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RESEARCH ARTICLE

Effect of Mass and Aspect Heterogeneity of Chitosan Nanoparticles on Bactericidal Activity

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Abstract

Introduction

Owing to the emergence of new causative agents coupled with development of multidrug-resistant strains, bacterial infections are still the major cause of death in the developing and developed world. Although, few antibiotics have proved effective against multidrug-resistant bacteria (Mandal et al., 2014), these pathogens have evolved effective mechanisms to counter the biocidal action of antibiotics. Hence, it becomes imperative to design and develop novel microbial agents in order to overcome these limitations. Nanoparticles are successfully being exploited as neo-generation delivery systems (Zhang et al., 2008), to reduce bacterial burden (Mohanty et al., 2007; Rai et al., 2009), in diagnostics for chronic disease (Hong et al., 2008; Verma et al., 2011), in the textile and food industries as antimicrobial agents (Vigneshwaran et al., 2007). Biopolymeric ChNPs encapsulating culture filtrate proteins (CFP) of *Mycobacterium tuberculosis* have been successfully used to enhance the immune response in mice (Verma et al., 2011).

Chitosan (α-(1–4)-2-amino-2-deoxy-β-D-glucan), the N-deacetylated derivative of chitin, is an abundant, a renewable, nontoxic and biodegradable carbohydrate polymer that is largely present in the exoskeletons of shellfish, insects and crustaceans like shrimp, crabs and lobsters (Panos et al., 2008). Various biological activities of ChNPs have been reported including immunoenhancing effects (Verma et al., 2013), antitumoral, antifungal, and antimicrobial activities (Dutta et al., 2004). The bacteriocidal action of chitin, chitosan and their derivatives against several groups of microorganisms, such as bacteria, yeast and fungi, have received considerable attention (Khanafari et al., 2008; Li et al., 1992; Kamala K, 2013). The antimicrobial activities of chitosan are greatly dependent on molecular weight (Mw) and degree of deacetylation (DD) (Khanafari et al., 2008; Li et al., 1992). The organism's size, shape, pH, presence of lipids and proteins affect the activity of chitosan and ChNPs (No et al., 2002; Rabea et al., 2003). Although, synthesis and activity of ChNPs has been reported, data on size-dependent antibacterial activity is surprisingly lacking.

This work aims to formulate chitosan nanoparticles of different sizes and to evaluate its potential against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive), and to further elucidate the mechanism of bacteriocidal activity of ChNPs, if any. The biocompatibility of the ChNPs was also investigated on HEK (Human Embryonic Kidney) and SiHa (Human cervical cancer) cell lines.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Chitosan (~85% deacetylated, Mw ~50 kDa), tri-polyphosphate (TPP), (3-(4,5-dimethyathiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulphoxide (DMSO), 1-Chloro,2,4-Dinitrobenzene (CDNB), Sodium bicarbonate, Sodium nitrite ($NaNO_2$) and HEPES (Hydroxy ethyl piperazine ethane sulphonic acid) were purchased from Sigma-Aldrich (USA). Nutrient Broth and Agarose were procured from Conda Laboratories (Madrid, Spain); Nutrient agar, Penicillin, Streptomycin, Proteinase K, Sodium acetate, Ethidium bromide and Gentamycin were purchased from Hi Media Lab. Pvt. Ltd. (Mumbai, India). Bacterial cultures (*E. coli*-DH5α) and (*S. aureus* ATCC 13709 native strain) were procured from PGI, Chandigarh. All the experiments were done using double-distilled and deionized water.

2.2 Preparation of nanoparticles

Chitosan nanoparticles were prepared by modifying our previously published protocol (Verma AK et al., 2011). Briefly, 0.1% chitosan (~85% Deacetylated) solution in 1% acetic acid was prepared and incubated with different concentrations (8 M, 3 M, 0.5 M, 0.05 M) of sodium nitrite (Dhawade and Jagtap, 2012; Suitcharit et al., 2011; Liu and Gao, 2009). The above solution was stirred slowly at 200rpm for 30 min at room temperature and the reaction was neutralized with 0.1 M NaOH. Subsequently, the chitosan solution was titrated with 1% aqueous solution of sodium salt of tri-polyphosphate (sTPP). The sTPP titrated chitosan solution was further stirred mildly (500rpm) for 24h at room temperature. After high speed centrifugation at 10,000 rpm for 20 min, the pellet was collected and resuspended in 0.5% of acetic acid (Fig 1). By altering the concentration of $NaNO_2$ and adjusting the mass ratio of chitosan and sTPP, heterogeneous nanoparticles were prepared. The nanoparticles were lyophilized and stored at room temperature for all further experiments.

2.3 Characterization of chitosan nanoparticles

The sizes of various ChNPs were observed by Zetasizer (Nano-ZS, Malvern USA) (Verma et al., 2005). Briefly, the sample solution was diluted with deionized water to isolate all individual nanoparticles from the aggregates to assess the distribution based on size and surface charge of nanoparticles. The migration voltage was fixed at 100 mV for all measurements. The instrument was calibrated against 10^{-4}M AgI colloidal dispersions.

2.4 Biological Applications

2.4.1 Preparation of bacterial culture

Both the bacterial cultures were maintained in nutrient agar at 37°C. Bacterial inoculations were prepared to 0.5 McFarland standards before performing the antimicrobial assays (Ip et al., 2006). Briefly, the primary inoculation was performed in nutrient broth at 37°C and was kept overnight at 180rpm in the shaker incubator. Secondary inoculation was further done and the flask was incubated at 37°C for 4h. The turbidity of the resulting suspension was observed and optical density (O.D) was measured at 600 nm by using UV spectrophotometer (Agilent technology, Germany). These bacterial cultures were used for further experiments.

2.4.2 Bactericidal assay

ChNPs of different sizes were tested for bacteriocidal activity by disc diffusion assay (Kirbey-bauer method) against *E. coli* and *S. aureus*. Briefly, hard agar using Luria agar (3.5 %) and Agar agar (0.8 %) were prepared and autoclaved. After cooling, the hard agar was poured onto the petri-plates and was allowed to solidify. Agar agar containing bacterial inoculum was prepared in the ratio of 1:100. This was evenly poured on the previously prepared solidified agar plates (2 ml/petri-plate). 20 µL of ChNPs were impregnated onto 3 mm sterile blank discs and allowed to dry before placing onto the culture plates on the marked areas. Plates inoculated with bacteria were incubated overnight at 37°C. Bacteriocidal activity was directly proportional to the diameter of inhibition zone. Gentamycin sulphate (1×10^{-3} µg/ml) was used as a positive control (Singhal et al., 2006, Rawat et al., 2014).

2.4.3 Determination of MIC

The minimum inhibitory concentration (MIC) of ChNPs was determined using turbidmetric method (Nester et al., 2003). Briefly, test tubes containing 5.0 mL of Muller–Hinton broth (MHB, Difco, England) were prepared and autoclaved. Chitosan nanoparticles of different sizes (5.0 ml; 1mg/ml) were added to these tubes separately and the mixture was further transferred to the second tube with serial dilutions. The experimental tubes were then inoculated under aseptic conditions with 50 µL of the bacterial suspension and were allowed to incubate at 37^0C for 24h. The lowest concentration of chitosan nanoparticles that inhibited the growth of bacteria in terms of turbidity was considered as MIC (Ruparelia et al., 2008), was calculated for all the samples.

2.4.4 Antimicrobial tolerance

The inhibitory and bactericidal activities of ChNPs were noted. The MBC/MIC ratios were calculated to determine the presence or absence of tolerance. Tolerance is generally characterized by two parameters, bactericidal and

bacteriostatic activity and is represented as a ratio of the minimum inhibitory (MBC)/MIC ratio. The effect was considered as bactericidal if MBC/MIC ratio ≤2, but if ratio was ≤4 the effect was defined as bacteriostatic (Gaillard et al., 1988**).**

2.4.5 DNA Fragmentation Assay

The effect of size variation of ChNPs on the mechanism of death of the bacteria was determined by DNA fragmentation (Jena et al., 2012). Briefly, genomic DNA was isolated by the phenol-chloroform extraction method after 2h, 4h 6h, 8h and 24h post treatment of ChNPs. Briefly, the pellet of the treated bacterial strains were washed with PBS, lysed by the addition of lysis buffer (10 mM EDTA, 0.5% Triton® X-100, 5 mM Tris-HCl, pH 8.0) and incubated on ice for 30 min. Samples were centrifuged and the supernatant was treated with ribonuclease A for 1h, followed by digestion with proteinase K (0.5 mg/mL) (Roche, Mannheim, Germany) for 3h at 50°C. Phenol-chloroform-isoamyl alcohol (25:24:1) extraction was performed and the DNA was precipitated with 2.5 volumes of 95% ethanol and one-tenth volume of 3 M sodium acetate (pH 5.2). DNA was resolved and electrophoresed in 1.5% agarose gel at 40 V (Tao et al., 2011; Qi et al., 2007). The gel was stained with ethidium bromide and DNA fragments were visualized under UV light.

2.4.6 Cell line and cell culture

Cervical cancer cells (SiHa) and Human Embryonic Kidney (HEK) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The cells were grown at 37ºC in a humidified atmosphere of 5% CO_2 in CO_2 incubator (Thermo).

2.4.7 *In vitro* Biocompatibility Evaluation

The biocompatibilities of ChNPs were measured by previously published protocol (Verma et al., 2005). Briefly, $5x10^3$cells/well were plated in a microplate, supplemented with 2.5% FCS and incubated with various concentrations of ChNPs i.e. 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml for 24h, 48h and 72h. After the requisite time period, 20 µl of MTT solution (5 mg/ml in PBS pH 7.4) was added to each well. 4 hours post incubation of ChNPs with MTT, formazan crystals were observed. The crystals were formed by the cellular reduction of MTT and were further dissolved in 100 µl of DMSO. After mixing with a mechanical plate mixer, the optical density was read at 540 nm wavelength on an ELISA-reader (SynergyHT, Biotek, USA).

All measurements were done in triplicates. The percent cytotoxicity values were determined by

$$\% \text{ Biocompatibility} = \frac{[A]\text{control} - [A]\text{test}}{[A]\text{control}} x100 \quad \text{..... (i)}$$

Where, [A]test is absorbance of the test sample and [A]control is the absorbance of the control sample.

3. RESULTS

The ChNPs were successfully prepared by conventional ionic gelation method using sTPP as a cross linker and various amounts of $NaNO_2$, i.e. 8 M, 3 M, 0.5 M, and 0.05 M (Table 1). The morphological assessment of different sized ChNPs was done by Dynamic Light scattering (DLS) experiments. The mean diameters of various nanoparticles were in the range ~50.59 nm, ~80 nm, ~111.4 nm and ~245.9 nm with a polydispersity index of 0.21, 0.213, 0.365 and 0.245, respectively (Fig 2). Zeta potential clearly indicates that the particles were positively charged having zeta potential ranging from +19.7 mV to +41 mV (Fig 3).

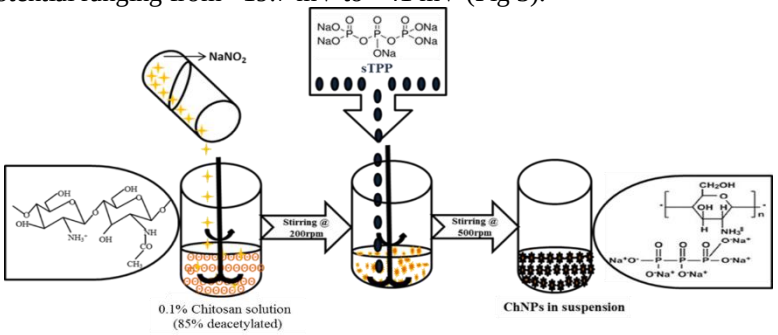

Fig.1 Schematic representation of preparation of Chitosan nanoparticles by ionic gelation method

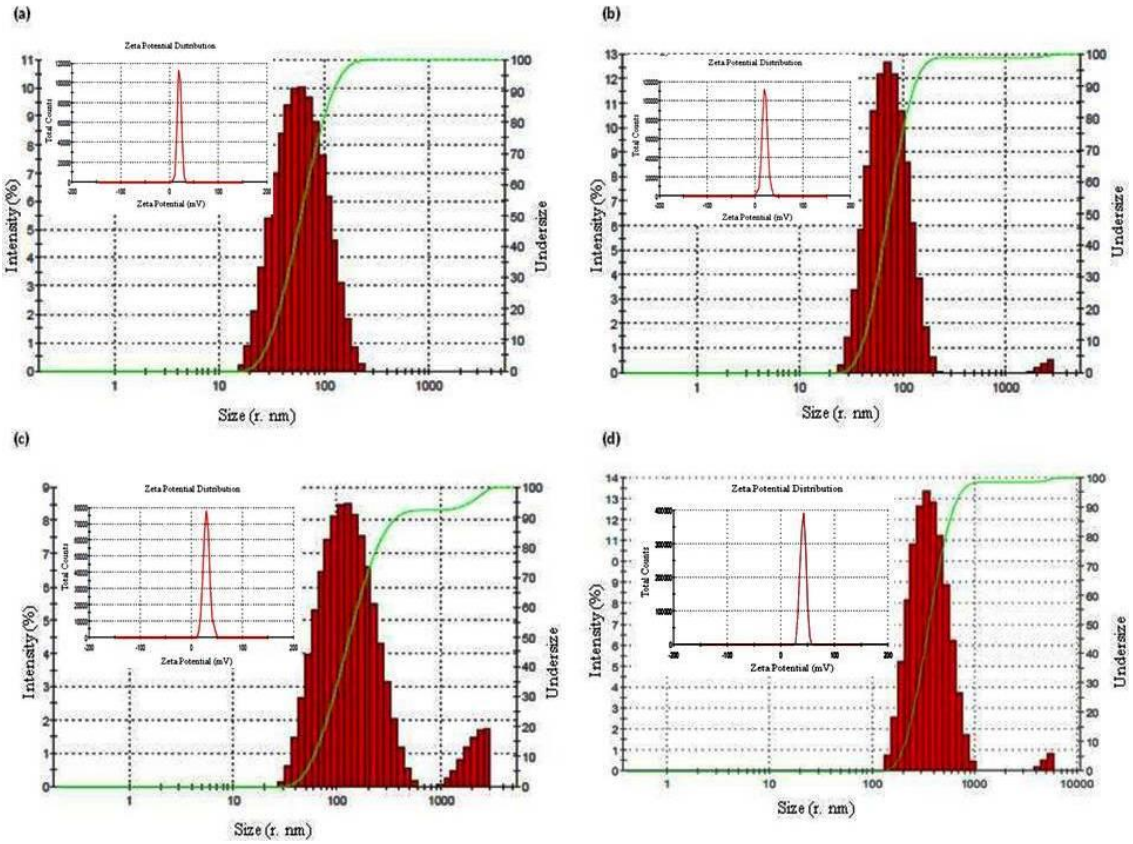

Fig 2. DLS measurements showing (a) mean diameter (51.97 nm) with polydispersity index 0.219 and ζ = +19.7 mV , (b) mean diameter (80 nm) with polydispersity index 0.213 and ζ = +20 mV, (c) mean diameter (111.4 nm) with polydispersity index 0.365 and ζ = +29.3 mV, and (d) mean diameter (245.9 nm) with polydispersity index 0.245 and ζ = +41 mV

Antimicrobial activity of Chitosan nanoparticles

The results of the antimicrobial activities of different sized ChNPs using disc diffusion assay and DNA fragmentation assay are represented in (Fig 4 and Fig 5).

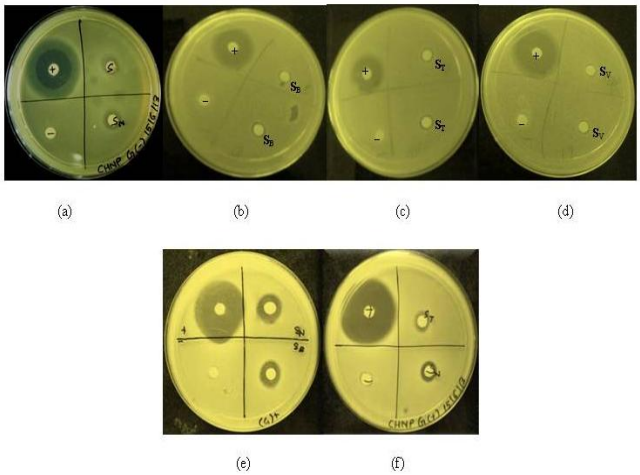

Fig 4. Antimicrobial activity of different nanoparticles against (a - d) Gram-negative and (e - f) Gram-positive bacteria, *E. coli* and *S. aureus* (a) sample (S_N) =80.0 nm, sample (S_B) =111.4 nm, sample (S_T) =245.9 nm, sample (S_V) = 51.97 nm, (-) negative control; distilled water, (+) positive control; Gentamycin.

The results in **Table 1** indicated that size variation in ChNPs exhibited interesting bacteriocidal effects. As, the size of the zone of inhibition was directly proportional to the bacteriocidal activity of ChNPs. The largest inhibition zone of 2±0.11 nm was observed in *E. coli* by ChNPs of 80nm followed by ChNPs of 111.4 nm, 51.97 nm and 245.9 nm with inhibition zones 1±0.77 mm, 1±0.56 mm and 1±0.12 mm respectively. But, in *S. aureus*, both 80.0 nm ChNPs and 111.4 nm ChNPs exhibited the highest zone of inhibition with 4±0.55 mm and 4±0.54 mm, followed by 245.9 nm and 51.97 nm with 2±0.21 mm and 1±0.23 mm inhibition zones respectively.

The results in **Table 2** indicated Minimum inhibitory concentration (MIC) of ChNPs wherein the MIC of 80 nm and 111.4 nm sized ChNPs were higher (i.e. ≤12.5 µg/mL for both) as compared to 51.97nm (i.e. <50 µg/mL) and 245.9 nm (i.e. ≤50 µg/mL) for *S. aureus*. Similar results were observed in *E. coli* where MIC exhibited by 80 nm and 111.4 nm sized ChNPs was 75 µg/mL and <75 µg/mL respectively followed by 51.97nm (i.e. <81.25 µg/mL) and 245.9 nm (i.e. 81.25 µg/mL).

Table 1: Size-dependent killing of bacterial strains by different Chitosan nanoparticles

Chitosan Nanoparticles Size(nm)	Concentration of $NaNO_2$(M)	PDI	Zeta Potential (ζ) (mV)	Zone of Inhibition (mm) *E. coli*	*S. aureus*	Positive Control
51.97	8	0.21	+19.7	1±0.56	1±0.23	10±0.11
80.0	3	0.213	+20.0	2±0.11	4±0.55	10±0.20
111.4	0.5	0.365	+29.3	1±0.77	4±0.04	10±0.34
245.9	0.05	0.245	+41.0	1±0.12	2±0.21	10±0.06

(-) = No zone of inhibition.
Values are given as means±SD of three experiments.
Positive control (Gentamycin, 1mg/ml); Negative control (Distilled water).

Table 2: Quantitative antimicrobial activity of chitosan nanoparticles

Test Organism	MIC (µg/ml)				MBC (µg/ml)				MBC/ MIC
	ChNP-51.97	ChNP-80	ChNP-111.4	ChNP-245.9	ChNP-51.97	ChNP-80	ChNP-111.4	ChNP-245.9	
E. coli	<150	75	<75	150	≥300	150	150	300	≤2
S. aureus	<50	≤12.5	≤12.5	≤50	≥100	25	25	100	2

DNA fragmentation assay

The mechanism of death has been assessed by DNA fragmentation assay (Fig 5) where the characteristic pattern of DNA ladder by intermediate sized nanoparticles clearly indicated significant DNA damage in both *E. coli* and *S. aureus*.

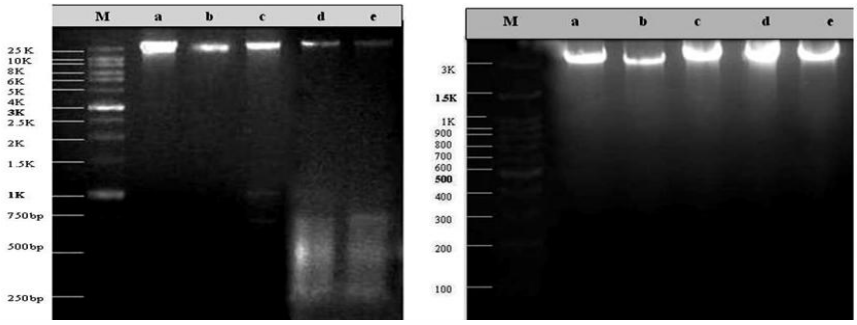

Fig 5. DNA fragmentation assay (A) showing *E. coli* and *S. aureus* treated with different ChNPs at 24h (M) 250-25K bp Marker, (a-b) *E. coli* treated with ChNPs of 80 nm and 111 nm size, (c-e) *S. aureus* treated with 50 nm, 80 nm, and 111 nm size ChNPs; (B) HEK and SiHa cells treated with different ChNPs at 24h (M) 100bp-3K Marker, (a) Control, (b) 50nm for 24h, (c) 80 nm for 24h, (d) 80 nm for 24h, (e) 111 nm for 24h.

Cell viability assay

Assessments of cytotoxicity of ChNPs were performed in HEK cell line and SiHa cell lines (Fig 6).

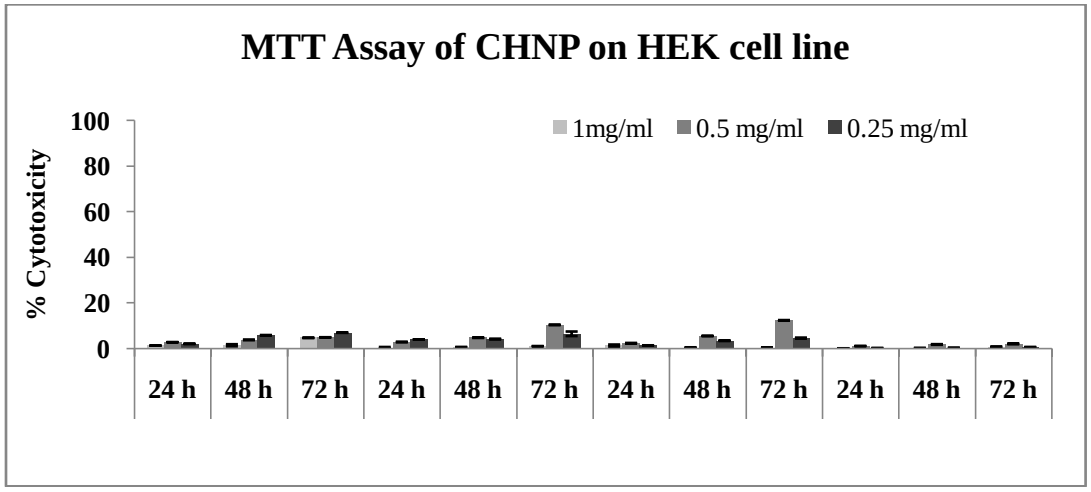

Fig 6a. Cytotoxicity of Chitosan nanoparticles on HEK (Human Embryonic Kidney) cell line in 24h, 48h and 72h MTT assay. ChNPs 51.97 nm showing ~5% of cytotoxicity, 80 nm = ~1%, 111.4 nm = ~0.2% and 245.9 nm = ~0.6%. Negligible cytotoxicity was observed by all the nanoparticles.

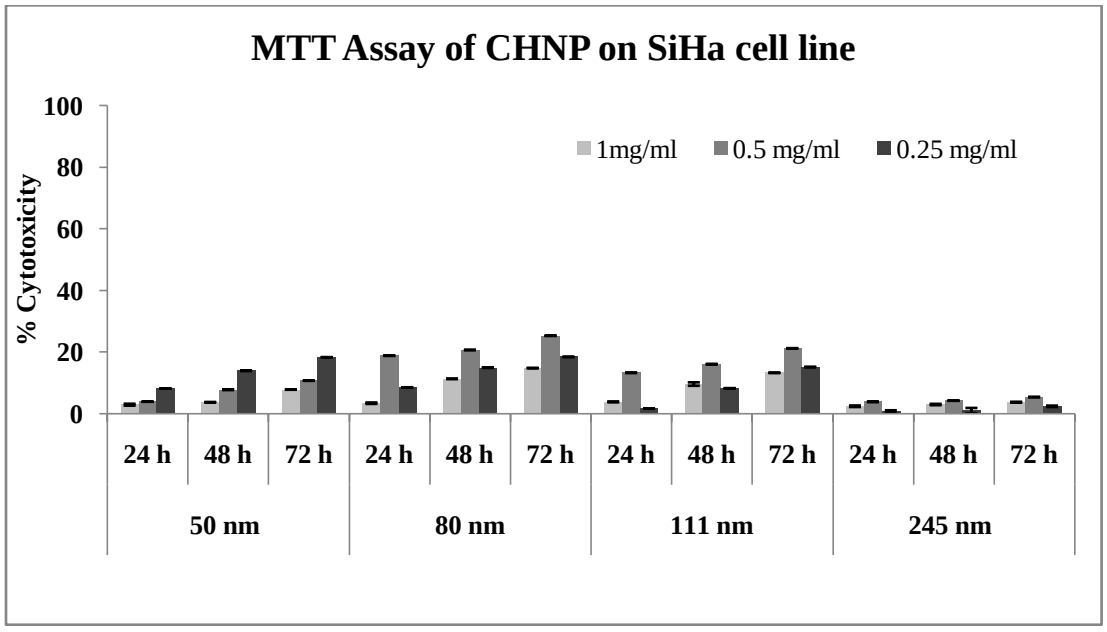

Fig 6b. Cytotoxicity of Chitosan nanoparticles on SiHa (Human cervical cancer) cell line in 24h, 48h and 72h MTT assay. ChNPs of 50 nm and 245 nm showed ~8% and ~4% cytotoxicity whereas enhanced cytotoxicity (~15%) was observed in 80 nm and (~13%) 111 nm. Maximum toxicity was observed in ChNPs of 80 nm.

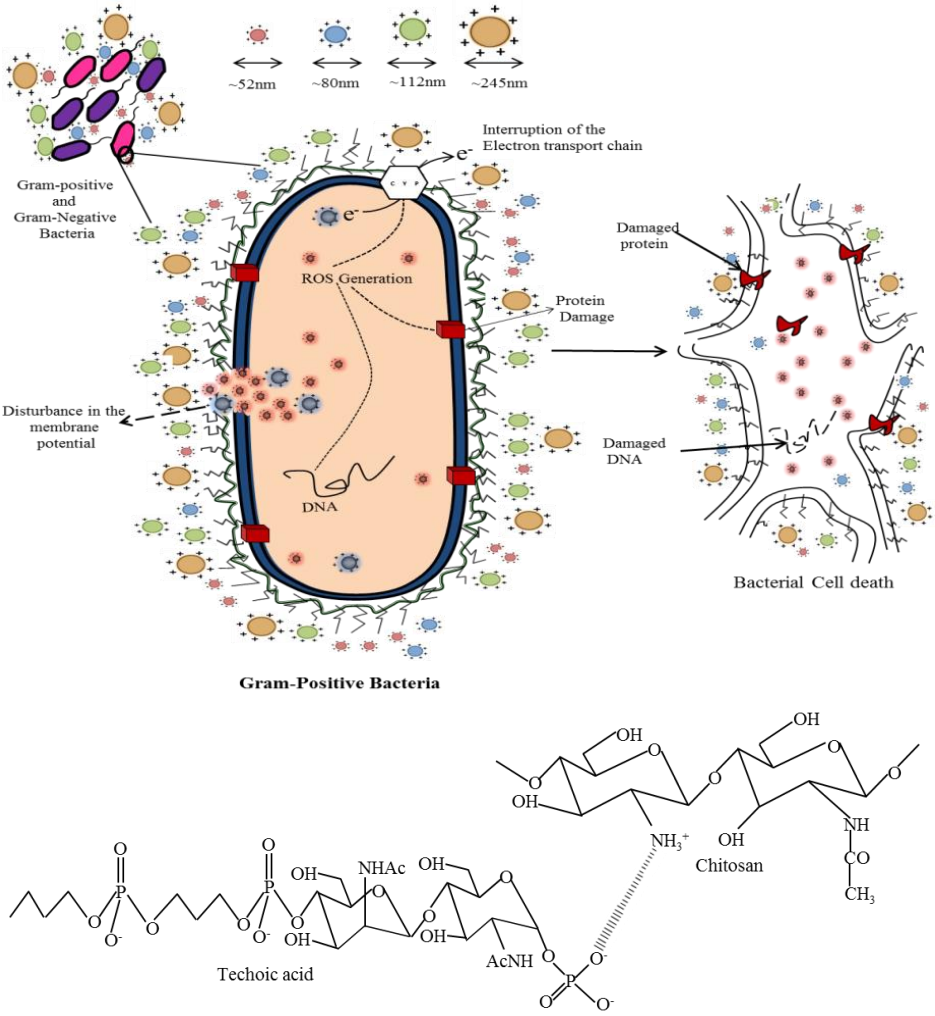

Fig 7. Cartoon illustrating antimicrobial potential of Chitosan nanoparticles on *S. aureus*

4. Discussion

Since pathogens have successfully infected humans over centuries by adapting various strategies to combat the effect of anti-infective agents, and constantly acquiring resistance to various drugs, novel and effective therapeutic strategies have to be evolved to overcome these challenges. In the recent decades, nanoparticles have received considerable attention, largely due to their broad range of bioactivities and distinctive mode of action (Verma et al., 2008). The bacteriocidal activity has been reported to be dependent on the molecular weight, ionic strength, temperature, pH and the methodology adopted for ChNPs preparations (Liu N et al., 2006; Chung et al., 2003). Owing to its polycationic nature, chitosan polymer possibly interferes with the metabolism of bacteria by electrostatic stacking at the cell surface of bacteria (Chung et al. 2004; Je and Kim, 2006) leading to inhibition of growth of the microbial cells. Moreover, ChNPs may also penetrate the cell membrane to block the transcription by interfering with the DNA, resulting in killing of the bacteria (Liu, XF et al, 2001). Since, the effect of size variability has not been evaluated based on the variation in salt concentration, we hereby report the bacteriocidal activity of various ChNPs by disc diffusion assays on both Gram-negative (*E. coli*) and Gram-positive bacteria (*S. aureus*).

Presuming that the exact mechanism of bactericidal activity of ChNPs was size dependent, we tried to predict a possible interaction pattern of polycationic ChNPs with the negatively charged teichoic acid of bacteria (Fig 7). At weakly acidic conditions, chitosan becomes protonated due to the presence of large number of C_2 amino groups having pKa values of ~6.5. The pKa values may also depend on the degree of deacetylation of chitosan. The interaction leads to decreased osmotic stability causing membrane disruption. This may effectively lead to the alterations in the cell permeability and cause perturbations in the membrane integrity (Chen et al., 1998; Lifeng et al., 2004). Also, the positively charged chitosan interacts with the negatively charged teichoic acids in the cell wall

of gram-positive microbes leading to the formation of a small pores with subsequent leakage of the intracellular components (Banergee et al., 2010,; Ma et al., 2008; Sanpui et al., 2008).

It has been repoted that at pH values above 6, the activity of ChNPs was lost due to protonation (Lifeng et al., 2004). This discrepancy may be due to the differences in the membrane structure and the composition of the cell wall. The cell membranes of gram-negative and gram-positive bacteria are negatively charged due to the presence of lipopolysaccharide (LPS) and teichoic acids respectively (Jena et al., 2012). The bactericidal activity of 80 nm and 110 nm ChNPs against *E. coli* and *S. aureus* could be due to the susceptibility of the bacteria to an optimum size of cationic ChNPs. The antimicrobial effect on *E. coli* and *S. aureus* was evident by the observed DNA ladder (Fig 5). But the susceptibility of *S. aureus* was particularly evident as a characteristic DNA ladder pattern was observed.

The cartoon illustrates the internalization of the ChNPs. The ChNPs of 50 nm are most likely to get entangled in the teichoic acid and lipopolysaccharide. The ChNPs of ~250 nm are much larger than the bacterial pores. They are internalized only when cell membrane gives way due to perturbations. The ~80 nm and 110 nm sized ChNPs are optimal sized and are able to penetrate the walls.

The biocompatibility studies of heterogeneous ChNPs were performed using tumor and non-tumor cell lines, SiHa and HEK cell lines, respectively. Our results clearly proved that the activity of ChNPs against the tumor cells were higher when compared to non tumor cells. The MTT endpoints showed low cytotoxic effects of ChNPs of all sizes ranging from 51.97 nm showing ~5% cytotoxicity, 80 nm = ~1% , 111.4 nm= ~0.2% and 245.9 nm= ~0.6% in HEK whereas enhanced cytotoxicity by up to ~8%, ~15%, ~13%, and ~4% SiHa cells, respectively. Hence, we can conclude that the non tumor HEK cell line was not susceptible to ChNPs with observed cytotoxicity higher than ~5%. Extermination of the target without altering the viability of the normal cells is the foremost requisite for any biomolecules to be used as a therapeutic agent. Despite being the most proposed as a biologically safe material, chitosan is restricted by FDA to be used as a therapeutic agent (Kean and Thanou, 2010), possibly due to its immunogenic activity (Verma et al., 2011). Moreover, it has been observed that intermediate sized ChNPs showed ~15% and ~13% cytotoxicity in SiHa cell line. ChNPs clearly had no effect on DNA, hence the mechanism of death if any, warrants further investigation.

5. Conclusion

We proposed that size heterogeneity may be considered as an important parameter to enhance the bacteriocidal activity of ChNPs. Our results clearly proved that ChNPs in the size range of ~50 nm to ~250 nm having charge range from +12 mV to +41 mV are effective anti-microbials. Moreover, antimicrobial activity was slightly enhanced in gram positive bacteria when compared to gram negative bacteria with 80 nm sized ChNPs as observed by zone inhibition assay which was further confirmed by obtained MIC (Kim et al., 2011). Significant DNA fragmentation was observed in both *E. coli* and *S. aureus*. Biological activity was assessed by MTT assay that conclusively proved the biocompatibility of the various ChNPs. The biocompatibilities of ChNPs were further confirmed by absence of DNA ladder as assessed by DNA fragmentation assay. The present study concludes that size heterogeneity of ChNPs exhibit potent bacteriocidal activity but does not show cytotoxicity on mammalian cells. Both prokaryotic cells and eukaryotic cells have interestingly exhibited size preferences wherein 80 nm and 110 nm ChNPs were optimally internalized when compared to 50 nm and 250 nm. These nanoparticles have the potential to be used as antimicrobial agents in membranes, surface coating of water tanks and sponges (Li D et al., 2008). This makes the mechanistic studies inevitable to exploit the potential of ChNPs.

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Conflicts of Interest:

None declared.

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