Synthesis and antibacterial activity of colloidal selenium nanoparticles in chitosan solution: a new antibacterial agent

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Keywords: selenium, chitosan, nanoparticles, antibacterial

Abstract
High incidence of bacterial infections and antibiotic resistance as a growing problem has urged the need for novel antibacterial agents. The main purpose of this in vitro study was to evaluate the antibacterial activity of the chitosan-based selenium nanoparticles (Cts-Se-NPs) solution against gram-positive and gram-negative bacteria. The Cts-Se-NPs solution was synthesized using a simple chemical reduction method and characterized using ultraviolet-visible spectrophotometry, a particle size analyzer, and atomic force microscopy. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the Cts-Se-NPs solution were determined against gram-negative (i.e., Pseudomonas aeruginosa, Salmonella typhimurium, and Escherichia coli) and gram-positive (i.e., Streptococcus sanguinis, Staphylococcus aureus and Enterococcus faecalis) bacteria using a broth microdilution method. According to the results, S. sanguinis, S. aureus, and E. faecalis showed MIC values of 0.068, 0.137, and 0.274 mg ml$^{-1}$, respectively. Moreover, the results revealed that the concentration of 0.274 mg ml$^{-1}$ had a higher bactericidal effect than the other concentrations. As the concentration of Cts-Se-NPs increased to 0.274 mg ml$^{-1}$, S. sanguinis, S. aureus, and E. faecalis were completely killed after 1, 2, and 6 h, respectively. Further, S. aureus and S. sanguinis were totally killed after 24 and 6 h, respectively at the concentration of 0.137 mg ml$^{-1}$. The Cts-Se-NPs solution did not elicit any significant antibacterial effect against P. aeruginosa, S. typhimurium, and E. coli. The Cts-Se-NPs solution can be further investigated for various antibacterial applications in the fields of medicine and dentistry, such as disinfection and sterilization of medical devices, as well as mouthwash in periodontal diseases and anti-caries agents.

Introduction

Selenium (Se) was discovered in 1817 by a Swedish chemist, Jons Jakob Berzelius. It is an essential trace element, and its low level in humans leads to an increased risk of various diseases, such as cancer and heart disease [1]. This micronutrient has been studied over the last years, and a great deal of evidence has revealed its vital role in the antioxidant defense system, thyroid hormone metabolism, and redox control of cell reactions [2–4].

The nanoform of Se is more interesting for researchers due to its lower toxicity and higher bioavailability compared to other forms [5]. This property results from using Se in zero oxidation state (SeO) [5–7], which is highly unstable and easily transformed into an inactive form [5]. Accordingly, researchers have focused on finding materials and methods to stabilize Se in SeO.

Chitosan (Cts) is one of the natural materials that can be used for this purpose [8]. This biopolymer was reportedly first discovered by Rouget in 1859. The important properties of Cts, such as antimicrobial activity, biocompatibility, and biodegradability, motivated researchers, and industrialists to discover more effective and
novel medical applications for it [9]. The Cts is used in a variety of biomedical fields, including drug delivery systems, wound dressings, as well as food and chemical industries [10].

Extremely few studies synthesized and investigated various aspects of chitosan-selenium nanoparticles (Cts-Se-NPs). In their recent study, Zhai et al [8] evaluated the antioxidant capacities of Se-NPs stabilized by Cts. To our knowledge, there have been no previous studies investigating the antibacterial properties of colloidal Se-NPs in Cts solution. Therefore, the main purpose of this in vitro study was to evaluate the antibacterial activity of the Cts-Se-NPs solution against gram-positive and gram-negative bacteria.

Materials and methods

Preparation and characterization

In total, 0.15 g Cts (low molecular weight, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50 ml double distilled water by adding some drops of acetic acid (1.0%) solution at room temperature. Afterward, 25 ml of ascorbic acid (0.01 g ml⁻¹, Sigma-Aldrich, St. Louis, MO, USA) solution was added to the Cts solution and stirred. Following that, 25 ml of sodium selenite solution (0.005 g ml⁻¹, Sigma-Aldrich, St. Louis, MO, USA) was added to the Cts-ascorbic acid solution. Finally, the solution was stirred to obtain a reddish-orange homogeneous colloid. The ultraviolet-visible (UV–vis) analysis of the solution was characterized using UV–vis spectrophotometry (Cecil, UK). The UV–vis wavelength covered a range from 200 to 700 nm.

The average particle size distribution was determined using a particle size analyzer (PSA) (Horiba Model SZ-100). Moreover, the surface charge of Cts-Se was determined using zeta potential measurements with the same equipment. In addition, the atomic force microscopy (AFM) images were taken using an Autoprobe CP Research AFM system (JPK NanoWizard II Instrument, Berlin, Germany).

Antibacterial activity

The minimum inhibitory concentration (MIC) of the Cts-Se-NPs solution was determined against gram-negative (i.e., Pseudomonas aeruginosa, Salmonella typhimurium, and Escherichia coli) and gram-positive (i.e., Streptococcus sanguinis, Staphylococcus aureus, and Enterococcus faecalis) bacteria using a broth microdilution method in sterile 96-well microplates (figure 1). The serial dilution with sterile Mueller Hinton broth (MHB) was transferred into wells of a microplate and inoculated with 100 μl of bacterial suspension (1–2 × 10⁸ CFU/ml). Afterward, the microplate was incubated at 37 °C for 24 h. Finally, the lowest concentration of the Cts-Se-NPs solution, which prevented the growth of bacteria, was defined as MIC. The positive control contained bacteria inoculated into MHB without Cts-Se, while the negative control included culture media without bacteria and Cts-Se-NPs. To determine the minimum bactericidal concentration (MBC) in various contact times (1, 2, 6, and 24 h), 10 μl of suspensions from wells without turbidity were inoculated into blood agar medium and incubated at 37 °C until sufficient growth was obtained. The lowest concentration that killed 99.9% (>3 log10) of the initial inoculum after 1, 2, 6, and 24 h was regarded as MBC in each contact time. Figure 1 demonstrates the MIC and MBC tests of Cts-Se-NPs.
Results

Figure 2 illustrates the UV–vis spectrum of the Cts-Se-NPs solution. It can be observed that the absorbance peak of the Se-NPs was located at 264 nm. The obtained result is in line with the findings of a study conducted by Chen et al who reported the UV–vis spectrum of Cts-Se-NPs [11]. Their results showed that the absorption peak of Se-NPs was located at 267 nm.

The PSA technique was utilized to determine the average size of small particles in suspension. As shown in figure 3, the size of Cts-Se-NPs varied in the range of 50–105 nm, and the mean diameter of the nanoparticles was approximately 69.6 nm. The zeta potential value for the obtained Cts-Se-NPs was 45.4 mV, indicating the suitable stability of the nanoparticles.

Figure 4 depicts a well-dispersed heterogeneous shape of the Cts-Se-NPs using AFM. The size distribution of the nanoparticles was less than 100 nm.

The Cts-Se-NPs revealed good antimicrobial activity against the gram-positive bacteria. Table 1 summarizes the MIC values of the Cts-Se-NPs solution against the gram-positive bacteria within the range of 0.068 to 0.274 mg ml$^{-1}$. As indicated in the table, S. sanguinis, S. aureus, and E. faecalis had the MIC values of 0.068, 0.137, and 0.274 mg ml$^{-1}$, respectively. The Cts-Se-NPs did not elicit any significant antibacterial effect against the gram-negative bacteria of P. aeruginosa, S. typhimurium, and E. coli.

Table 2 shows the MBC values of the Cts-Se-NPs against the tested bacteria after 1, 2, 6, and 24 h. The results revealed that the concentration of 0.274 mg ml$^{-1}$ had a higher bactericidal effect. As the concentration of the Cts-Se-NPs increased to 0.274 mg ml$^{-1}$, S. sanguinis, S. aureus, and E. faecalis were completely killed after 1, 2, and 6 h, respectively. Moreover, S. sanguinis and S. aureus were respectively killed after 6 and 24 h at the concentration of 0.137 mg ml$^{-1}$. However, the concentration of 0.068 mg ml$^{-1}$ did not show any significant bactericidal effect.
In this study, we utilized a simple synthesis method using ascorbic acid as a green reducing agent. The advantage of Se-NPs is the possibility of using Se\textsuperscript{0}, which presents low toxicity and excellent bioavailability compared to other oxidation states such as Se\textsuperscript{IV} and Se\textsuperscript{VI}\textsuperscript{[6, 7]}; however, it is highly unstable and easily transformed into an inactive form. Although, as can be observed in several studies, Se-NPs can be stabilized by Cts\textsuperscript{[5, 12–14]}. Therefore, we used Cts as a stabilizer for Se\textsuperscript{0} and evaluated the antibacterial activity of Se-NPs stabilized by Cts.

The results showed the antibacterial effects of the Cts-Se-NPs solution against the various gram-positive bacteria, but not the gram-negative bacteria. Several studies found that Cts generally had stronger effects on gram-positive bacteria than on their gram-negative bacteria\textsuperscript{[15]}. Tran \textit{et al}.\textsuperscript{[2]} reported that Se-NPs did not show any significant effect against \textit{E. coli}.

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Gram-negative bacteria are surrounded by lipopolysaccharide molecules, which carry a negative charge\textsuperscript{[16]}. Therefore, there is a strong electrostatic repulsion between Se-NPs and the outer bacterial membrane\textsuperscript{[2]}. The results of the current study revealed that the Cts-Se-NPs solution had a significant effect on the gram-positive bacteria (i.e., \textit{S. sanguinis}, \textit{S. aureus}, and \textit{E. faecalis}).

Table 1. MIC values of the Cts-Se-NPs against different bacteria.

| Bacteria name               | MIC (mg ml\textsuperscript{−1}) |
|-----------------------------|----------------------------------|
| \textit{Pseudomonas aeruginosa} | NA\textsuperscript{□}              |
| \textit{Salmonella typhimurium} | NA\textsuperscript{□}              |
| \textit{Escherichia coli O157} | NA\textsuperscript{□}              |
| \textit{Enterococcus faecalis} | 0.274                             |
| \textit{Staphylococcus aureus} | 0.137                             |
| \textit{Streptococcus sanguinis} | 0.068                             |

NA\textsuperscript{□}: Not killed at any concentration in the range of examination.

Table 2. MBC values of the Cts-Se-NPs against bacteria after 1, 2, 6, and 24 h.

| Bacteria name               | MBC after 1h (mg ml\textsuperscript{−1}) | MBC after 2h (mg ml\textsuperscript{−1}) | MBC after 6h (mg ml\textsuperscript{−1}) | MBC after 24h (mg ml\textsuperscript{−1}) |
|-----------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| \textit{Pseudomonas aeruginosa} | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  |
| \textit{Salmonella typhimurium} | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  |
| \textit{Escherichia coli O157} | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  |
| \textit{Enterococcus faecalis} | NA\textsuperscript{□}                  | 0.274                                  | 0.274                                  | 0.274                                  |
| \textit{Staphylococcus aureus} | NA\textsuperscript{□}                  | 0.274                                  | 0.137                                  | 0.137                                  |
| \textit{Streptococcus sanguinis} | 0.274                                  | 0.274                                  | 0.137                                  | 0.137                                  |

NA\textsuperscript{□}: Not killed at any concentration in the range of examination.

### Discussion

In this study, we utilized a simple synthesis method using ascorbic acid as a green reducing agent. The advantage of Se-NPs is the possibility of using Se\textsuperscript{0}, which presents low toxicity and excellent bioavailability compared to other oxidation states such as Se\textsuperscript{IV} and Se\textsuperscript{VI}\textsuperscript{[6, 7]}; however, it is highly unstable and easily transformed into an inactive form. Although, as can be observed in several studies, Se-NPs can be stabilized by Cts\textsuperscript{[5, 12–14]}. Therefore, we used Cts as a stabilizer for Se\textsuperscript{0} and evaluated the antibacterial activity of Se-NPs stabilized by Cts.

The results showed the antibacterial effects of the Cts-Se-NPs solution against the various gram-positive bacteria, but not the gram-negative bacteria. Several studies found that Cts generally had stronger effects on gram-positive bacteria than on their gram-negative bacteria\textsuperscript{[15]}. Tran \textit{et al}.\textsuperscript{[2]} reported that Se-NPs did not show any significant effect against \textit{E. coli}.

Gram-negative bacteria are surrounded by lipopolysaccharide molecules, which carry a negative charge\textsuperscript{[16]}. Therefore, there is a strong electrostatic repulsion between Se-NPs and the outer bacterial membrane\textsuperscript{[2]}. The results of the current study revealed that the Cts-Se-NPs solution had a significant effect on the gram-positive bacteria (i.e., \textit{S. sanguinis}, \textit{S. aureus}, and \textit{E. faecalis}).

Figure 4. AFM image of the Cts-Se-NPs (a) and its size histogram (b).

Table 1. MIC values of the Cts-Se-NPs against different bacteria.

| Bacteria name               | MIC (mg ml\textsuperscript{−1}) |
|-----------------------------|----------------------------------|
| \textit{Pseudomonas aeruginosa} | NA\textsuperscript{□}              |
| \textit{Salmonella typhimurium} | NA\textsuperscript{□}              |
| \textit{Escherichia coli O157} | NA\textsuperscript{□}              |
| \textit{Enterococcus faecalis} | 0.274                             |
| \textit{Staphylococcus aureus} | 0.137                             |
| \textit{Streptococcus sanguinis} | 0.068                             |

NA\textsuperscript{□}: Not killed at any concentration in the range of examination.

Table 2. MBC values of the Cts-Se-NPs against bacteria after 1, 2, 6, and 24 h.

| Bacteria name               | MBC after 1h (mg ml\textsuperscript{−1}) | MBC after 2h (mg ml\textsuperscript{−1}) | MBC after 6h (mg ml\textsuperscript{−1}) | MBC after 24h (mg ml\textsuperscript{−1}) |
|-----------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| \textit{Pseudomonas aeruginosa} | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  |
| \textit{Salmonella typhimurium} | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  |
| \textit{Escherichia coli O157} | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  | NA\textsuperscript{□}                  |
| \textit{Enterococcus faecalis} | NA\textsuperscript{□}                  | 0.274                                  | 0.274                                  | 0.274                                  |
| \textit{Staphylococcus aureus} | NA\textsuperscript{□}                  | 0.274                                  | 0.137                                  | 0.137                                  |
| \textit{Streptococcus sanguinis} | 0.274                                  | 0.274                                  | 0.137                                  | 0.137                                  |

NA\textsuperscript{□}: Not killed at any concentration in the range of examination.

### Discussion

In this study, we utilized a simple synthesis method using ascorbic acid as a green reducing agent. The advantage of Se-NPs is the possibility of using Se\textsuperscript{0}, which presents low toxicity and excellent bioavailability compared to other oxidation states such as Se\textsuperscript{IV} and Se\textsuperscript{VI}\textsuperscript{[6, 7]}; however, it is highly unstable and easily transformed into an inactive form. Although, as can be observed in several studies, Se-NPs can be stabilized by Cts\textsuperscript{[5, 12–14]}. Therefore, we used Cts as a stabilizer for Se\textsuperscript{0} and evaluated the antibacterial activity of Se-NPs stabilized by Cts.

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Gram-negative bacteria are surrounded by lipopolysaccharide molecules, which carry a negative charge\textsuperscript{[16]}. Therefore, there is a strong electrostatic repulsion between Se-NPs and the outer bacterial membrane\textsuperscript{[2]}. The results of the current study revealed that the Cts-Se-NPs solution had a significant effect on the gram-positive bacteria (i.e., \textit{S. sanguinis}, \textit{S. aureus}, and \textit{E. faecalis}).
S. aureus is a major human pathogen that causes a wide range of clinical infections [17]. It is a leading cause of multiple human infections, including bacteremia, infective endocarditis, skin and soft tissue infections, osteomyelitis, septic arthritis, device-related infections, pulmonary infections, meningitis, toxic shock syndrome, and urinary tract infections [18].

The results of the present study also showed that the Cts-Se-NPs solution had a remarkable effect on S. aureus. Tran et al [2] found that Se-NPs showed strong growth inhibition toward S. aureus. The cell wall in gram-positive bacteria contains a thick layer of peptidoglycan without any outer lipopolysaccharide membrane, and their net surface charge is much less negative compared to that of gram-negative bacteria [19]. Therefore, Se-NPs could penetrate much more easily through the peptidoglycan layer of S. aureus, reducing bacterial cell division. This consequently inhibits the growth of S. aureus [2, 20].

Guisbiers et al [20] observed that Se-NPs had a significant inhibitory effect on S. aureus. Moreover, Goy et al [21] found that Cts was more active against S. aureus compared to the gram-negative E. coli. In the same line, Tao et al [22] studied the effect of Cts on the membrane permeability and cell morphology of S. aureus. Their results showed that Cts had a significant effect against S. aureus and performed its antibacterial activity via increasing the permeability of cell membranes. Therefore, in the present study, Cts and Se might have a synergic antibacterial effect on the gram-positive bacteria, such as S. aureus.

E. faecalis is a gram-positive bacterium that colonizes human gastrointestinal tracts. It is one of the main causing agents of hospital-acquired infections, which is regarded as a major concern in public health [23]. In our study, the Cts-Se-NPs solution showed noticeable antibacterial activity against E. faecalis. Khiralla et al [24] found that Se-NPs demonstrated an antimicrobial effect against E. faecalis. Furthermore, Perelshtein et al [25] observed that Cts-NPs had more effects on E. faecalis than on E. coli. This is probably attributed to this property that E. faecalis only has a single membrane and is more susceptible to the effects of surface disruption than E. coli, which has a double outer membrane [26].

S. sanguinis is a member of the viridans streptococcal group and is a leading cause of infective endocarditis, which is a life-threatening infection of the cardiovascular system [26, 27]. It is also one of the pioneering tooth colonizers and is one of the most abundant species in oral biofilm, dental plaque, dental caries, and periodontal diseases [28].

Further, the results of the present study revealed that the Cts-Se-NPs solution had a significant effect on S. sanguinis. In a similar vein, Aliasghari et al [29] showed that Cts and nano Cts had anti-growth and anti-adherence effects against cariogenic bacteria, such as S. sanguinis.

In addition, Archana et al [30] found that chlorhexidine along with Cts combination mouthrinse was superior in antimicrobial activity than chlorhexidine alone. The effect of Se against S. sanguinis has not been so far studied. In a study performed by Tran et al [2], it was demonstrated that Se-NPs had an extremely low hemolysis rate with only 18% of maximal hemolysis. Other studies determined the hemolytic tendency of other nanoparticles, such as silver and gold, at 100% and 60%, respectively [31, 32]. Therefore, Se-NPs have low hemolytic activity and can be potentially used for medical devices having direct contact with blood [2].

MIC and MBC of an antibacterial substance are the basic and one of the most important criteria for antibacterial activity. Although, the MIC and MBC levels are not a confirmation of the antibacterial activity on the bacterial cell wall. NPs need to be in contact with bacterial cells to accomplish their antibacterial role [33]. With respect to existing studies, the main processes underlying the antibacterial activity of NPs are as follows: (1) contact with bacterial cell walls (various forms of contact include electrostatic attraction [34], Vander Waals forces [35], receptor–ligand [36], and hydrophobic interactions [37]), (2) disruption and penetration of the bacterial cell membrane, (3) generation of reactive oxygen species (ROS) and interact with basic component of microbial cell (i.e., DNA and mitochondria), protein deactivation, electrolyte balance disorder, enzyme inhibition, and modify gene expression levels [38–40].

Conclusion

In summary, in this study, the Cts-Se-NPs solution exhibited excellent antibacterial activity against the gram-positive bacteria. This product can be investigated further for various antibacterial applications in the fields of medicine and dentistry, such as disinfection and sterilization of medical devices, as well as a mouthwash in periodontal diseases and anti-caries agents.

Acknowledgments

We would like to thank the Research Vice-Chancellor of Mashhad University of Medical Sciences for financial support (grant no. 961533) to conduct this study.
Conflicts of interest

The authors report no conflicts of interest in the present work.

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