Synthesis and screening of N-acyl thiolated chitosans for antibacterial applications

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Abstract

Low-molecular weight chitosan-thioglycolic acid has shown significant antibacterial properties against different microorganisms. In order to explore the potential and structure-activity relationships of newly synthesized alkyl thiomers, chitosan has been functionalized with a series of thio-acids with increasing alkyl chain length. All thiomers were characterized with special emphasis on the determination of their degree of deacetylation and substitution, as well as on their molecular weight and amount of thiol groups. The pre-screened chitosan-thiomers were

further investigated with plate counting on *Pseudomonas aeruginosa*, *Streptococcus sobrinus* and *Streptococcus mutans*. Furthermore, LIVE/DEAD assays supported the efficiency of chitosan-thiomers against the above microorganisms. All fully characterized chitosan-thiomers showed comparable or enhanced antimicrobial activity compared to pristine chitosan. Our comprehensive approach paves the way to detailed explorations of much sought-after structure activity relationships in the complex chitosan parameter room, starting from correlations between alkyl chain length and antimicrobial activity.

Keywords

Chitosan-thiomers, structure-activity relationship, antimicrobial agents, microbiological characterization

1. Introduction

Chitosan is a polymer of β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, which is obtained from deacetylation of chitin as the second most abundant biopolymer worldwide (Rinaudo, 2006) and an inexpensive side product of seafood industry (Ravi Kumar, 2000). The antibacterial properties of chitosan keep attracting increasing research interest, especially with respect to its high potential for application in health technology (Anitha et al., 2014; Jayakumar, Prabakaran, Sudheesh Kumar, Nair, & Tamura, 2011), agriculture (El Hadrami, Adam, El Hadrami, & Daayf, 2010), and pharmaceuticals (Illum, 1998).

Although numerous studies clearly established the efficiency of chitosan against different bacterial strains (No, Park, Lee, & Meyers, 2002; Sette-de-Souza et al., 2015), its mechanisms of action are still intensely debated. The following hypotheses are currently prevailing: (a) chelation of essential metal ions (Jassal & Raut, 2013), (b) cellular penetration by low molecular weight chitosan and chitooligomers followed by inhibition of protein synthesis through attachment to

nucleic acids (Liu, Song, Li, Li, & De Yao, 2007), and, most widely accepted, (c) destruction of bacterial cell wall structures (Liu, Du, Wang, & Sun, 2004; Raafat, von Bargaen, Haas, & Sahl, 2008; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Additionally, a combination of the above pathways was proposed (Li et al., 2013).

Concerning the structure-activity relationship (SAR) of chitosan, manifold studies indicate that the molecular weight (MW) and the degree of deacetylation (DD) are among the most influential parameters (Omura et al., 2003; Younes, Sellimi, Rinaudo, Jellouli, & Nasri, 2014). However, the impact of MW on the antimicrobial activity appears to be different for gram-negative and gram-positive bacteria (No et al., 2002; Seo, Mitsuhashi, & Tanibe, 1992). While low MW (LMW)-chitosan has been reported as superior for eliminating gram-negative-bacteria, higher MWs exert stronger effects against gram-positive bacteria (Omura et al., 2003; Younes et al., 2014; Zheng & Zhu, 2003), probably due to the different cell wall types. Chitosans with high DD values display higher positive charge density of the protonated amine group in acidic media, which enhances their antimicrobial efficiency (Fernandes et al., 2010; Omura et al., 2003; Zheng & Zhu, 2003) mainly against gram-negative strains, and to a lower extent vs. gram-positive strains (Younes et al., 2014). Generally, pH is another key factor for antimicrobial chitosan activity, which is higher against many microorganisms at low pH (No et al., 2002; Park, Nah, & Park, 2011). Preceding studies in their entirety illustrate that further systematic elucidation of the correlations between chitosan properties and their resulting antimicrobial efficiency is crucial to fully understand the underlying mechanisms of action for future targeted optimization of chitosan derivatives.

Chitosan thiomers, i.e. functionalized chitosans with mercapto-substituents attached to the amine group, have recently been developed as drug carriers to enhance the interaction with cysteine residues in the mucosa, thereby increasing the retention times *in situ* (Kumar & Sinha, 2013; Sarti & Bernkop-Schnürch, 2011). Moreover, our previous studies revealed a strong effect

of chitosan-thioglycolic acid (TGA) on different microorganisms, including *Streptococcus sobrinus* (gram-positive bacteria), *Neisseria subflava* (gram-negative bacteria) and *Candida albicans* (fungus) (Geisberger et al., 2013). Interestingly, gram-positive bacteria, which are usually less sensitive toward LMW-chitosan and its derivatives, were selectively and efficiently attacked by LMW-chitosan-TGA, thus identifying this compound as a promising and versatile antimicrobial agent.

The present study aims to further investigate the SAR of antimicrobial chitosan derivatives through a systematic investigation of the antibacterial activity of different chitosan-thiomers with increasing alkyl chain length. While a modification of the lipophilicity of chitosan derivatives was reported to affect their antimicrobial properties (Huang, Du, Zheng, Liu, & Fan, 2004; Inta, Yoksan, & Limtrakul, 2014), the underlying structure-activity relationships still remain to be defined, as evident from previous results on quaternary alkyl chitosans. Studies of Kim and Choi and Wiarachai et al. described stronger antimicrobial activity for longer alkyl chains (Kim & Choi, 2002; Wiarachai, Thongchul, Kiatkamjornwong, & Hoven, 2012), while related work of Vallapa et al. demonstrated an opposite trend (Vallapa et al., 2011). Recently, Sahariah et al. showed that the antimicrobial activity of N-alkyl chain depended on the tested strain, so that no general SARs could be derived to date (Sahariah et al., 2015).

Consequently, further investigations on the effect of lipophilic substituents on the antibacterial activity of chitosan derivatives are required. We therefore applied a series of mercapto carboxylic acids with systematically increasing chain length (i.e. lipophilicity, including several constitutional isomers) for amide bond formation via carbodiimide activation. All of the mercapto carboxylic acid precursors are commercially available to facilitate possible large-scale production at a later stage. The following functionalized chitosan derivatives were isolated and characterized in detail: thioglycolic acid (TGA), thiolactic acid (TLA), mercaptopropionic acid (MPA), 2-

methyl-3-sulfanylpropanoic acid (MSPA), mercaptobutyric acid (MBA), mercaptohexanoic acid (MHA) and mercaptooctanoic acid (MOA) (Figure 1). Special emphasis is placed on the comprehensive analytical characterization of all chitosan thiomers in light of reliable SAR trends for drug design guidelines. Among the target compounds of this study, antibacterial activity tests have only been performed before for chitosan-TGA (Geisberger et al., 2013), which was particularly effective against *Streptococcus sobrinus*, and for chitosan-MPA (Radhakumary, Antonty, & Sreenivasan, 2011) that was combined with poly(N-isopropyl acrylamide) and ciprofloxacin to constitute films, wherein the biopolymers contributed no additional antimicrobial effect. To the best of our knowledge, all other members of the series are novel. Here, we report on the antimicrobial activity of the new chitosan thiomers against *Pseudomonas aeruginosa*, *Streptococcus sobrinus*, and *Streptococcus mutans*, applying complementary assay techniques.

Figure 1. Synthetic approach to new alkyl thiolated chitosans and survey of characterized and tested alkyl thiolated chitosans.

2. Experimental

2.1 Materials

Unless otherwise specified, reagents were purchased from Sigma-Aldrich (Buchs, Switzerland) and used as received. Chitosan with different DD and MW was purchased from Heppe Medical Chitosan (Halle (Saale), Germany). In the following they are referred to as 85/5, 85/10, 85/20, and 85/100, with the first number indicating the DD and the second number indicating the viscosity of the material at 1% w/v in 1% acetic acid at 20 °C measured in mPas. Thioglycolic acid (97%), mercaptopropionic acid (99%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC 98%, 99%, and crystalline) and tris(2-carboxyethyl)phosphine hydrochloride (TPH 99%) were purchased from abcr GmbH (Karlsruhe, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Alfa Aesar (Heysham, UK).

2.2 Thiomers synthesis

Published synthesis protocols (Hornof, 2003; Kast & Bernkop-Schnürch, 2001) were applied to achieve different degrees of substitution (DS) and characteristics. The conditions are summarized in Table S1 in the supporting information, and a representative procedure is described below in detail. 500 mg chitosan were dissolved in 4 mL 1 M HCl for 15 min, and 46 mL water were added to obtain a 1% (w/v) chitosan solution. After chitosan was completely dissolved, the functionalizing reagent was added to the solution. EDC was added after 10 min while maintaining the temperature of the system for the applied reaction time (cf. Table 2). The pH of the reaction was adjusted to 5. 300-400 mg reducing agent (TPH) were added during the final 30 min. The product was dialyzed in cellulose membrane tubing with a molecular cutoff of 12 kDa

in the dark at 4 °C against different media, as described below, for at least 75 h. The following media were used and changed twice a day: 5 mM HCl (day 1), 5 mM HCl (day 2 and day 3) + 1% (w/v) NaCl, and 1 mM HCl (day 4). The product was then freeze dried for 48 h and stored in the dark at -20 °C until further use. A chitosan sample processed without the addition of any reagents was used as a reference in the antimicrobial tests.

2.3 Characterization

2.3.1. ¹H-NMR

¹H-NMR spectra were recorded at 75°C on a 300 or 400 MHz Bruker Avance Spectrometer (Billerica, USA) with CP-MAS. The DD of chitosan and the thiomers was calculated according to *Hirai* (Hirai, Odani, & Nakajima, 1991). Samples were prepared in D₂O at a concentration of ≥ 10 mg/mL.

2.3.2. Zeta potential measurements

All samples with a concentration of 4 mg/mL PBS (pH 7.3) were analyzed with a Zetasizer Nano ZS90 (ZEN3690, Malvern Instruments Ltd, Malvern, UK) using Malvern disposable folded capillary cells.

2.3.3. Aqueous Size Exclusion Chromatography

MW determination was performed in acetate buffer (0.15 M, pH 4.6-4.7) using a Viscotek GPC Max system equipped with a VE 2001 GPC solvent/sample module (Malvern). The separation was achieved with one A Guard (Malvern) and two GMPWXL 08025 (Tosoh Bioscience LLC, King of Prussia, USA) columns.

The detectors used were Viscotek 270 Dual Detector for light scattering and viscosimetry measurements, Viscotek VE 3580 RI detector and Viscotek UV detector 2600 (Malvern) for refractive index and UV measurements, respectively. Methods were calibrated with

polyethyleneoxide and dextran Malvern standards. For each chitosan-thiomer weight average and number average MW were determined and the polydispersity index (PDI) calculated. Each sample was dissolved in the buffer within a concentration range of 4.5-5.5 mg/mL and filtered through a 0.22 μ m filter before injection.

2.3.4. Elemental analysis

Elemental analysis was performed on a Truespec CHNS-microanalyzer (LECO Corporation, St. Joseph, USA). To evaluate the DS of the chitosan-thiomers, the molar sulfur amounts were correlated with molar nitrogen contents, dividing their percentage in weight by their MW.

2.3.5. Thiol group determination

5,5'-dithiobis-(2-nitrobenzoic acid), namely DTNB or Ellman's reagent, was used to determinate thiol group concentrations according to literature protocols (Bravo-Osuna, Teutonico, Arpicco, Vauthier, & Ponchel, 2007). Cysteine (concentration range 75-350 μ M) was used as standard (calibration curve reported in Fig. S1). Absorbance at 405 and 450 nm was measured with a plate reader (Synergy 2 Multi-Mode Reader, BioTek Instruments, Winooski, USA).

2. 4. Antibacterial activity determination

2.4.1. Bacterial strains and media

Pseudomonas aeruginosa (ATCC 27853, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and was cultivated in Luria-Bertani (LB) broth pH 7.0 $\pm$ 0.2, 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride. *Streptococcus sobrinus* and *Streptococcus mutans* (OMZ 176, OMZ 918, respectively, Institute of Oral Microbiology, University Hospital Zurich, Zurich, Switzerland) and were cultivated in Brain Heart Infusion (BHI) broth pH 7.4 $\pm$ 0.2 (5 g/L beef heart, 12.5 g/L calf brains, 2.5 g/L disodium hydrogen 2 g/L phosphate, D(+)-glucose, 10 g/L peptone, 5 g/L sodium chloride) at 37 °C under constant rotation

(220 rpm). Plate Counting Agar (PCA) containing 5.0 g/L tryptone, 2.5 g/L yeast extract, 1.0 g/L D(+)-glucose and 12.0 g/L bacteriological agar was used for plate counting experiments.

MTT viability assays. A log-phase bacteria suspension containing 10^8 colony forming units (CFU)/ml was incubated with chitosan-thiomers (dissolved in medium/0.9% NaCl (1:1) to reach 2 mg/mL or 4 mg/mL) at 37 °C and 220 rpm. After 30 or 60 min, aliquots were taken and mixed to a ratio of 1:10 with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 1 h at 37 °C, 220 rpm, samples were centrifuged for 10 min at 13300 rpm, the insoluble formazan was dissolved in 1 mL DMSO and the absorbance was measured at 550 and 570 nm with a plate reader (Synergy 2 Multi-Mode Reader, BioTek). To ensure sterility of chitosan samples, aliquots of chitosan solutions were plated onto LB agar as described below. Assays were performed as biological triplicates with technical quadruplicates.

2.4.2. Plate Counting

Bacterial suspensions were adjusted to 0.5 McFarland turbidity standard with medium as described above and incubated for 30 or 60 min with chitosan-thiomers (final concentrations 20 and 40 μ M, in 1:1 medium/0.9% NaCl) at 37 °C and constant rotation. After preparation of serial 10-10²-fold dilutions aliquots were plated onto PCA. Bacterial cultures that received culture medium instead of chitosan-thiomers served as positive controls and were serially diluted to 10⁶-10⁸-fold. All plates were incubated for 24 h at 37 °C before colony counting. Only plates containing between 300 and 10 colonies were used for enumeration. Experiments were performed in triplicates. Statistical calculation was performed with GraphPad Prism using one-way ANOVA including Tukey's test and Mann-Whitney t-test ($p < 0.05$).

2.4.3. LIVE/DEAD assays

LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Molecular Probes, Oregon, USA) was used according to the provided protocol. Briefly, bacterial suspensions containing 10⁸ CFU/ml were incubated with 40 μ M chitosan-MPA (in 0.9% NaCl) for 30 minutes at 37 °C, washed twice (13000 rpm for 2 min with 0.9% NaCl) and the dye mixture of the kit was added. After 15 min in the dark 10 μ L of the sample were immobilized on a glass slide coated with 2% agarose. Imaging was performed with a confocal laser scanning microscope (CLSM SP5 Mid UV-VIS Leica, Wetzlar, Germany). Imaris (Bitplane, Zurich, Switzerland) was used as software for image processing.

3. Results and discussion

3.1. Thiomers synthesis and characterization

Different thiomers were synthesized by functionalizing chitosan with mercapto compounds via amide formation activated by EDC carbodiimide. Starting from thioglycolic acid, the alkyl chain length was increased to 2-4, 6 and 8 carbon atoms to investigate the resulting antibacterial activity, including two branched substituents (Figure 1). In the present study, the majority of the title thiomers were newly synthesized and screened to explore the SAR of this versatile chitosan type.

On a wider scale, chitosan starting materials of different MW and DD were first screened to produce functionalized products with different characteristics (cf. Table S1 for detailed characterization). The antibacterial properties of the obtained thiomers were screened with MTT tests to select the most promising compounds for in-depth studies. We found that chitosan-TGA and -MPA obtained from chitosan 85/20 (DD $\sim$ 93%, M_w $\sim$ 80-100 kDa) showed the highest

antimicrobial activity (Figures S10-S13). Therefore, this starting material was used for the standardized preparation of the chitosan thiomers with increasing alkyl chain lengths.

All materials including the pristine chitosan standard were characterized in detail with different spectroscopic and analytical techniques to correlate their specific structural properties with the observed antibacterial behavior. Special emphasis was placed on the quantification of the amount of sulfur present in the samples through Ellman's reagent assay and elemental analyses. As Ellman's reagent reacts only with –SH groups, the actual sulfur contents might be underestimated due to the formation of disulfide bridges. S/N molecular ratios, indicating the amount of sulfur introduced into the chitosan backbone, were calculated from elemental analysis results and range from 3 to 5%, except for chitosan-TGA and -MBA (Table 1). Multiple attempts to synthesize chitosan-TGA with a higher DS via novel or modified literature protocols remained unsuccessful. For all compounds, the DD, the MW, PDI and the zeta potential were determined together with other key parameters (cf. Table 1 for a summary).

Table 1. Analytical characterization of chitosan thiomers for antimicrobial screening.

Substituent	DD (%)	M _w (kDa) ± SD	PDI	S/N (%)	SH (μmol/g) ± SD	ξ pot. (mV) ± SD
TGA	93.7	103.2 ± 3.4	1.31	0.5	90.2 ± 0.5	27.6 ± 1.4
TLA	93.3	124.4 ± 4.4	1.1	3.1	186.7 ± 2.4	29.6 ± 0.9
MPA	93.2	97.6 ± 2.3	1.25	5.1	144.1 ± 2.7	32.5 ± 0.9
MSPA	93.1	104.6 ± 8.5	1.22	3.7	206.6 ± 1.7	38 ± 1.0
MBA	94.2	87.6 ± 3.9	1.32	1.0	168.3 ± 0.5	32.2 ± 0.8
MHA	92.8	96.2 ± 2.4	1.23	4.5	253.7 ± 14	30.3 ± 1.4
MOA	93.2	117.8 ± 2.6	1.14	4.3	254.6 ± 24	30.2 ± 0.3
Chitosan	93.1	92.5 ± 1.1	1.06	n.d.	n.d.	29.9 ± 1.0

(DD = degree of deacetylation; M_w = average molecular weight; PDI = polydispersity index; S/N = ratio between moles of S and N; SH = concentration of free thiol groups; ζ- pot. = zeta potential; n.d. = not detectable).

The DD of the polymers was determined with ^1H -NMR spectroscopy which also proved useful to determine the functionalization of the chitosan backbone (Figures S2-S8). For chitosan-MSPA, -MBA, -MHA and -MOA the chemical shifts related to the alkyl chain were clearly visible in the ^1H -NMR spectra. The spectrum of chitosan-MSPA is shown as a representative example (Figure 2) displaying a well-defined peak at δ 1.76 related to the methyl group in alpha position.

Figure 2. Chitosan-2-methyl-3-sulfanylpropanoic acid ^1H -NMR (400 MHz, D_2O , 348 K, with suppression of water): δ 1.76 (d, $J = 6.3$ Hz, $3\text{H}_{2'}$), 2.56 (s, N-acetyl), 3.14, 3.58 (m, $2\text{H}_{3'}$), 3.42 (m, $\text{H}_{1'}$), 3.71 (s, $\text{H}_{2 \text{ deac.}}$), 4.25-4.42 (br, $\text{H}_{2 \text{ ac.}}$, H_3 , H_4 , H_5 , $\text{H}_{6\text{a,b}}$), 5.12 (d, $J = 6.5$ Hz, $\text{H}_{1 \text{ ac.}}$), 5.40 (d, $J = 7.9$ Hz, $\text{H}_{1 \text{ deac.}}$).

Gel permeation chromatography was used to determinate the MW of the thiomers. The number average and the weight average MW were used to calculate the PDI. The resulting PDI values in the range of 1.1-1.3 indicate a good uniformity of the polymer chain mass. All thiomers had MW in the 90 to 120 kDa range, in line with the MW of the starting material.

As mentioned above, the pH exerts a major influence on the antimicrobial efficacy of chitosan and its derivatives. In order to exclude the pH influence on the screening of chitosan-thiomers, samples for zeta potential measurements were dissolved in PBS to remain as close as possible to the biological conditions. The pH values after dissolution were around 5.5-6.0 with measured zeta potentials around + 30 mV due to the residual protonated ammonium groups. Finally, we aimed to minimize the influence of DS, DD, MW and positive charge density as far as possible in order to focus on the antimicrobial activity as a function of the mercapto-substituent as the main tuning parameter.

3.2. Antimicrobial activity

After detailed analytical characterizations, chitosan-thiomers were screened for their antimicrobial activity against one representative gram-negative (*Pseudomonas aeruginosa*) and two representative gram-positive (*Streptococcus sobrinus* and *Streptococcus mutans*) strains. The first round of chitosan-thiomers screening still covered a wider range of MW and DD to obtain a broader overview of the influential parameters.

For this purpose, an easy and low-cost screening assay was set up by adapting and optimizing the well-known MTT-based cytotoxicity assay (Wang, Cheng, Wang, Wei, & Wang, 2010). In contrast to previously described MTT protocols, we established a 96-well platform that permits to quickly process multiple compounds and replicates detecting the absorbance with a standard plate

reader. This protocol moreover avoids time consuming steps by treatment and MTT metabolism in the same reaction tube, which improves the throughput of the method. As a further advantage the dissolution of formazan crystals requires small solvent volumes. Our modified MTT assay allows for quantitative monitoring of alive bacteria after treatment with chitosans, because the formazan crystals produced by MTT metabolism are directly proportional to the number of viable cells. The linearity of the method was calibrated under experimental conditions (cf. Figure S9).

Using this assay, chitosan-TGA, -TLA and -MPA derivatives synthesized from different starting materials (cf. Table S1) were tested under different conditions, i.e. incubation times of 30 min and 60 min, and concentrations of 2 mg/mL and 4 mg/mL, respectively (Figures S10-S13). The MTT assay results highlighted the remarkable improvements in the antimicrobial effect of chitosan-TGA and -MPA (and to a lower extent of chitosan-TLA) synthesized from chitosan 85/20 in comparison to chitosan 85/5, 85/10, and 85/100. The DD of the 85/20 chitosan-thiomers, as determined by $^1\text{H-NMR}$, was around 93%, suggesting that this starting material affords chitosan-thiomers with higher DD, which is well known to enhance the antimicrobial activity. Therefore, chitosan 85/20 was used as starting material for the synthesis of chitosan-MSPA, -MBA, -MHA and -MOA.

The MTT results generally indicated that all our compounds showed a clear antimicrobial activity under the experimental conditions (Figures S10-S13). Both gram-positive strains were strongly affected by the chitosan-thiomer treatment, i. e. the percentage of alive gram-positive bacteria of most of the samples fell below the detection limit of the plate reader. While the percentage of alive gram-negative cells was also considerably reduced after treatment, effects were not as pronounced.

Whereas the MTT assay allows for a rapid and convenient screening, the quantitative assessment of the antimicrobial efficiency requires more precise methods. We therefore used the plate

counting method for the following in-depth tests of one representative chitosan-thiomer batch per substituent generated from functionalization of the same starting material (chitosan 85/20, cf. above) vs. pristine chitosan. In our experimental setting, the post-treatment capability of bacterial cells to form colonies after 24 h of incubation with 20 μ M and 40 μ M chitosan for 30 min and 60 min was determined.

Figure 3. Plate counting results: colony forming units (CFU) per mL for *Pseudomonas aeruginosa* (A), *Streptococcus sobrinus* (B) and *Streptococcus mutans* (C) (20 μ M, 30 min incubation).

The results revealed that for all strains tested higher concentrations and prolonged treatment times did not exert a significantly stronger antimicrobial effect than the milder conditions. Therefore, results after treatment for 30 min with 20 μ M chitosan thiomers are discussed in the following (Figure 3) to highlight the differences between the performances of chitosan-thiomers compared to pristine chitosan more clearly. The results obtained for the other conditions are summarized in the Supporting Information (Figures S14-S22). Generally, plate counting confirmed the results of our MTT assays where all compounds had a strong impact on the bacteria tested. Beyond MTT assays that measure the cell's metabolic activity, colony counting provides information on the proliferative capacity after chitosan exposure as a parameter of clinical importance. We found that, under the above-mentioned milder conditions, incubations of both our gram-positive and gram-negative bacteria with pristine chitosan led to an at least 4.5-log reduction of colonies, but not to complete eradication. In previous reports on *P. aeruginosa*, however, chitosan inhibition rates were not as high as in our study, even at higher concentrations or longer incubation times (e.g. 94.9%, (Lee, Park, & Ahn, 2003) with chitosan (30 kDa and 120 kDa) or 99.9% (Kumar, Surendan, & Thankappan, 2006; Tsai, Su, Chen, & Pan, 2002) with chitosans 49 kDa (DD 53%), 285 kDa (DD 76%) and 1080 kDa (DD 98%)). Others (Kanatt, Chander, & Sharma, 2008) achieved a reduction of 8.5-log in 24 h with 0.5 mg/mL chitosan (DD 78-82%) on *P. fluorescence*, which is, however, not directly comparable to *P. aeruginosa*. Furthermore, minimal inhibitory concentration (MIC) values of chitosan on *S. mutans* have been reported to range from 1.25 to 5 mg/mL (Bae, Jun, Lee, Paik, & Kim, 2006; Costa, Silva, Pina, Tavarria, & Pintado, 2012; Ji et al., 2009). The variance of these results illustrates their strong dependence on the tested chitosan and on the applied methodology.

Whereas treatment with pristine chitosan already significantly reduced the number of alive bacteria in our study, this effect could be further enhanced through mercapto-functionalization. In line with previous works, the thiol-alkyl chains of the chitosan substituents can enhance the antimicrobial activity of the pristine compound through hydrophobic interactions between the alkyl chain and the bacterial wall proteins (Inta et al., 2014). Furthermore, reaction of the thiol-moiety with the cysteine groups of their proteins could exert a detrimental effect on bacteria (Sarti & Bernkop-Schnürch, 2011). These combined features of the chitosan-thiomers could lead to a prolonged interaction with the bacterial cell wall, thus enhancing the antibacterial activity. All thiomers compounds of our study induced at least a 5.6-log colony reduction compared to untreated controls. Since a 5-log decrease is often accepted as a sufficient benchmark for antibacterial efficiency, all the investigated mercapto-functionalized chitosans may be regarded as highly active against both gram-positive and gram-negative strains. However, the extent of antimicrobial activity of individual thiomers differs among tested strains, as observed for pristine chitosan (No et al., 2002; Sette-de-Souza et al., 2015) and for quaternary N-alkyl derivatives (Sahariah et al., 2015). Even apparently small differences may have strong implications (such as on the repopulation of infected sites) due to the short generation times of most bacteria.

As shown in Figure 3a, chitosan-TLA, chitosan-TGA, chitosan-MHA and chitosan-MSPA worked equally excellent for the gram-negative *P. aeruginosa*. All of these functionalized chitosans were statistically significant ($p < 0.05$) compared to pristine chitosan (Figure 3a). The latter leads to 6.4-log reduction with respect to the control, while chitosan-MSPA enhanced this effect by 100-times (8.4-log reduction).

The gram-positive *streptococci* displayed a less consistent pattern. Chitosan-MOA, chitosan-TLA and chitosan-MBA always led to complete eradication of *S. sobrinus* (Figure 3b), while chitosan in a third of the experiments was unable to do so. Advantages of chitosan thiomers were clearly

visible in *S. mutans* (Figure 3c), where chitosan-MSPA incubations resulted in 8.3-log reduction (complete killing), thereby enhancing the antimicrobial activity of chitosan approx. by a factor of 10^4 (4.5-log reduction). Chitosan-MHA, chitosan-MBA, and chitosan-MSPA all displayed a statistically significant effect ($p < 0.05$) compared to pristine chitosan (Figure 3c). However, even if certain thiomers effects were not statistically significant, elimination of all present bacteria in 30 min is key to prevent a recolonization from surviving cells. Our findings support our previous work, where chitosan-TGA exerted a 5-log CFU reduction on *S. sobrinus* (Geisberger et al., 2013). In contrast, previous studies on *E. coli* and *S. aureus* found chitosan-TGA (Han, Wei, Jia, Xu, & Li, 2012) and chitosan-acetylcystein (Fernandes, Francesko, Torrent-Burgués, & Tzanov, 2013) to be less effective than pristine chitosan. As mentioned above, numerical differences in the antimicrobial activity often arise from different tested strains, chitosan characteristics and test protocols, which render direct comparisons difficult.

In vitro cytotoxicity tests performed so far showed low toxicity of N-acyl thiomers towards mammalian cells.

The promotion of bactericidal effects by chitosan-thiomers was further investigated microscopically with a viability assay (Chavez de Paz, Resin, Howard, Sutherland, & Wejse, 2011; Shi, Neoh, Kang, & Wang, 2006). Two fluorescent dyes are used to discriminate between alive and dead cells, with SYTO 9 generally labeling all bacteria, regardless of intact or damaged membranes. In contrast, propidium iodide (PI) penetrates only bacteria with damaged membranes, causing a reduction in SYTO 9 fluorescence when both dyes are present.

Chitosan-MPA was tested as an example for the chitosan-thiomer series. In all strains investigated, untreated cells showed only the incorporation of SYTO 9, whereas PI fluorescence was detected after treatment with chitosan-MPA. With killing rates of 99-100% (Figures 4f, 5f, and 6f) our microscopic analyses support our results from plate counting experiments. They

provide further evidence that chitosan derivatives eliminate bacteria by disrupting the membrane as one major cause of death. The exclusive presence of agglomerates (Sarasam, Brown, Khajotia, Dmytryk, & Madihally, 2008; Virga, Landa, Beltramo, Ausar, & Dorronsoro, 2003) after chitosan-MPA treatment was detected for both gram-positive bacteria (Figures 5d and 6d).

Figure 4. Effects of chitosan-MPA treatment on *Pseudomonas aeruginosa* analyzed with confocal laser scanning microscopy: untreated control (A-C), treated (D-F); SYTO 9 fluorescence (A, D); propidium iodide fluorescence (B, E), and overlay (C, F).

Figure 5. Effects of chitosan-MPA treatment on *Streptococcus sobrinus* analyzed with confocal laser scanning microscopy: untreated control (A-C), treated (D-F); SYTO 9 fluorescence (A, D); propidium iodide fluorescence (B, E), and overlay (C, F).

Figure 6. Effects of chitosan-MPA treatment on *Streptococcus mutans* analyzed with confocal laser scanning microscopy: untreated control (A-C), treated (D-F); SYTO 9 fluorescence (A, D); propidium iodide fluorescence (B, E), and overlay (C, F).

4. Conclusions

In this study, we present the first systematic investigation on antimicrobial effects of new N-acyl thiolated chitosans with mercapto-substituents bearing increasing alkyl chain lengths. All functionalized chitosans were characterized with respect to key parameters for SAR (e.g. MW, DD, zeta potential). Comparative tests of representative gram-negative and gram-positive bacteria with various methods point to an enhanced bactericidal effect of chitosan thiomers compared to pristine chitosan. Whereas no complete eradication of all cells of a given species was observed for pristine chitosan under the applied experimental conditions, at least one chitosan thiomers per strain eliminated the complete bacterial population. This feature of chitosan

thiomers is very promising for clinical applications, because even minor amounts of remnant bacteria may turn out detrimental over time due to their rapid growth.

Concerning synthesis and screening procedures, we aimed for consistent parameter standardization, including MW, DD, sulfur content and positive charge of the chitosan-thiomers, to investigate the effect of the alkyl chain length on the antimicrobial properties. This systematic approach paves the way to SAR requested for future rational chitosan design. However, further experiments are necessary at this point to define general relationships between alkyl chain length and antimicrobial activity. Our results are in line with the strongly strain-dependent SAR observed for other chitosan derivatives before, and at this point, the presence of thiol-bearing substituents may be the most important common denominator. Therefore, our investigation highlights N-acyl thiolated chitosans as a rewarding target group for highly efficient, biocompatible and cost-effective antimicrobial compounds. Metabolism and mechanistic studies are now under way to optimize this versatile compound class for clinical applications.

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