Thiolate chitosan nanoparticles capable to delivery nitric oxide: synthesis, characterization and antibacterial potential

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Abstract. Nitric oxide (NO) is a small but powerful molecule. It is involved in several biological pathways such as vasodilatation, wound healing and toxicity towards pathogens. At high concentrations, NO has a toxicity towards bacteria, virus and fungi which has a great biomedical potential. However, NO has a small half-life of just a few seconds and this hazards its clinical application. In this scenario, the combination of nanotechnology with NO donors can create new strategies to load and deliver NO. Polymeric nanomaterial intrinsically have advantages such as low toxicity, biodegradability and low-cost. In this study, we used chemically modified chitosan (CS) to prepare nanoparticles capable of loading and releasing NO with antibacterial activity. CS was chemically modified to add a thiol group (-SH) to its structure. This modification was performed by the reaction with thioglycolic acid (TGA) in the presence of a carbodiimide (EDC). The thiol groups in CS structure serve a double function: create an anchorage site for NO and increase polymer mucoadhesivity. The synthesis of thiolated chitosan nanoparticles (TCS NP) occurred by ionotropic gelation method using sodium tripolyphosphate (TPP) as counter ion. The NO donor precursor molecule, mercaptosuccinic acid (MSA), was encapsulated into TCS NP to increase loading capacity of NO. To identify the best parameters of the synthesis we used the ratios 1:3, 1:4, 1:5, 1:6 and 1:7 for TCS:TPP. TCS NPs were characterized by dynamic light scattering (DLS), microscopy electron transmission (MET) and nanoparticle tracking analysis (NTA). The release of NO was characterized by a kinetic using UV-vis spectroscopy. Finally, the antibacterial potential was evaluated by minimum inhibitory concentration (MIC) assay against Staphylococcus aureus, Streptococcus mutans and Escherichia coli strains. The ratio 1:5 showed the most adequate size parameters and the other analysis were performed using it. The hydrodynamic size was found to be 113.0 ± 1.6 nm, PDI of 0.292 ± 0.035 and zeta potential of 27.1 ± 0.9. The MET images indicated small and spherical nanoparticles. The kinetic profiles showed a linear release of NO reaching the 100% after 150 min. The antibacterial effect was tested for E. coli, S. aureus and S. mutans. The MIC values was 50 μg mL for NO-CS NP, this result was 50% lower compared to TCS NPs for S. mutans and E. coli. The TCS NP has suitable properties for the biomedical field with potential for antibacterial application.

1. Introduction

Nitric oxide (NO) is an endogenously produced signaling molecule involved in several physiological and pathophysiological processes [1–3]. NO is synthesized in vivo by the nitric oxide synthase (NOS) enzyme, which catalyzes the oxidation of L-arginine to L-citrulline [4]. NO is a free radical, devoid of charge, of small size that freely diffuses in biological environments, crossing most physiological barriers...
and performing communication between cells [5]. NO is well known for its cardiovascular effects [6], however, its effects goes far beyond that and includes inhibition of platelet aggregation [7], promoting healing skin and tissue regeneration [8,9], antimicrobial action [10,11] and antitumor [12,13].

In a physiological environment, NO has a relatively short half-life (t½) of 1-5 s, it is quickly deactivated by oxidation reactions generating the species of nitrite (NO2-') and/or nitrate (NO3-) [1–3,5]. In addition, it can react with proteins heme groups and with thiol groups (RSH) [7]. Thus, donors and carriers of NO capable of increasing their t½ have great potential to make their various applications feasible [7].

There are several molecules that act as NO donors by preserving their bioactivity [11]. Among them, we highlight the S-nitrosothiols (RSNOs) which are a class of NO donors generated from the nitrosation of RSHs [7]. RSNOs spontaneously decomposes and releases NO through homolytic cleavage of covalent bond S-NO [7]. RSNOs decomposition can be catalyzed by temperature, light (visible or ultraviolet (UV)) and presence of copper ions [7].

Due to the various biochemical actions of NO, there is a great interest in the development of agents capable of releasing NO in a controlled manner in living systems, where NO can play its therapeutic effects [14,15]. These NO carriers can be used in target tissues to locally modulate the NO release kinetics [12,14–16]. Up to the present moment, the strategies for the controlled release of NO have presented some limitations for therapeutic use, such as the insufficient duration of the release of NO at the site of interest, the rapid inactivation of the precursor molecule of NO, the low biocompatibility or low solubility of the NO donor vehicle and the release of NO before it reaches the place of interest [17].

In order to overcome these limitations and increase the usefulness of NO donor systems, the current research trend is geared towards combining NO donors (such as RSNOs) with polymeric nanoparticles [12,15].

This strategy has a promising potential for NO biomedical applications. In this study, we used polymeric nanoparticles of thiolated chitosan as releasing systems of NO [18]. CS is a biopolymer composed of the repetition of the monomer ß-1,4-glucosamine [19]. The production of CS occurs from the deacetylation of chitin, a structural element found in the shell of crustaceans [20]. The properties of CS as biocompatibility, susceptibility to enzymatic hydrolysis, intrinsic physiological activity (such as healing and antimicrobial properties) and mucoadhesiveness make it widely used by the pharmaceutical industry [20]. Its use depends on the degree of deacetylation (amount of deacetylated monomers in their chains), which can vary between 40 and 95%. CS can be chemically modified to add a thiol group to its structure [21], henceforward, called thiolated chitosan (TCS). This modification was performed by the CS reaction with thioglycolic acid (TGA) in the presence of a carbodiimide (EDC) [22].

Thiolated chitosan nanoparticles (TCS NP) were synthesized by ionic crosslink method. Among different protocols of nanoparticles synthesis, the ionic crosslink method has gained much attention because it is a simple, convenient and controllable method [20]. The synthesis of TCS NPs is based on the electrochemical interaction of protonated amino groups (NH3+ of TCS and phosphate (PO4-) negatively charged groups of sodium tripolyphosphate (TPP) [23]. The thiol groups had a doble effect by increasing the mucoadesivity and offering an anchoring site for NO [21]. The NO donor precursors, mercaptosuccinic acid (MSA), was encapsulated into TCS NPs to increase their NO loading capability.

Thus, this study main objective was to obtain TCS NPs capable of carrying and releasing NO in a sustained manner that combine the advantages of CS, such as biocompatibility and mucoadhesiveness, to the therapeutic properties of NO as antibactericidal effect.

2. Methods

2.1. Chemicals
Chitosan (CS, low molecular weight 51-190 kDa, 75% deacetylation, viscosity of 20-300 cps), sodium tripolyphosphate (TPP), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), thioglycolic acid (TA), acetic acid, ammonium hydroxide (NH4OH), sodium nitrate (NaNO3), 5,5-dithiobis(2-nitrobenzoic acid), ethylenediaminetetraacetic acid (EDTA), phosphate buffer (pH 7.4) and
mercaptosuccinic acid (MSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All experiments were carried out with analytical grade water from Millipore Milli-Q gradient filtration system (resistivity below 18.2 MΩ cm⁻¹ at 25.0 °C).

2.2. Synthesis of thiolated chitosan (TCS)
The synthesis of TCS was described in detail elsewhere [21,24]. In brief, EDC (2.44 g mL⁻¹) and TGA (754 μL) were added to CS (10 mg mL⁻¹ in 1% acetic acid) and kept under magnetic stirring for 4 h. TCS was precipitated by adding NH₄OH. The precipitated was filtered and washed twice with cold Milli-Q water. The purified material was frozen and lyophilized for 24 hours.

2.3. Synthesis of TCS nanoparticles (TCS NP) and encapsulation of MSA (MSA-TCS NPs)
TCS nanoparticles (TCS NPs) were synthesized by ionotropic gelation process [12,25]. First, a TCS at 1 mg mL⁻¹ in 1% acetic acid was homogenized alone or in the presence of MSA (66.6 mmol L⁻¹) by magnetic stirring for 90 min. Second, a TPP solution (0.6 mg mL⁻¹) was dropwise added to TCS or TCS/MSA solution. This step was performed using a peristaltic pump at 123 μL per min. The TCS:TPP ratio were 1:3, 1:4, 1:5, 1:6 and 1:7. Third, the final mixture was magnetic stirred for 45 min. Then, an aqueous suspension of TCS NPs and MSA-TCS NPs were formed at 1 g L⁻¹ of TCS.

2.4. Dynamic light scattering measurements (DLS)
The hydrodynamic size diameter, polydispersity index (PDI) and zeta potential of TCS NP (using different TCS:TPP ratios) and MSA-TCS NP were evaluated by DLS (Nano ZS Zetasizer, Malvern Instruments Co, UK) [12,25]. Measurements were performed at a fixed angle of 173°, using a disposable capillary cuvette (DTS1070). The results were reported as an average of three independent experiments with the error bar values expressed by their standard error of the mean (SEM).

2.5. Nanoparticle tracking analysis (NTA)
The hydrodynamic size and concentration of particles were evaluated by NTA LM-20 (NanoSight Ltd. UK). The size distribution of nanoparticles was obtained on particle-by-particle basis. The NTA enables separation of particles population by size and intensity, microscopical visualization of individual nanoparticles in suspension and simultaneously determining their Brownian motion (Kanhed et al., 2014).

2.6. Transmission electron microscopy (TEM)
The morphology of TCS NPs was obtained by transmission electron microscopy (TEM) at 80 kV (Carl Zeiss 120 TEM, Zeiss International, Oberkochen, Germany) [26].

2.7. Encapsulation efficiency of MSA into TCS NP
The encapsulation efficiency of MSA into TCS NPs was measured [13,25]. Non-encapsulated MSA was separated from encapsulated MSA by centrifugal filter device (MWCO 10,000, Millipore). The non-encapsulated MSA was titrated with DTNB (0.7 mmol L⁻¹) in the presence of EDTA (10.3 mmol L⁻¹) in phosphate buffer (pH 7.4). The absorption band at 412 nm (Ɛ = 14.15 mmol⁻¹ L cm⁻¹) was measured by Uv-vis spectrophotometer (Agilent 8454, Palo Alto, CA, USA). The analysis was performed in duplicates.

2.8. Nitrosation of MSA-TCS NP
Thiol group were nitrosated by the addition of NaNO₂ (50 mmol L⁻¹) to aqueous suspension of MSA-TCS NPs. The suspension was homogenized by magnetic stirring for 60 min at room temperature. The nitrosation was evaluated by the absorbance bands at 545 nm (Ɛ = 18.4 mol⁻¹ L cm⁻¹) using UV–vis spectrophotometer (Agilent 8454, Palo Alto, CA, USA). The MSA-TCS NPs after the nitrosation were called NO-TCS NPs.
2.9. NO release profile from NO-TCS NP
The kinetics of NO release from NO-TCS NPs was determined by monitoring the spectral changes at 545 nm (nN → π* transition) [12,25]. This absorption band decay is associated with the decomposition of S-nitrosothiols and free NO release. The initial concentration of S-nitroso-MSA encapsulated was 50 mmol·L⁻¹ and the kinetic data were collected in 15 min intervals at 25°C for 5 h of monitoring. Each point in the kinetic curves represents the average of three independent experiments, with the error bar values expressed by their standard error of the mean (SEM).

2.10. Antibacterial activity
The broth microdilution method and time-kill curves were performed in triplicate to evaluate the antimicrobial activity of (i) TCS NP, (ii) NO-TCS NP and (iii) S-nitroso-MSA. The broth microdilution method was used to achieve the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), based on the CLSI guidelines (2018) [27].

Broth microdilution was performed in 96-well microplate, using MH broth, testing i-iv samples at 12.5, 25, 50 and 100 μg mL⁻¹ against 1.5 × 10⁵ CFU mL⁻¹ (initial inoculum) of each bacterium. The plate was incubated at 37 °C for 24 h, and MIC was determined based on the observation of wells that did not present medium turbidity. The lowest concentration lacking broth turbidity was determined as MIC. The volume of 10 μL of each well was plated in Muller Hinton (MH) agar (Difco, USA) and plates were incubated at 37°C for 18-24 h, to enable bacterial–colony counting. The lowest concentration lacking bacterial growth in the MH agar plates was determined as MBC. 100 μL of each material was individually diluted in MH broth, at previously determined MIC value.

3. Results and Discussion

3.1. TCS NPs characterization
CS has been widely used in biomedical and pharmaceutical fields due to its biocompatibility and biodegradability [19,28]. CS can be used to form films, hydrogels and nanoparticles [19]. CS NPs synthesis involves the interaction between negatively charged TPP phosphate groups to positively charge CS amino groups [23]. The modification of CS has been an interesting approach to add new properties to the polymer or to enhance their intrinsic properties [21]. For example, modification with PLGA for cancer treatment [29,30], addition of thiol groups for increased mucoadhesivity [21,31] and modification using other nanoparticles [32–34]. In this study, we add thiol groups to CS structure to increase mucoadhesivity and to provide an anchor site for NO.

Table 1 shows the hydrodynamic size, PDI and zeta potential values for TCS NPs synthesized using different TPP:TCS ratios. The data showed a wide-range hydrodynamic sizes from 113 – 1060 nm. The biggest value was observed for 1:4 ratio and the smaller for 1:5 ratio. The SD/ Mean (%) indicates the variance of the sample within different measurements and data showed a range of 1.4-29.7%. The biggest value was observed for 1:7 ratio and the smaller for 1:5 ratio. PDI values relates to nanoparticles dispersion homogeneity and they showed a range between 0.292-0.627. The biggest value was observed for 1:7 ratio and the smaller for 1:5 ratio. Zeta potential values varied between 0.1-27.1 mV, the biggest value was for 1:5 ratio and the smaller for 1:7 ratio. The TPP:TCS ratio is a crucial parameter to control overall nanoparticle size, its homogeneity and colloidal stability. Due to small size, good homogeneity and high zeta potential, the 1:5 rate was determined the most suitable for biomedical applications. The 1:5 ratio was used in the next analysis and procedures.

Table 1. Parameters of TCS NPs synthesized using different TPP:TCS ratios (1:3, 1:4, 1:5, 1:6 and 1:7).

| TPP:TCS ratio (v/v) | Hydrodynamic size (nm) | SD / Mean (%) | PDI | Zeta potential (mV) |
|--------------------|------------------------|---------------|-----|---------------------|
| 1:3                | 338.7 ± 82.1           | 24.2          | 0.484 ± 0.132 | 23.3 ± 0.5          |
The Figure 1A,B shows representative images of TCS NPs (ratio 1:5) acquired by TEM. The images showed spherical and dispersed nanoparticles. The NTA result corroborate with TEM data and it shows small nanoparticle with low aggregation (Multimedia file). Wang et al. 2018 showed a similar TEM image for CS and hyaluronic acid nanoparticles with spherical shape and well dispersed [35]. The encapsulation efficiency (EE%) of MSA was 91% which is considerable high and indicate a successful formation of nanoparticle.

![Figure 1](a) and (b).

### 3.2. NO release profile from NO-TCS NP

MSA-TCS NP was nitrosated to form NO-TCS NP. The nitrosation reaction occurred in the thiol groups of MSA and TCS, they were nitrosated by adding equimolar amount of NaN0₂ to nanoparticle dispersion. The kinetic of NO-TCS NP decomposition showed a linear profile (Fig. 2). Using this data, it was possible to calculate the theoretical amount of NO release (Fig. 2 dotted red line). NO level reached 100% after 160 s of kinetic at 25°C.
3.3. Antibacterial activity

The antibacterial activity of TCS NP and NO-TCS NP was tested against three bacterial strains: *E. coli*, *S. aureus* and *S. mutans*. NO-TCS NP decreased the value of TCS NPs necessary to reach the MIC by 50% for *E. coli* and *S. mutans* (Table 1). However, the MBC results were similar to each other (Table 2).

These results indicate that TCS NP has intrinsically bactericidal effects, the TCS NP alone showed a MIC value of 5 μg mL⁻¹ for all bacterial strains tested. The CS is well known to show bactericidal effect because of its protonate amino groups [19]. The MIC of CS alone was found to be 25.0 μg mL⁻¹ for *E. coli*, as previous reported [36]. The combination of CS and NO in a nanomaterial decreased 50% of need nanoparticle concentration to achieve the inhibitory effect of bacteria. Anitha et al. 2009 showed similar results for nanoparticles made of chitosan and chitosan modified [37] and Luo et al. 2019 also showed similar results and they observed that the effect of *E. coli* could be seen until 8h [38].

**Table 1.** MIC values for experimental groups.

|           | TCS NPs | NO-TCS NPs |
|-----------|---------|------------|
| *E. coli* | 5 μg mL⁻¹ | 2.5 μg mL⁻¹ |
| *S. aureus* | 5 μg mL⁻¹ | 5 μg mL⁻¹ |
| *S. mutans* | 5 μg mL⁻¹ | 2.5 μg mL⁻¹ |
### Table 2. MBC values for experimental groups.

|                | TCS NPs | NO-TCS NPs | S-nitroso-MSA |
|----------------|---------|------------|---------------|
| *E. coli*      | >5 μg mL⁻¹ | 5 μg mL⁻¹ | 5 μg mL⁻¹     |
| *S. aureus*    | 5 μg mL⁻¹  | 5 μg mL⁻¹ | 5 μg mL⁻¹     |
| *S. mutans*    | 5 μg mL⁻¹  | >5 μg mL⁻¹ | >5 μg mL⁻¹    |

#### 4. Conclusion

The use of TCS to synthesize a nanomaterial was successful and the most efficient TCS:TPP ratio was 1:5. The ratio showed good size, polydispersity and colloidal stability. TEM and NTA data showed spherical and well dispersed nanoparticles. The NO-TCS NP was efficient to release NO in mmol range during the first 3h. The TCS NP showed antibacterial activity against *E. coli*, *S. aureus* and *S. mutans* and NO-TCS NP decreased the MIC value by 50% for *E. coli* and *S. mutans*.

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