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Article

Assessment of the quorum sensing inhibition activity of a non-toxic chitosan in a AHL-based *E. coli* biosensor

Xiaofei Qin [1], Jana Emich [1], Francisco M. Goycoolea [1,2,*]

[1] Institute of Plant Biology and Biotechnology, University of Münster, Schlossplatz 8, 48143 Münster, Germany; qxf200459420@163.com

[2]School of Food Science and Nutrition, University of Leeds, LS2 9JT, Leeds, United Kingdom

* Correspondence: F.M.Goycoolea@leeds.ac.uk; Tel.: +44 113 343 1412

Abstract: New approaches to deal with drug-resistant pathogenic bacteria are urgent. We studied the antibacterial effect of chitosans against an *E. coli* quorum sensing biosensor reporter strain, and selected a non-toxic chitosan to evaluate its QS inhibition activity and its effect on bacterial aggregation. To this end, chitosans of varying DA (12 to 69%) and M_w (29 to 288 KDa) were studied. Only chitosans of low DA (~12%) inhibited the bacterial growth, regardless of the M_w. Chitosan MDP DA30 (DA 42% and M_w 115 kDa) was selected for further QS inhibition and SEM imaging studies. MDP DA30 chitosan exhibited QS inhibition activity in an inverse dose-dependent manner (≤12.5 μg/mL). SEM images revealed that this chitosan, when added at low concentration (≤30.6 μg/mL), induced substantial bacterial aggregation, whereas at high concentration (234.3 μg/mL), it did not. Aggregation explains the QS inhibition activity as the consequence of retardation of the diffusion of AHL.

Keywords: Chitosan; quorum sensing; antibacterial activity; quorum sensing inhibition

1. Introduction

Chitin, poly (*β*-(1-4)-*N*-acetyl-D-glucosamine), is the second most abundant polymer in the biosphere. It occurs in the exoskeleton of crustacean and insects, in squid pen, the cell wall of fungi, in diatoms, among other organisms. Currently, chitin is sourced at industrial scale from shrimp and crab shell waste. Chitosan is a linear polycationic heteropolysaccharide biopolymer that is produced by partial alkaline N deacetylation of chitin [1]. It is mainly composed of two kinds of (1->4), linked structural units, namely 2-amino-2-deoxy-D-glucose and N-acetyl-2-amino-2-deoxy-D-glucose. However, since it is technically difficult to completely deacetylate chitin and also that highly deacetylated chitosan can be re-acetylated, what is usually known as *chitosan*, rather refers to a family of polysaccharides with different degrees of acetylation and polymerization. Therefore, it is best to refer to this family of polymers as *chitosans*. The capacity of chitosans to dissolve in dilute aqueous solutions is the commonly accepted criterion to differentiate them from chitin. Chitosans dissolve in dilute aqueous acids, when the pH is below its pK_a (around 6.1) [2]. Common acids in this regard include acetic, formic, succinic, lactic, and hydrochloric acid. The characteristics of chitosans are mostly defined by its molecular weight (Mw) or degree of polymerization (DP), degree of

acetylation (DA) and pattern of acetylation (PA). There is not a unique precise criterion to distinguish chitosan polymers from oligomers. The DP can vary over a wide range DP~20 to > ~1000; the DA from 0 up to 70%, and the PA from purely random to blockwise. The variation in these three parameters give rise to a vast number of different possible chitosans.

Research on chitosan has burgeoned in the recent decades, due to the large number of material and bioactive properties, namely antimicrobial [3-9], anti-inflammatory [10], mucoadhesive [11-12], immuno-adjuvant [13], capacity to complex and condense nucleic acids [14] among other. A broad-spectrum antibacterial activity of chitosan in solution, or as nano- and microparticles, or as films, has been demonstrated against Gram-positive and Gram-negative bacteria [7, 15-20]. The antimicrobial properties of chitosan have found applications in the food industry, as a pharmaceutical agent in medical industry and is used by the textile industry, cosmetics, agriculture and wastewater purification [1, 5, 15].

Antibiotic resistance is serious problem that currently poses major unmet challenges and gaps in global health [21]. There are several mechanisms whereby bacteria intrinsically resistant or acquire resistance to antibiotic treatment. They can inhibit the antibiotics access to drug targets, change the structure of antibiotic targets or direct modify the antibiotics that make them inactivation [21]. Nowadays it is urgent to pursuit alternative strategies for the control of bacterial infections. Quorum sensing (QS) is a form of cell-density bacterial regulation mechanism controlled by a chemically exquisite signaling system that effects communal traits (*e.g.*, biofilm formation, bioluminescence, motility, toxin production) in both Gram-negative and Gram-positive bacteria [22]. In large number of Gram-negative species, QS is mediated by conserved small molecule signals that act as autoinducers, termed acylated homoserine lactones (AHLs). At high cell densities, AHLs can bind to specific receptors that act as transcriptional activators of the gene cascades involved in the virulence responses [22, 23]. Hence, inhibition of QS can be considered as a novel promising strategy to deal at large scale with bacterial pathogens while potentially circumventing antibiotic-resistance bacteria [24].

So far, the antimicrobial activity of chitosan has been extensively studied, but only limited information focuses on the effect of chitosans on bacterial QS activity. To the best of our knowledge, only one recent paper has reported that chitosan showed a potential to disturb biofilm formation by QS inhibition and to inhibit *C. violaceum* violacein production [25]. In two parallel studies conducted in our laboratories [26, 27], we have documented the QS-inhibiting of chitosan-based both oil-core nanocapsules and crosslinked matrix nanoparticles. In the present work, complementary to the two parallel ones, we have addressed the antibacterial activity of ten samples of chitosans of varying MW and DA. This enabled us to select one non-toxic chitosan to explore in further detail its anti-QS activity using the *E. coli* Top 10 reporter strain that we have used in previous works [26, 35-37].

2. Results

Ten chitosans with varying M_w and DA (Table 1) were part of the same series of samples that we have addressed in the frame of the Nano3Bio EU project [28]. The range of both DA and M_w of the samples spans a very wide range. This range of DA and M_w envelops very well the most common properties of commercial chitosans. Notice in Table 1 that we have included a column with the concentration (μg/mL) of each chitosan to afford an constant equivalent charge [$-NH_3^+$]

concentration of 0.389 mM. We have performed the biological antibacterial activity assays by controlling the charge equivalent concentration, rather than the mass per unit volume one. Of note, two chitosan samples missed the experimental evaluation of their DA (%), namely LDP DA 20 and LDP DA 30. However, in Table 1, we have added a column with the estimated DA, as calculated from the stoichiometry of the N-acetylation reaction. Hence, the experimental DA values of these samples can be expected to have lied close to the expected ones. Also, we were not able to determine the M_w and I_p for the sample with code LDP DA 60. However, given that this sample corresponds to the same series of N-acetylated chitosan that were prepared by N-acetylation from the same depolymerized chitosan sample of low DA (1.6%), we are confident that the degree of polymerization of this material lied close to the rest of the samples in the "LDP" series. Despite the fragmented data for these three samples, we decided to include them in the study.

2.1. Antibacterial assay

Two distinct methods were used to investigate the antibacterial activity of the different chitosans, namely by measuring the growth rate during the exponential phase from the time evolution of the optical density (OD_{600}), and by a plating assay to estimate the number of viable bacteria (CFU/mL). Notice in Figure 1a that regardless of the M_w of the different chitosans, only the samples with DA 12% clearly reduced significantly the relative growth rate by < ~50%. In turn, the agar gel plating assay (Fig 1b) revealed that exclusively MDP and LDP of low DA (DA=12%) reduced the number of CFUs per mL. This reduction exhibited a dose-dependent response, as we noticed upon treatment with the two different doses (0.389 and 0.778 mM). Also, LDP DA12 chitosan was more effective than MDP DA12 as revealed by the agar plate assay. Interesting, the highest DA chitosan sample (LDP DA60, DA 69%) did also reduce the plate count number in a dose-dependent manner with respect to the control. None of the other chitosans had antibacterial activity when dosed at the same charge equivalent concentrations.

2.2. *E. coli* Top10 biosensor assay with different concentrations of chitosan

Based on the results of antibacterial activity above, we selected one of the chitosan samples that did not exhibited any effect on growth rate as per the two antibacterial assays, namely chitosan MDP DA30 (DA 42% and M_w 115 kDa), to further investigate its QS inhibition activity. To this end, we evaluated the effect of varying concentrations of chitosan (1.25 to 50 μg/mL) on the response of the *E. coli* top 10 strain. Given that the QS response operates with AHL concentrations orders of magnitude lower than those tested for chitosan in the antimicrobial assays, the concentrations of chitosan tested in this assay were 160- to 4-fold lower than those tested in the antibacterial assays. This strain is the same one that we have used in parallel studies [26, 27, 29]. It is a GFP-reporter that does not synthesize its own AHL, namely $3OC_6HSL$, and responds by producing GFP (that is detected by fluorescence spectroscopy) upon addition of extremely low concentrations of $3OC_6HSL$ (< 1 nM).

Figure 2a and c illustrate the influence of adding varying concentrations of chitosan on the evolution and end-point values of OD_{600}, respectively. Close inspection of Figure 2a reveals that the addition of chitosan in low doses of 1.25 and 2.50 μg/mL did not modify whatsoever the growth curve with respect to the control. However, as the concentration of chitosan increased to 4.17 μg/mL a

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Table 1. Chitosan with different Mw and DA and mass concentration are tested in antibacterial

Chitosan name	M_w (kDa)[1]	Ip[1]	Calculated DA (%)[2]	Experimental DA (%)[3]	Chitosan concentration in antibacterial bioassays (μg/mL)[4]
LDP DA12	29	1.6	12	12	73.5
LDP DA20	35	2.1	20	n.a	82.5
LDP DA30	37	1.7	30	n.a	96.7
LDP DA40	58	2	40	44	115.6
LDP DA60	n.a	n.a	60	69	181.7
MDP DA12	85	1.6	12	14	73.5
MDP DA30	115	1.7	30	42	96.7
MDP DA50	91	2.5	50	n.a	142.0
HDP DA12	288	2.1	12	16	73.5
HDP DA60	185	1.9	60	68	181.7

[1] Weight average molecular weight (M_w) and polydispersity index ($I_p = M_w / M_n$) values as determined from GPC-MALLS-DRI; [2] Degree of acetylation (%) values as estimated from the stoichiometry of the N-acetylation reaction; [3] Experimental DA (%) values as determined by ^{1}H NMR; [4] Concentration (μg/mL) equivalent to 0.389 mM [-NH_3^+] charge; n.a. = not available data.

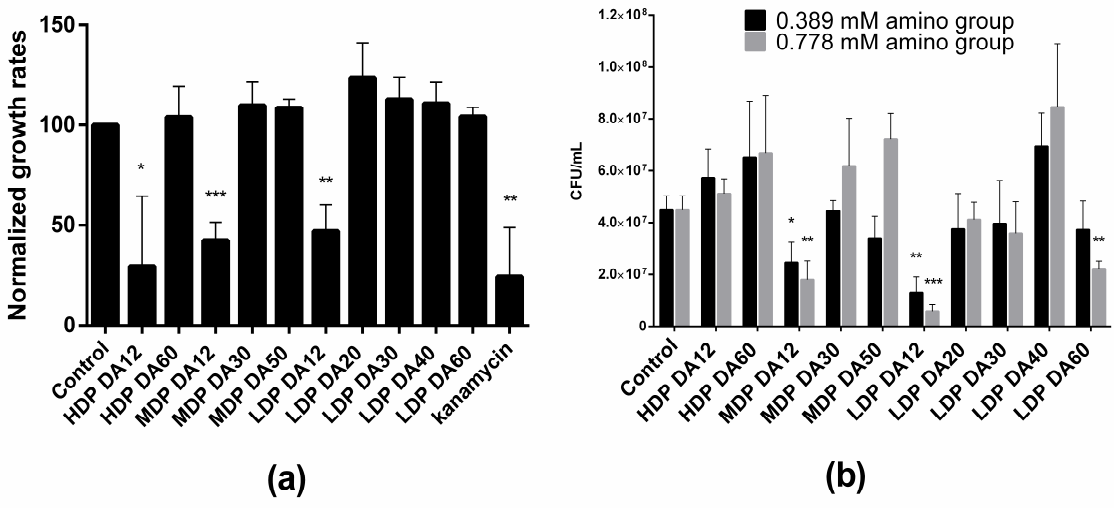

Figure 1. (a) Relative growth rate after re-seeding of *E. coli* Top 10 bacteria treated with identical equivalent charge concentrations (0.389 mmol) of chitosans of varying degree of acetylation and

polymerization (for sample details refer to Table 1). In the first cycle, the bacteria were incubated during 2:38 h in M9 medium at 37 °C, whereas in the second cycle, an aliquot of the bacterial culture was reseeded in fresh M9 medium and incubated during 2:38 h. Absorbance, as measured at λ=600 nm, as a function of chitosan concentration added in the M9 medium with *E. coli*. One (negative) control corresponds to bacteria cultivated in M9 medium , while the other (positive) control was kanamycine (50 μg/mL) added in the M9 medium; (b) Effect of chitosans of varying degree of acetylation and MW with two different amino group concentration (0.389 and 0.778 mM) on the growth of *E. coli* Top 10 incubated in a 96-well-plate at 37 °C and 100 rpm for 1 h and tested for antibacterial activity using colony-forming unit (CFU) agar plate assay. The control corresponds to untreated bacteria in the CFU assay (*p < 0.05, **p<0.01, ***p<0.001 and ****p<0.0001; values represent averages ± SD, n≥3; three technical replicates).

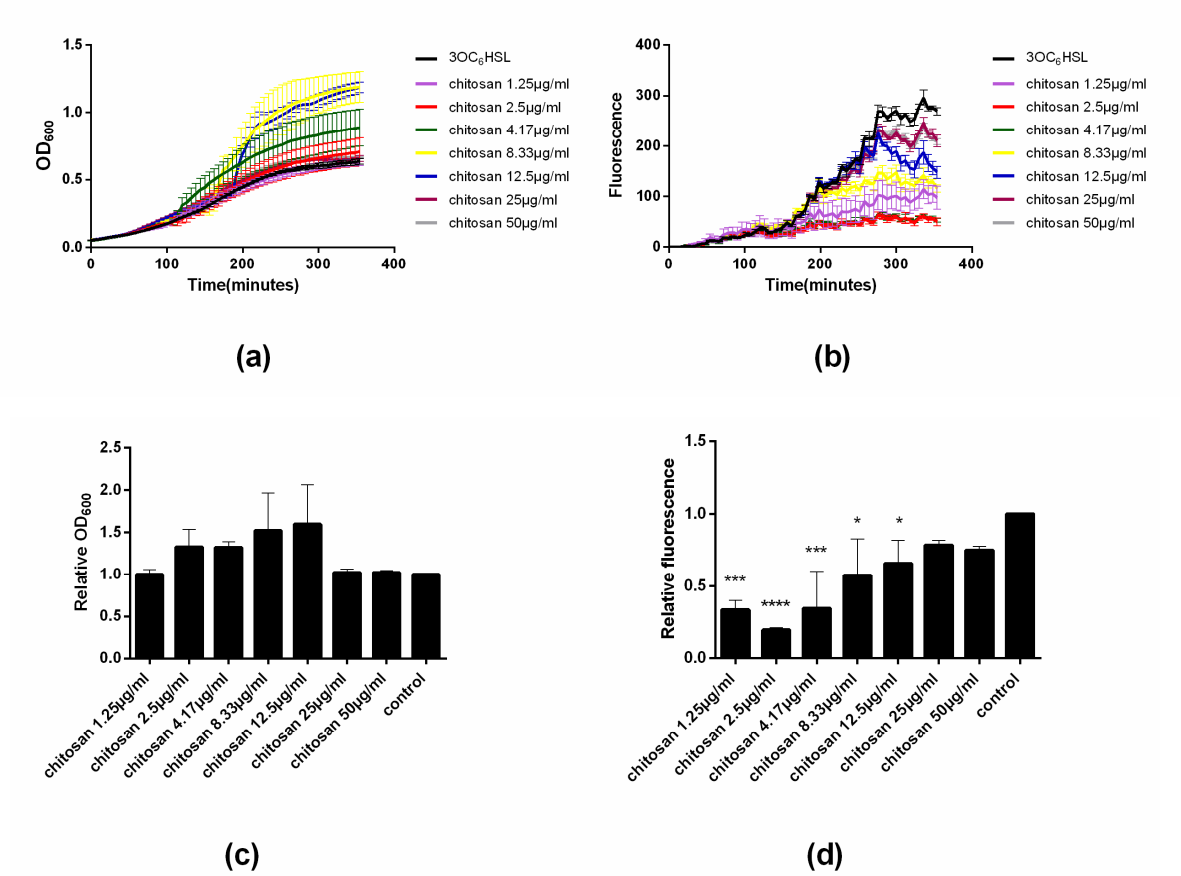

Figure 2. (a) Effect of varying concentrations of chitosan MDP DA30 (DA 42% and M_w 115 kDa) on the growth of *E. coli* Top 10 measured from the absorbance, as measured at λ=600 nm; (b) Effect of different concentrations of chitosan on bacterial fluorescence intensity; (c) Effect of varying concentrations on endpoint OD_{600} relative to the control corresponding with the data shown in panel a; (d) Effect of varying concentrations on fluorescence/OD_{600} relative to the control corresponding with the data shown in panel b; $3OC_6HSL$ concentration: $2.5x10^{-10}$ M (*p < 0.05, **p<0.01, ***p<0.001 and ****p<0.0001; values represent averages ± SD, n≥3; three technical replicates)

sudden rise in OD_{600} occurred at time ~110 min. The same effect occurred also at 8.33 and 12.5 μg/mL, though at later onset times, namely ~150 and ~180 min, respectively. Upon addition of even greater concentrations of chitosan (25 and 50 μg/mL), the rise in OD_{600} is no longer appreciated and the traces mirror those of the control. The results after 350 min ("end point") summarized in Figure 2c illustrate that up to a concentration of 12.5 μg/mL, chitosan leads to an increase on the final OD_{600} values; beyond this concentration, there is no effect on the bacterial growth. Figure 2b depicts the fluorescence intensity data corresponding to the same time points of the OD_{600} measurements during the microtiter plate assay. A detailed inspection of the fluorescence intensity traces, reveals a rather peculiar dependence of the GFP expression on the concentration of added chitosan. At low concentrations of chitosan, namely 1.25-4.17 μg/mL the fluorescence intensity is severely attenuated after ~120 min, and attains a minimum value at 2.5-4.17 μg/mL. At greater concentrations of chitosan (≥8.33 μg/mL), the intensity increases until almost the same value as the untreated control. Figure 3d shows the fluorescence intensity end-point values that clearly summarizes the results. Notice that the minimal fluorescence intensity was assessed at a concentration of chitosan of 2.5 μg/mL.

2.3. SEM imaging of E. coli Top 10 stains mix with different concentration of chitosan

With the aim to gleaning understanding on the observed effects of chitosans on the *E. coli* top 10 strain, we recorded SEM images after incubating a fixed number of bacteria (OD_{600}=0.2) with varying concentrations of MDP DA30 chitosan (2.95, 5.88, 8.79, 28.7 and 226.6 μg/mL). Figure 3a shows the image of *E .coli* top 10 bacteria alone that appear as isolated rods. However, upon addition of chitosan at concentrations ≤ 28.7 μg/mL, bacteria invariably appeared aggregated in conglomerates (Figure 3 b-e). At the highest concentration of chitosan (226.6 μg/mL) though, bacteria again appeared as isolated entities (Figure 3f).

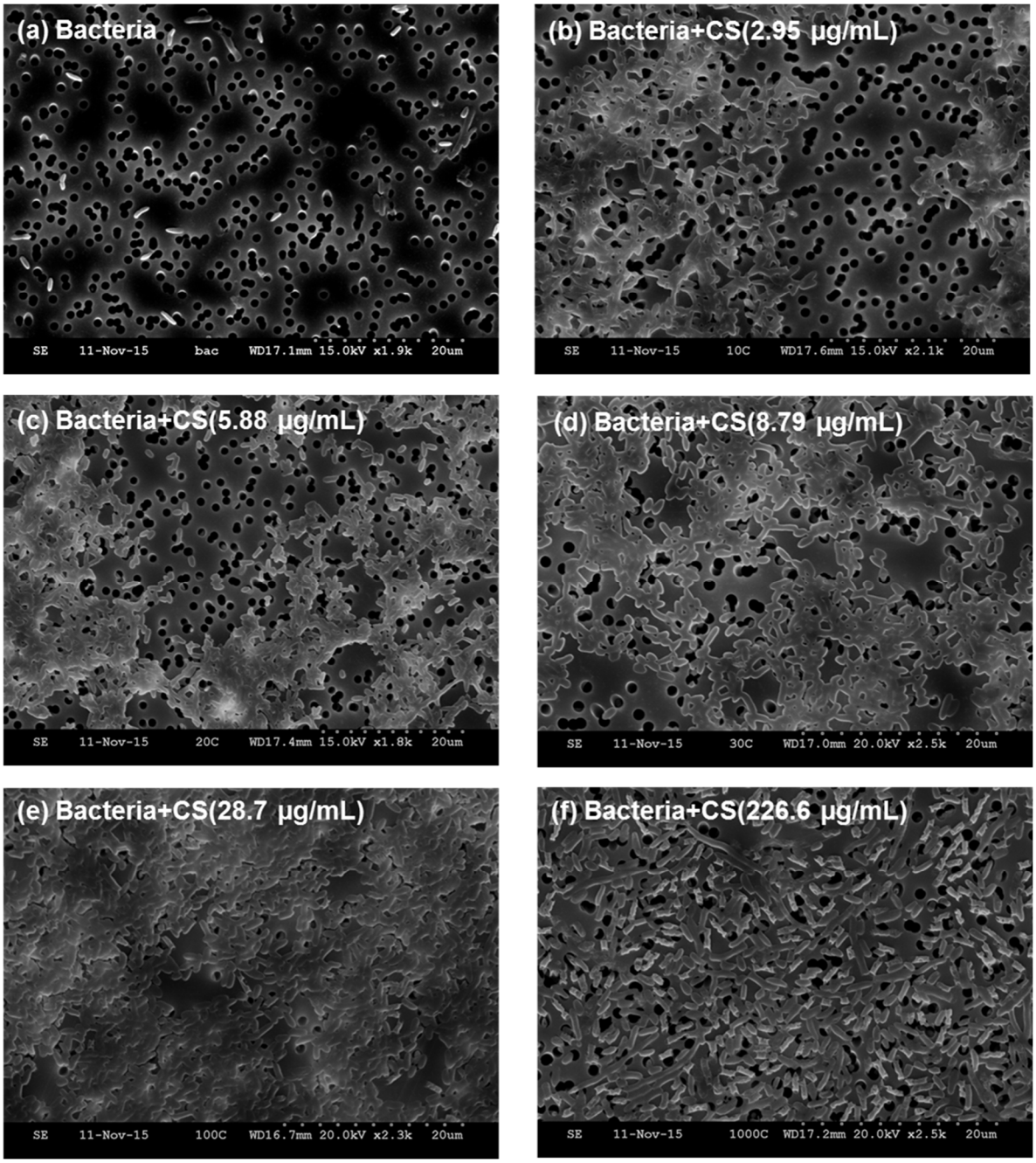

Figure 3. SEM images of (a) *E.coli* Top 10 bacteria alone; and after incubating in the fridge (4 ºC) and slightly shaking for 1 hour, mixed an equivalent number of bacteria (OD_{600}=0.2) with varying concentrations of MDP DA30 chitosan, namely (b) 2.95 µg/mL; (c) 5.88 µg/mL; (d) 8.79 µg/mL; (e) 28.7 µg/mL; (f) 226.6 µg/mL supported on track-etched polycarbonate membranes. Under there imaging conditions bacteria appear as bright rod objects. Polycarbonate support appear as a gray background, and sharply-defined track-etched pores appear as dark circles of ca. 1.2 µm in diameter.

3. Discussion

The actual mode of action whereby chitosan exerts its antimicrobial activity is not fully understood. In the case of antibacterial activity, several hypotheses have been put forward to explain the mechanisms at play. One is related to the ability of the available NH_3^+ functions in chitosan to bind to negatively charged proteins of the outer membrane of Gram-negative bacteria leading to the

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imbalances of internal osmotic and/or the leakage of proteinaceous and other intracellular constituents [17, 18]. The other proposed mechanism argues that chitosan penetrates in the cytosol and binds with DNA, or mRNA, thus interfering with protein synthesis [4, 5, 9, 30]. It has also been proposed that chitosan mode of action would involve chelating trace elements leading to growth and metabolism arrest [5]. The antimicrobial activity of chitosan is known to depend on several factors either associated to the type of microorganism, the characteristics of the cell envelope, to intrinsic features of chitosan (*e.g.*, DA, M_w, chelating capacity and hydrophilic/hydrophobic characteristic), or to environmental factors (*e.g.*, pH of the medium, temperature, time) [9, 15].

In an attempt to account to examine in detail the influence of the charge density of chitosan (*i.e.*, given directly by the DA), in the present work, we applied the same [NH_3^+] charge equivalent concentration of chitosans of varying DA and M_w. Notice that in these series of experiments to assess identical charge equivalent concentrations, the greater the DA, the greater was the added mass concentration (Table 1); and yet only the chitosans of lower DA (*i.e.*, dosed at the lowest mass concentrations) exhibited antibacterial activity, as evidenced on the growth rate in liquid culture and in the agar plate number counts. Regarding the M_w, in the case of growth rate studies, the three chitosans of low DA namely LDP DA12, MDP DA12, and HDP DA12 had a strong effect on suppressing the growth. However, in the plating assays, carried out in agar gels, only the medium and low Mw chitosans reduced the number of CFU/mL.

A possible explanation to the apparently discrepant results between the two assays, based purely on physical grounds, could be the differences in the diffusion rates of chitosan when dissolved either in the liquid or in the agar gel media for the growth rate and plating assays, respectively. In the former case, the chitosans in solution are able to diffuse freely and interact with bacteria, whereas in the agar plate assay, presumably only the chitosans of low M_w were able to diffuse through the agar dense molecular gel network and interact with bacteria, while the high M_w was not. This is consistent with the fact that LDP DA12 chitosan, of the lowest M_w, had stronger activity than that of MDP DA 12. Also, these results are in overall agreement with previous studies that have documented that chitosans of low M_w exhibit stronger antibacterial activity due to their greater mobility and amenability to interact with the cell membrane surface [4, 30]. This could be explained as the consequence of stronger electrostatic interactions between charged NH_3^+ groups in deacetylated D-glucosamine and the bacterial cell membrane. In previous studies, it has been suggested that the low antibacterial effect of chitosans of high DA is the result of greater inter-chain interactions due to H-bonds in addition to the hydrophobic character of the acetyl group in N-acetyl glucosamine, thus leading to a more densely overlapping coiled conformation; those interactions have been suggested to inhibit the high DA chitosan binding to the bacterial cell walls [2, 7]. We noticed that also chitosan LDP DA 60 (DA 69%) also reduced the plate count number in a dose-dependent manner. However, this same chitosan had no effect on growth rate in the liquid suspension assay. We do not have a convincing explanation at present for this result, particularly because the other chitosans also of high DA, namely LDP DA 30 (DA 30% M_w 37 kDa) and LDP DA 40 (DA 44% M_w 58kDa), did not reduce the number of viable bacteria.

Contrary to our expectations, only the chitosan of lower DA had a consistent effect on arresting the bacterial growth, even when the bacteria were treated with identical doses of charge equivalent. From this experiment, it stands to reason that not only the effective amount of charged NH_3^+ groups

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in chitosan is what determines the antibacterial effect of chitosan, but also other structural features must also be at play. Otherwise, a similar antibacterial activity would have been exhibited by all chitosans, when the same equivalent concentration of charged NH_3^+ was given to the bacteria. Whether a minimum block length of contiguous deacetylated D-glucosamine residues in chitosan is needed for the interaction with the bacterial cell surface is yet to be established (*i.e.*, as a highly cooperative binding process). This would resemble a highly cooperative binding process. Moreover, the pattern of acetylation (PA), that might vary from block over random to an alternating pattern, should also have significant impact on biological and physicochemical activity as the distribution of the acetyl group along the chitosan chain, which probability also affect the chitosan conformation and the ability attach on bacterial membrane [2, 31, 32]. Ongoing studies in our laboratories are currently testing the validity of these proposals using well-characterized chitosan oligomers.

For the further QS inhibition experiments, we selected only one chitosan that had no antibacterial activity, namely sample MDP DA 30 (DA 42% and M_w 115 kDa). In the QS inhibition studies, both the evolution of OD_{600} and fluorescence intensity were recorded upon treating the bacteria with varying concentrations of chitosan (1.25 to 50 μg/mL). The OD_{600} traces confirmed that this chitosan did not reduced the growth rate with respect to the control. However, sudden increases in OD600 were observed at three concentrations, namely 4.17, 8.33 and 12.5 μg/mL. In paralell studies [26, 38, 39], we have observed very similar "bumps" in the growth curves (OD_{600}) upon addition of increasing number of chitosan-coated nanocapsules to the same *E. coli* strain as in this study. The appearance of such sudden "bumps" observed in the growth traces, are explained as the consequence of attaining specific chitosan/bacteria ratios that result in the aggregation of bacteria, and thus in the sudden increase in OD_{600} values. Our SEM results are consistent with this explanation. In the case of chitosan-based nanocapsules [26], we have found that there is an optimal ratio of nanocapsules per bacterium at which the electrical charge of both is fully compensated (i.e., the ζ-potential ~ 0). At this so-called "stoichiometric" ratio, the bacteria form large conglomerates and aggregation is maximal, and this behavior dependent on the material concentration and the cell density. Interestingly, the "bumps" were only observed for three concentrations ratios. It seems that only in these cases, the optimal stoichiometric ratio, was hit in the course of growth. At lower or greater concentrations, this optimal ratio was not attained, either because of defect or surplus of chitosan was present.

The MDP DA30 chitosan was found to have the capacity to inhibit QS as detected on the *E. coli* top 10 strain, particularly when dosed at concentrations of 1.25 to 4.17 μg/mL. At greater doses, the QS inhibition was less accentuated. The doses of maximal QS inhibition coincide closely with those at which the "bumps" in the growth traces appear. Hence, it seems reasonable to argue that the inhibition of QS is the result of the maximum in agglomeration of bacteria, which results in the retardation of the diffusion of $3OC_6HSL$ to the cytosol, and hence in the attenuation of GFP expression. The SEM images recorded after incubation of bacteria with varying concentrations of chitosan showed that substantial bacterial aggregation occurs when chitosan is added at low concentrations (Figure 3b–e). These results are in full agreement with the results recorded in the QS inhibition assays, namely with the occurrence of "bumps" in OD_{600} growth traces and with the greatest attenuation in QS activity. However, when added at the highest concentrations, bacteria appear as isolated species (Figure 3f). In this case, we reasoned that the high concentration of

chitosan leads to an excess of net positive charge at the bacterial surface, so as to prevent their aggregation [5].

4. Conclusion

Out study demonstrates that chitosan of low DA (≤12%) can be characterized as a bacteriostatic biopolymer rather than bactericidal. Only chitosan with DA 12% showed ability to inhibit bacterial growth even when all the chitosans were tested at the same charge equivalent concentration. Structural features of chitosan, hydrogen bond and hydrophobic interactions could affect the antibacterial activity of chitosan. The MDP DA30 (DA 42% and M_w 115 kDa) chitosan when dosed at low concentration has QS inhibition activity of our *E.coli* bioreporter because of the bacterial agglutination. The practical implications of these results, are yet to be fully realized.

5. Materials and Methods

5.1. Materials

The parent chitosan with a molecular weight of 288 kDa and DA of 16% was provided by Mahtani Chitosan Pvt. Ltd., India (brand name: Chitosan 132; batch no. SCCF 20140609). The chitosan samples derive all of them from the same parent chitosan. They have been depolymerized and if needed, re-acetylated, both by chemical methods, and purified. This has been conducted in our laboratory according with the methods described in our previous study [33]. The DA was determined by ^{1}H NMR by dissolving the chitosans in D_2O with DCl (at pD 3–4) according with the method by Lavertu *et al* [34]. The molecular weight distribution and corresponding M_w, M_n and I_p parameters were determined via HPSEC-MALLS-DRI (Polymer Standards Service GmbH, Germany) using an Agilent 1200 system with isocratic pump and Novema columns (30Å, 3000Å, 3000Å and guard column; I.D.: 8 mm; PSS, city, country) coupled online with a refractive index detector (Agilent 1200 RID, city, country) and multi-angle-laser-light-scattering (SLD 7000 MALLS, Brookhaven Instruments, Warwick, England) equipped with a 5 mW He/Ne laser operating at λ = 632.8 nm. The degassed mobile phase consisted of ammonium acetate 0.2 M/acetic acid 0.15 M buffer pH 4.5 at a flow of 0.7 mL/min at 35°C. Data were evaluated using the software WinGPC 7.0.1 (PSS Polymer, city, Germany). Chitosans with M_w of ~185-~288, ~85-~115, and ~29-~58 were regarded here as of high (HDP), medium (MDP), and low degree of polymerization (LDP), respectively. The rest of the identification code was given by the DA (e.g. HDP DA12, etc.). 3OC_6HSL and all other chemicals were of analytical grade and unless otherwise stated were from Sigma (Sigma-Aldrich, Hamburg, Germany).

5.2. Chitosan samples preparation

The chitosan samples in powder form were fully dissolved in aqueous 5% stoichiometric excess of hydrochloric acid by gentle magnetic stirring (~10 h). In order to test the antibacterial activity of different chitosan samples, the same concentration of amino group of chitosan were prepared and applied in this assay. The final concentration of amino group was defined as 0.389 or 0.778 mM for all samples. However, the mass concentration of samples were different according to the DA as were shown in Table 1

5.3. Bacterial strains

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E. coli Top10 was transformed with the plasmid pSB1A3-BBa_T9002, carrying the BBa_T9002 genetic device (Registry of Standard Biological Parts: http://parts.igem.org/Part:BBa_T9002), kindly donated by Prof. Anderson Lab (UC Berkeley, USA). The sequence BBa_T9002 was introduced by chemical transformation (Invitrogen, Life Technologies Co., UK) and stored as a 30 % glycerol stock at –80 ºC. The transformed strain is a biosensor that can respond to the N-(3-oxohexanoyl)-L-homoserine lactone (3OC6HSL) and is the same strain used in accompanying studies [29]. The sequence BBa_T9002, comprised the transcription factor (LuxR), under the control of the lux pR promoter from *Vibrio fischeri*, which is constitutively expressed but it is active only in the presence of the exogenous cell-cell signaling molecule 3OC6HSL.

5.4. Growth media and conditions

Bacterial strains were cultivated using on Luria-Bertani (LB) and M9 minimal medium purchased from Becton, Dickinson and company, Germany. We inoculated 10 mL of LB broth supplemented with 200 μg/mL ampicillin with a single colony from a freshly streaked plate of Top10 containing BBa_T9002 and incubated the culture for 18 h at 37ºC, shaking at 100 rpm. Each culture was then diluted 1:1000 into 20 mL M9 minimal medium supplemented with 0.2% casamino acids and 1 mM thiamine hydrochloride plus 200 μg/mL ampicillin (AppliChem GmbH, Germany). The culture was maintained under the same conditions until the OD_{600} reached 0.15 (~5 h). Then we mixed 500 μL overnight culture and 500μl 30% sterile glycerol together in the white plastic vials and store them in the -80 degree freeze. Before the biosensor assay, we prepared the bacteria cultivation that we took the 40μL bacterial from the white plastic vials and cultivated it with 20 mL M9 medium plus 200 μg/mL ampicillin, the culture will be used for biosensor assay until the OD_{600} reached 0.04~0.07 (~4 h).

5.5. Assays for antibacterial activity

The antibacterial activity of chitosan was tested by two different methods. The first consisted in recording the time evolution of the absorbance intensity (=600 nm, OD_{600}) during the incubation of *E.coli* in culture medium. Briefly, bacteria were cultivated in M9 medium to reach an OD_{600} ~0.04-0.07. In a flat bottomed 96-well-plate (Greiner Bio-One), a 180 μL aliquot of *E. coli* culture was mixed with 20 μL of the different chitosan solutions with final amine group concentrations of 0.389 mM. As control, 20 μL of the chitosan solutions were mixed with 180 μL M9 medium. Three different blanks were also prepared, namely 180 μL *E. coli* added with 20 μL water as a negative control, 180 μL M9 medium added with 20 μL water to measure the absorbance background, and 180 μL *E. coli* added with 20 μL kanamycin (AppliChem, 0.5 mg/mL) as a positive control. The OD_{600} of the microtiter plate was measured with a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim) every 158 s for 60 cycles (2:38 h total time) at 37 °C. Afterwards, aliquots of 20 μL were sampled from the microplate wells and remixed with fresh M9 medium and further measured the evolution of A_{600} during 2:38 h. Data were processed with software XFLUOR4 version 4.51. The obtained data were corrected by subtracting the corresponding chitosan control and the difference between the bacterial blank and the medium blank. The growth rate of control and samples was obtained and analyzed from the original data. Using a log-linear model to regress OD_{600} (log phase) with respect to time (0-100 mins), and thus obtain the growth rate of OD_{600} directly from the slope of the estimated regression equation. Relative data were expressed as the ratio of each measurement to the corresponding control group.

The second assay aimed to estimate the number of colony forming units (CFU). To this end, a culture was prepared by mixing 20 mL LB medium, 20 µL ampicillin (200 µg/mL, AppliChem, Darmstadt) and 40 µL of *E. coli* glycerol stock, incubated overnight at 37 °C with shaking at 100 rpm. The OD_{600} was adjusted to ~0.1 using LB medium, and 180 µL of the diluted *E. coli* culture were incubated with 20 µL of one each chitosan solutions of identical equivalent charge concentration (0.389 or 0.778 mM) in a 96-well-plate at 37 °C and 100 rpm for 1 h. Afterwards, a serial dilution was performed with 180 µL M9 medium. Then, all the dilutions were plated in LB-ampicillin-agar-plates by adding a drop of 10 µL. The plates were incubated overnight at 37 °C and the colonies were counted the next day. A weighted average was calculated from the dilutions.

5.6. E. coli Top10 biosensor assay

The $3OC_6HSL$ was dissolved in acetonitrile to a stock concentration of 100 mM and stored at 20ºC. Prior to each experiment, serial dilutions from the stock solution were prepared in water to produce solutions with a concentration ranging from 100 mM to 10 nM. For a typical biosensor assay, 40 µL of the bacterial glycerol stock were cultivated with 20 mL M9 medium added with 200 µg/mL ampicillin until an OD600 between 0.04 and 0.07 (~4 h). The growth of the bacteria was monitored by measuring the optical density at λ = 600 nm (OD_{600}) on a Microplate Reader Safire F129013 (Tecan, Crailsheim, Germany).

We then mixed 10 µL $3OC_6HSL$ solution with 10 µL of the MDP DA 42% chitosan at varying concentration (1.25, 2.5, 4.17, 8.33, 12.5, 25 and 50 µg/mL) in the wells of a flat-bottomed 96-well plate (Greiner Bio-One, cat. # M3061) and each well was then filled with 180 µL aliquots of the bacterial culture to test for QS inhibition activity. Several controls were also set up. Blank 1 contained 180 µL of M9 medium and 20 µL of miliQ water to measure the absorbance background. Blank 2 wells contained 180 µL of bacterial culture and 20 µL of milliQ water to measure the absorbance background corrected for the cells. Finally, positive control wells contained 10 µL of water plus 10 µL $3OC_6HSL$ solution and 180 µL of the bacterial culture to measure the fluorescence background. In order to remove the effect of samples themselves with OD_{600} and fluorescence, we added 10 µL $3OC_6HSL$ solution with 10 µL of the samples in the wells and each well was then filled with 180 µL M9 medium to test the samples control in biosensor assay.

The plates were incubated in a Safire Tecan-F129013 Microplate Reader (Tecan, Crailsheim, Germany) at 37ºC and fluorescence measurements were taken automatically using a repeating procedure (λ_{ex}=480 nm and λ_{em}=510nm, 40 µs, 10 flashes, gain 100, top fluorescence), absorbance measurements (OD_{600}) (λ=600 nm absorbance filter, 10 flashes) and shaking (5 s, orbital shaking, high speed). The interval between measurements was 6 min. For each experiment, the fluorescence intensity (FI) and OD_{600} data were corrected by subtracting the values of absorbance and fluorescence backgrounds and expressed as the average for each treatment. All measurements were taken in triplicate.

5.7. Scanning electron microscopy (SEM).

A five mL suspension of bacteria (OD_{600} = 0.2) was mixed with different concentrations of MDP DA30 chitosan (2.95, 5.88, 8.79, 28.7 and 226.6 µg/mL). The mixture was kept at 4°C and under 100 rpm shaking for one hour. Subsequently, the mixtures were filtered through a polycarbonate membrane with 1.2 µm track-etched pores mounted to a funnel assembly connected to a vacuum pump. After filtering, the material was fixed in 2.5 % aqueous glutaraldehyde for 1 h; then the

membranes were washed with PBS one time for 10 min after fixing. Finally, the samples were dehydrated through a graded series of aqueous ethanol solutions (10, 30, 50, 70, 90, and 100 % v/v) for 10 min and kept in a sealed desiccator until complete dehydration. Dilute samples of NC and NE for SEM were dropped on a smooth and sterile glass and then kept in a sealed desiccator for two days. All samples were sputter coated with gold and imaged in the SEM (S-3000N, Hitachi, Tokio). Micrographs were recorded digitally.

5.8. Statistical analysis

Statistical analysis was carried out using Prism v6.0c (GraphPad Software Inc., La Jolla, USA). All the experiments were performed in triplicates to validate reproducibility and the *P* values were calculated statistically by Student's *t* test. Values were expressed as mean ±SD. Comparison analysis was performed between tests and control.

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