fold dilutions of the inoculum in saline. Two times 10 μl of the dilutions were plated on blood agar, incubated in the same conditions as the tests and the colonies counted. The results of the counts were then used to calculate the 99.9% killing of the bacteria, which is the criterion for MLC. The criterion for acceptance of results for measurements of bacterial activity was that all controls should be within acceptable limits.

2.11. Killing curves

Five milliliters suspension of the chitosan derivatives were made in their 8- or 16-fold MIC in Mueller–Hinton broth and inoculated with the bacterial strains; approximately 5×10^7 CFU/ml. Suspensions were made without the chitosan derivatives, but with the same strains and in the same media, for testing normal growth curves of the organisms. They were incubated in ambient air at 35 °C. Viable cell count was performed immediately and after 1, 2, 4 and 8 h. Killing curves and normal growth curves were plotted and compared.

3. Results and discussion

The compounds were prepared in a five- to six-step synthesis procedure, involving protection and deprotection of the chitosan 2-NH_2 and 6-OH groups, as described earlier (Holappa et al., 2005, 2006b). Structures and molecular weights (M_w) and polydispersity (M_w/M_n) are given in Table 1. Compounds **1a–c** are substituted with *N*-1-carboxymethyl-2-(4-methylpiperazinyl) moieties, which is the common basic structure for the derivatives. Compounds **2a–c** are mono-quaternized by methylation on the *N*-1 position on the piperazinyl ring system, whereas compounds **3a–c** are mono-quaternized by methylation on the *N*-4 position. Compounds **4a–c** are di-quaternized by methylation on the *N*-1 and *N*-4 positions. The average M_w was in the range of 17–67 kDa. The chitosan starting material had molecular weight average of 201 kDa but the synthesis procedure resulted in significant degradation of the polymer backbone, as has previously been observed for similar multi-step synthesis procedures (Holappa et al., 2006a, 2005; Kurita et al., 1998).

3.1. Antimicrobial activities

Gram-positive *S. aureus* ATCC 29213, and Gram-negative *E. coli* ATCC 25922 were used in the screening of all compounds. Some typical bacterial strains that are more resistant to antibiotics, such as the Gram-positive *E. faecalis* ATCC 29212, the Gram-negative *P. aeruginosa* ATCC 27853 and the Methicillin resistant *S. aureus* (*MRSA*) strain, ATCC 43300, which has altered penicillin-binding proteins, were also used in the assay of all compounds (Table 2).

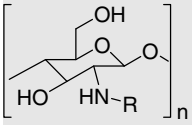
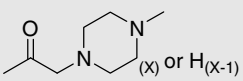
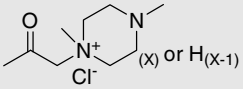
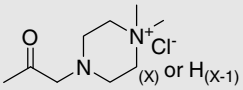
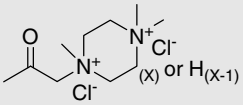
In the current study we used an approved and well known standard for measurements of MIC and MLC values (CLSI, 2006).

In the standard method, the pH of the bacterial broth is 7.2. Unmodified chitosan is not soluble at this pH (Rabea et al., 2003) and therefore was generally inactive. Investigations of the antimicrobial activity of chitosan are therefore usually done under acidic conditions (pH < 6). In this work, we have therefore also used the recommended broth, with pH adjusted to 5.5. The same conditions were also used in the previous investigations of the antibacterial activity of chitosan betainates (Holappa et al., 2006a) and *N,N,N*,-trimethylchitosan (Rúnarsson et al., 2007).

Growth rates of *S. aureus*, *E. coli* (Holappa et al., 2006a) and *P. aeruginosa* (Gudmundsson, Erlendsdottir, Gottfredsson, & Gudmundsson, 1991) are not affected by this pH change.

Compounds **1a–c** are *N*-1-carboxymethyl-2-(4-methylpiperazinyl) derivatives of chitosan. The second pK_a of piperazine is 9.8 (Weast, Aslte, & Beyer, 1985), and these compounds are therefore expected to be protonized and cationic at pH 5.5 and at pH 7.2. These compounds were the least active in the series but they showed some activity, *S. aureus* ATCC 29213, *E. faecalis* and *P. aeruginosa* at pH 5.5, but were inactive at pH 7.2. These results indicated that the charge on the protonized 2-amino group on the chitosan polymer backbone is necessary for activity and that the 4-methylpiperazinyl group does not contribute to activity. Compounds **2a–c**, were 1-amino group of the 4-methylpiperazinyl moiety, methylated and thus quaternary, were somewhat active at pH 5.5 against *S. aureus* ATCC 2913 and *E. faecalis*. The cationic charge is placed further away from the polymer backbone in the (mono-) quaternary compounds **3a–c**, and the range in the degree of substi-

Table 1
Piperazine derivatives of chitosan used in the study

General structure	Compound	R	Degree of substitution (X)	Weight average molecular weight (M_w)	Polydispersity index (M_w/M_n)
	1a		0.3	42,900	1.73
	1b		0.6	35,000	2.09
	1c		0.9	49,400	2.03
	2a		0.40	67,400	2.23
	2b		0.46	39,200	1.92
	2c		0.85	34,200	1.57
	3a		0.15	48,000	2.01
	3b		0.42	34,500	1.98
	3c		0.87	28,600	1.66
	4a		0.34	31,400	1.78
	4b		0.54	26,300	1.55
	4c		0.65	17,500	1.56

tution is larger than for the other compounds. In this case, the compound **3c** with high degree of substitution (*ds* = 0.87) was inactive, compound **3b** (*ds* = 0.42) had some activity, whereas compound **3a** (*ds* = 0.15) was active against *S. aureus* ATCC 29213 at pH 5.5 and 7.2, *E. faecalis* and *P. aeruginosa* at pH 5.5 with MIC equal to 64, 4096, 256 and 512 μg/ml, respectively. The results for compounds **1**–**3** are similar to what has been previously observed for chitosan *N*-betainates. With chitosan *N*-betainates, the activity is inversely correlated with the degree of substitution in these compounds. Chitosan *N*-betainates with low degree of substitution (*ds* ⩽ 0.15) have similar activity against *S. aureus* ATCC 2913 as compound **3a** and unmodified chitosan (Holappa et al., 2006a). Chitosan *N*-betainates and chitosan are active against *E. coli*, whereas the compounds **1**–**3** in the current series were not active. Activity is mostly limited to pH 5.5 which suggest that the antimicrobial activity of these compounds is due to protonization of the 2-amino group in the polymer backbone. The derivatives with a high degree of substitution are less active because amino groups are blocked by derivation with the cationic group. Cationic charge is therefore not a sufficient condition for antimicrobial activity.

In compounds **4a**–**c** the amino groups of the piperazinyl moiety are trimethylated and the substituent is therefore di-quaternary. The lowest MIC values for theses compounds at pH 5.5 were 16, 128, 4096 and 256 μg/ml for *S. aureus* ATCC 29213, *E. faecalis*, *E. coli* and *P. aeruginosa*, respectively. These compounds were more active than other compounds in the current series at this pH. The MIC and MLC range for each condition was within a relatively narrow range of the 1–2 dilutions and thus the degree of substitution seemed to have limited effect on the activity of these compounds. The di-quatenary compounds were, in general, more active at pH 7.2 than at pH 5.5. Compounds **4a**–**c** were active at pH 7.2 against *S. aureus* ATCC 4330, with MIC and MLC in the range 32–128 μg/ml and *E. faecalis*, with MIC in the range 512–2048 μg/ml, whereas other compounds in the series were inactive. Similar results have been obtained for *N*,*N*,*N*-trimethylated chitosan, where trimethylation contributed to activity against *S. aureus* ATCC 29213 at pH 7.2, but has the opposite effect at pH 5.5 (Rúnarsson et al., 2007).

Previously we have reported that the MIC for the activity of unmodified chitosan, against *S. aureus* ATCC 29213 is in the range 64–256 μg/ml and 1024–2048 μg/ml at pH 5.5 and 7.2, respectively (Holappa et al., 2006a). Unmodified chitosan is also active against *E. coli* ATCC 25922 with MIC in the range 128–256 μg/ml and 1024–2048 μg/ml at pH 5.5 and 7.2, respectively. Thus compounds **1**–**3** were found to be less active against *S. aureus* than unmodified chitosan, whereas compounds **4a**–**c** were more active. The modification did not contribute to activity against *E. coli.* As discussed in the introduction section various derivatives with fixed cationic charge, such as *N*,*N*,*N*-trialkyl chitosan or neutral and zwitter-ionic derivatives, have also been investigated for the antimicrobial effect. Many have been found to be active against microorganisms, but the methods used vary considerably. Often the MIC values have not been reported, determined according to procedures that vary from the CLSI standard or the bacterial species or strains used are different from the recommended quality control strains for susceptibility testing reported here. Thus we can only directly compare the results of the current investigation of piperazine derivatives with our previous investigation of *N*,*N*,*N*,-trimethylchitosan (Rúnarsson et al., 2007) and chitosan betainates (Holappa et al., 2006a). Highly substituted *N*,*N*,*N*-trimethylchitosan, with 69%, trimethylation has similar activity against *S. aureus* ATCC 29213, as compound **4c** with MIC equal 256 μg/ml and 8 μg/ml at pH 5.5 and 7.2, respectively. Chitosan betainates are less active than unmodified chitosan as mentioned above.

Compounds **3a** and **4a**–**c**, which showed some activity against *S. aureus* at pH 7.2, were further tested against three

Table 2
Activity of the compounds against five strains of bacteria

Compound	*S. aureus* ATCC 29213				*S. aureus* ATCC 43300				*E. faecalis* ATCC 29212				*E. coli* ATCC 25922				*P. aeruginosa* ATCC 27853			
	pH 5.5		pH 7.2		pH 5.5		pH 7.2		pH 5.5		pH 7.2		pH 5.5		pH 7.2		pH 5.5		pH 7.2	
	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC[μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]
1a	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	256	512	⩾8192	⩾8192	2048	4096	⩾8192	⩾8192	512	⩾8192	⩾8192	⩾8192
1b	512	512	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	2048	2048	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
1c	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
2a	512	1024	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	1024	1024	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
2b	2048	4096	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
2c	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
3a	64	64	4096	4096	⩾8192	⩾8192	⩾8192	⩾8192	256	256	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	512	⩾8192	⩾8192	⩾8192
3b	1024	1024	4096	4096	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	4096	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
3c	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192
4a	16	32	16	16	⩾8192	⩾8192	128	128	128	128	1024	1024	⩾8192	⩾8192	4096	4096	256	⩾8192	⩾8192	⩾8192
4b	32	64	8	8	⩾8192	⩾8192	32	64	256	512	512	1024	4096	4096	⩾8192	⩾8192	512	⩾8192	⩾8192	⩾8192
4c	64	128	8	8	⩾8192	⩾8192	64	64	128	1024	2048	⩾8192	⩾8192	⩾8192	⩾8192	⩾8192	2048	⩾8192	⩾8192	⩾8192

Table 3
Activity of selected compounds, at pH 7.2, against 3 strains of *S. pneumoniae*

Compound	*S. pneumoniae* ATCC 49619		*S. pneumoniae* ATCC 6306		*S. pneumoniae* ATCC 6303	
	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC [μg/ml]	MIC [μg/ml]	MLC[μg/ml]
3a	Unreadable	**256**	Unreadable	4	Unreadable	128
4a	Unreadable	128	Unreadable	⩽1	Unreadable	256
4b	Unreadable	128	Unreadable	⩽1	Unreadable	32
4c	Unreadable	128	Unreadable	⩽1	Unreadable	128

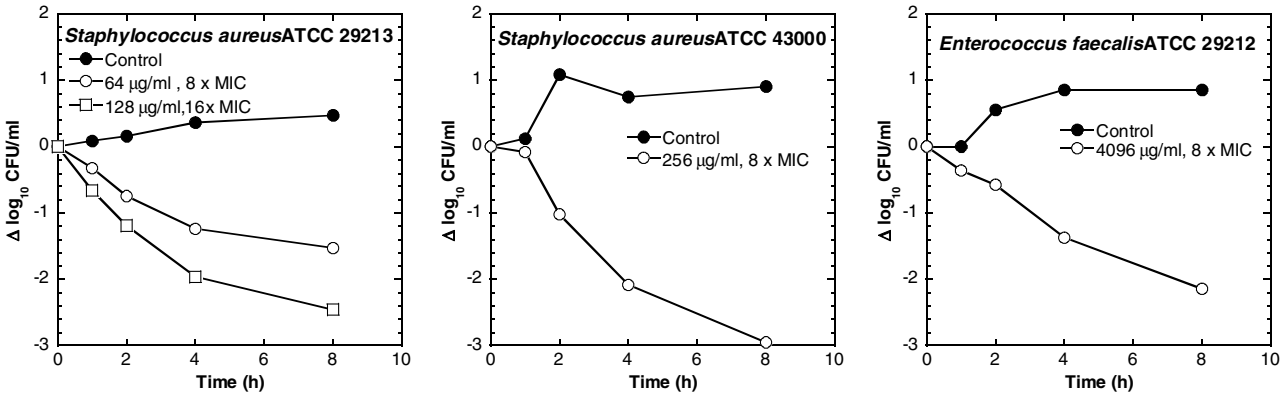


Fig. 1. Killing curves for compound **4b**.

strains of *S. pneumoniae* (Table 3). Chitosan derivatives could be considered for local treatment of inner ear infections which are commonly caused by this bacteria. The combination of the chitosan derivatives with *S. pneumoniae* caused some precipitation in the growth medium, also when bacteria was effectively killed. The MIC could therefore not be read, but MLC could be determined. All the compounds where effective against the three strains of *S. pneumoniae*.

In general, the MLC values were the same as the MIC values or within 1–2 dilutions. This indicates that these materials are bacteriocidal rather than bacteriostatic. The most active material, compound **4b**, was further investigated to determine the kill rate, with different bacterial strains of Gram-positive bacteria (Fig. 1). This compound was tested at 8× MIC and 16× MIC against the sensitive *S. aureus* ATCC 29213. In both cases, there is a gradual kill of the bacteria with a slightly increased rate at the higher concentration. This compound is also similarly effective against the *MRSA* ATCC 43300 strain. A similar pattern is also observed for the kill of *E. faecalis* at 8× MIC.

4. Conclusion

Chitosan derivatives with *N*-[1-carboxymethyl-2-(4-methylpiperazinyl)], *N*-[1-carboxymethyl-2-(4,4-dimethylpiperazinium)] or *N*-[1-carboxymethyl-2-(1,4-dimethylpiperazinium)] substitution on the 2-amino group on the polymer backbone can be active against bacteria at low pH when degree of substitution is low. These results are consistent with the interpretation that the main contribution to activity is the protonization of the free amino groups in the polymer backbone and that, in this case, substitution will reduce activity. Chitosan derivatives with the *N*-[1-carboxymethyl-2-(1,4,4-trimethylpiperazi-1,4-dium) substituents can be highly active against various strains of Gram-positive bacteria and are generally more active at pH 7.2 than at pH 5.5. The di-quaternary group contributes to the activity of these compounds.

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