Nitric oxide-releasing polymeric nanoparticles against *Trypanosoma cruzi*

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**Abstract.** Chagas disease, also known as American trypanosomiasis, is a potentially life-threatening illness caused by the protozoan parasite, *Trypanosoma cruzi* (*T. cruzi*), and the disease remains a major health problem in many Latin American countries. Several papers report that the killing of the parasite is dependent on the production of nitric oxide (NO). The endogenous free radical NO is an important cellular signalling molecule that plays a key role in the defense against pathogens, including *T. cruzi*. As *T. cruzi* is able to compromise host macrophages decreasing endogenous NO production, the administration of exogenous NO donors represents an interesting strategy to combat Chagas disease. Thus, the aims of this study were to prepare and evaluate the antimicrobial activity of NO-releasing polymeric nanoparticles against *T. cruzi*. Biocompatible polymeric nanoparticles composed of chitosan/sodium tripolyphosphate (TPP) were prepared and used to encapsulate mercaptosuccinic acid (MSA), which is a thiol-containing molecule. Nitrosation of free thiols (SH) groups of MSA were performed by the addition of equimolar amount of sodium nitrite (NaNO₂), leading to the formation of S-nitroso-MSA-containing nanoparticles. These polymeric nanoparticles act as spontaneous NO donors, with free NO release. The results show the formation of nanoparticles with average hydrodynamic diameter ranging from 270 to 500 nm, average of polydispersity index of 0.35, and encapsulation efficiency in the range of 99%. The NO release kinetics from the S-nitroso-MSA-containing nanoparticles showed sustained and controlled NO release over several hours. The microbialic activity of S-nitroso-MSA-containing nanoparticles was evaluated by incubating NO-releasing nanoparticles (200 - 600 µg/mL) with replicative and non-infective epimastigote, and non-replicative and infective trypomastigote forms of *T. cruzi*. In addition, a significant decrease in the percentage of macrophage-infected (with amastigotes) and NO-releasing nanoparticle-treated cells was observed. Taken together, our results reveal a potent toxic effect of NO-releasing polymeric nanoparticles against different life cycle forms of *T. cruzi*, indicating that the encapsulation of the NO donor S-nitroso-MSA represents an interesting approach to combat and to prevent Chagas disease.
1. Introduction

Chagas disease, caused by the heteroxenic trypanosomatid *Trypanosoma cruzi* (*T. cruzi*) [1] is endemic in Latin America with an estimated 7-8 million people infected [2]. However, the intense migration of people from endemic areas for non-endemic countries has contributed for the globalization of this disease [3,4].

The life cycle of *T. cruzi* involves a vertebrate and invertebrate hosts, alternating between replicating (epimastigote and amastigote) and infectious forms (trypomastigote). The natural transmission of the protozoan to mammalian host occurs through the excreta containing metacyclic trypomastigotes of an infected hematophagous reduviid bug of the subfamily Triatominae. Within vertebrate host, the parasite can invade a wide range of nucleated cell. After intracellular multiplication as amastigotes, they turn into trypomastigotes, which are released and can invade adjacent cells or spread hematogenously to distant sites. Once in the bloodstream, the trypomastigotes may be ingested by the invertebrate host, turn into epimastigotes which multiply actively and transform in metacyclic trypomastigotes in its gut [5]. Alternatively, the bloodstream trypomastigotes may be transmitted by blood transfusion [6].

In Brazil, benznidazole is currently available for the specific treatment of Chagas disease. The effectiveness of this drug is dependent on various factors such as the disease stage, dose, age, geographical origin of the patient and parasite drug susceptibility. In addition, this substance can produce serious side effects, which may lead to discontinuation of the treatment [7]. Thus, there is an urgent need for development of new strategies for the control of Chagas disease.

The endogenous found molecule nitric oxide (NO) is not only a physiological mediator of important process such as the dilation of blood vessels [8,9], but also plays a key role in the immunological system against several pathogens [10-12]. Upon pathogen infection, activated macrophages and other immune cells inhibit pathogen replication by releasing several effector molecules, including NO [13]. Therefore, NO is an important defense molecule against infectious organisms, including *T. cruzi* [13-15]. As a free radical, NO is unstable and it can be readily inactivated in the biological system. In order to increase the life-time of NO, several works describe the preparation of small molecule weight S-nitrosothiols (RSNOs), an important class of NO donor, used in several biomedical applications [11,16-18]. However, RSNOs are thermally unstable in aqueous solutions, leading to free NO release [19]. In this context, RSNOs have been successfully incorporated in biocompatible polymeric nanoparticles to promote a sustained NO release over several hours [12,20-23].

In this context, the present work describes the preparation of polymeric biocompatible and biodegradable nanoparticles composed by chitosan/ triopolyphosphate (TPP) containing S-nitroso-mercaptosuccinic acid (S-nitroso-MSA), an RSNO, which act as spontaneous NO donor. The antimicrobial efficacy of NO-releasing polymeric nanoparticles was evaluated against different forms of *T. cruzi*. The results indicate the potential of NO-releasing nanoparticles in the treatment and combat of Chagas’ diseases.

2. Methods

2.1. Synthesis of MSA-containing chitosan/TPP nanoparticles

Chitosan nanoparticles containing MSA were prepared through ionotropic gelation process using chitosan and sodium tripolyphosphate (TPP), as previous described [12]. Briefly, 0.1 g of chitosan was dissolved in 100 mL of 1% acetic acid. Through magnetic stirring at room temperature, 5 mL of the TPP (0.6 mg/mL) solution was added dropwise into 15 mL of a 1 mg/mL chitosan solution. The final mixture was stirred to prepare an aqueous solution with chitosan/TPP particles. MSA-containing chitosan nanoparticles were prepared by adding MSA into the chitosan solution. The final
concentration of MSA in the chitosan/TPP nanoparticles was 400 mmol/L, which corresponds to 60 mg/mL.

2.2. Synthesis of S-nitroso-MSA-containing chitosan/TPP nanoparticles
Free thiol groups of MSA-containing chitosan/TPP nanoparticles were nitrosated by adding equimolar amount of sodium nitrite (NaNO₂) directly into the polymeric aqueous dispersion (pH 4.0). The final solution was homogenized with magnetic stirring for 30 min and protected from light. S-nitroso-MSA-chitosan/TPP nanoparticle formation was characterized by the S-NO group absorption bands at either 336 nm (ε = 922 mol L⁻¹ cm⁻¹) or 545 nm (ε = 18 mol L⁻¹ cm⁻¹) using a UV–Visible spectrophotometer (Agilent, model 8453, Palo Alto), as previously described [24].

2.3. The average size and size distribution for polymeric particles in aqueous medium
The average size for MSA-containing chitosan/TPP nanoparticles were measured using photon correlation spectroscopy (PCS) (Nano ZS Zetasizer, Malvern Instruments Co.) at 25 °C in polystyrene cuvettes with a 10 mm path length.

2.4. MSA encapsulation efficiency in chitosan/TPP nanoparticles
The encapsulation efficiency of MSA in chitosan/TPP nanoparticles were measured by the UV–vis method, as already described [12]. Briefly, free MSA was separated from polymeric nanoparticles by ultracentrifugation, by using a Microcon centrifugal filter device containing ultrafiltration membranes (MWCO 10,000, Millipore). The amount of free MSA in the ultrafiltrates was measured by titration with the thiol-reacting 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), based on the absorbance band at 412 nm (ε = 14.15 mmol L⁻¹ cm⁻¹) of the 2-nitro-5-thiobenzoate anion, which is generated in the reaction of MSA with DTNB. The percentage of MSA encapsulation was determined by the described equation:

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\% = \frac{\text{mass of MSA encapsulated}}{\text{mass of MSA total}} \times 100
\]  
(Eq. 1)

2.5. Kinetics free NO release from S-nitroso-MSA-chitosan/TPP nanoparticles
The kinetics for NO release from S-nitroso-MSA-chitosan/TPP nanoparticles were monitored by following the spectral changes at 336 nm and 545 nm, which are solely associated with S-N bond cleavage and free NO release [12,19] by using an Uv-Visible spectrophotometer (Agilent 8453). The kinetics curves were monitored at 28 and 37 °C for 24 h. The amount of NO released was calculated from the amount of S-nitroso-MSA decomposed, as previously described [25,26]. The initial rates of NO release through S-nitroso-MSA decomposition were determined through linear regression of the curve slopes, as previously described [27].

2.6. Parasite and mammalian cell culture
Epimastigote forms of T. cruzi Y strain [28] were obtained from four-day incubation culture in liver infusion tryptose (LIT) medium [29] supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), at 28 °C. Trypomastigote forms were obtained from the supernatant of infected LLC-MK2 cell line after 120 h post-infection at 37 °C. LLC-MK2 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) of FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin B, in 5% CO₂ at 37 °C. The peritoneal cells were prepared from BALB/c mice obtained from the breeding colonies of the animal facility of the Centro de Ciências Biológicas of Universidade Estadual de Londrina, Londrina, Paraná, Brazil. The experimental protocol was approved by the Ethics Animal Experimentation Committee of the Universidade Estadual de Londrina. Mice were injected intraperitoneally with 1.5 mL of 3% thioglycollate and peritoneal cells were collected four days later by injecting sterile cold 10 mL of 50 mmol/L sodium phosphate buffer pH 7.4 containing 0.15 mol/L NaCl (PBS). The obtained cells were
centrifuged at 500 g for 10 min at 4°C and resuspended in RPMI 1640 medium (Invitrogen-Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen-Gibco, USA), 50 µg/mL gentamicin, 100 IU/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L glutamine.

2.7. Antitrypanosomal activity
Epimastigotes of \textit{T. cruzi} in logarithmic growth phase (1 x 10^6 cells/mL) were added in 24-well plates (Techno Plastic Products, Switzerland) containing LIT medium with different concentrations of S-nitroso-MSA nanoparticles (0 – 600 µg/mL). The cultures were incubated at 28°C, and cell growth was estimated by direct counting in a hemocytometer (Improved Double Neubauer) after 48 h. Trypomastigotes (1 x 10^6 cells/mL) were added in 96-well plates (Techno Plastic Products, Switzerland) containing RPMI 1640 medium with different concentrations of S-nitroso-MSA nanoparticles (0-400 µg/mL) and the plates were incubated at 37 °C. Parasites were counted, as above, after 10, 30, 60 and 120 min. Tests containing medium alone, medium plus MSA-containing nanoparticle (without the NO donor S-nitroso-MSA), medium with sodium nitrite (NaNO_2) were employed as controls. The experiments were performed in triplicate in two different occasions. The results were expressed as the minimal concentration of the fraction that causes 50% (IC_{50}, inhibitory concentration) growth inhibition of epimastigotes, and 50% (EC_{50}, effective concentration) lysis of trypomastigotes.

2.8. Interaction with macrophage cells
Murine peritoneal macrophages were added to a 24-well culture plate (Techno Plastic Products, Switzerland), containing a round cover glass, at a density of 5 x 10^5 cells/well, and incubated for 24 h, in 5% CO_2 at 37°C. Trypomastigotes (at a parasite to macrophage ratio of 5:1) were inoculated into the monolayer and the plate was incubated for 3 h. After this period, the plates were washed with sterile PBS to remove the extracellular parasites. The fresh culture medium containing S-nitroso-MSA nanoparticles (50-400 µg/mL) were added to the cell culture, and the plates were incubated as above. Additionally, the macrophages monolayer was: (i) pre-treated with S-nitroso-MSA nanoparticles and then infected with trypomastigotes; (ii) and the infection and treatment were carried out simultaneously. The cover glasses of controls (medium alone, medium plus MSA-chitosan nanoparticles, and medium plus sodium nitrite) were tested and fixed with methanol (Merck, Brazil), stained with Giemsa after 24, 48 and 72 h incubation and permanently prepared in ERV-Mount resin. The percentage of infected host cells and the mean number amastigotes/500 cells were determined by direct counting in light microscopy.

3. Results and Discussion

3.1. Synthesis of MSA or S-nitroso-MSA-chitosanTPP nanoparticles
In order to investigate the role of NO against \textit{T. cruzi} protozoa, this unstable molecule need to be delivered to an application site in a controlled and sustained manner. Thus, biodegradable and biocompatible polymeric nanoparticles are interesting vehicles for drug delivery [21,22]. In particular, chitosan is natural polysaccharide broadly used in pharmacological applications [30]. Due to the presence of free amino groups, chitosan has a positive charge and can react with negatively charged biomolecules, such as DNA [30,31]. In addition, chitosan is reported to have antimicrobial properties [30], and thus, chitosan with the NO donor, S-nitroso-MSA, might potentiate antimicrobial activity in the nanoparticles. In this work, chitosan/TPP nanoparticles containing MSA were successfully prepared through ionic gelation. Cationic chitosan readily reacts with multivalent counter ions, such as TPP, to yield a network structure with spherical shape nanoparticles [12, 31].

In aqueous solution, the hydrodynamic diameter of the MSA-chitosan/TPP nanoparticles were found to be in the range of 270 to 550 nm with a polydispersity index of 0.35, which are in accordance with our previous results [12, 20]. The encapsulation efficiency of MSA in chitosan/TPP nanoparticle solution was found to be 99 ± 1%, indicating the high affinity of MSA to the nanoparticle.
S-nitroso-MSA-chitosan/TPP was obtained by nitrosating of free thiol groups of MSA-containing nanoparticles. An equimolar amount of sodium nitrite (NaNO$_2$) was added to the acidified MSA-containing polymeric nanoparticle solution. In acidified aqueous solution, sodium nitrite will form nitrous acid (HNO$_2$), which is the nitrosating agent, leading to the formation of S-nitroso-MSA, according to Figure 1.

![Nitrosation of MSA (Mercaptosuccinic acid)](image)

**Figure 1.** Schematic representation of the nitrosation of MSA yielding S-nitroso-MSA.

### 3.2. NO release profile from S-nitroso-MSA-containing chitosan nanoparticles in aqueous solutions

The NO release profile from S-nitroso-MSA-chitosan/TPP nanoparticles in aqueous solution were monitored by following the spectral changes at 336 and 545 nm absorption bands, associated the S-N bound cleavage with free NO release [12]. The kinetic curves were monitored for 24 h at 28 and 37°C, since these two temperatures were used in the *in vitro* experiments with *T. cruzi*. Figure 2 shows the NO release profile from S-nitroso-MSA encapsulated in chitosan.

![Kinetics of NO release from S-nitroso-MSA-containing chitosan/TPP nanoparticles at 28 and 35 °C.](image)

**Figure 2.** Kinetics of NO release from S-nitroso-MSA-containing chitosan/TPP nanoparticles at 28 and 35 °C.

Figure 2 shows that NO is spontaneously released from S-nitroso-MSA-containing chitosan nanoparticles. The kinetic curves show an initial burst of NO release in the first 5 hours, followed by a
progressively increase at lower rates, for both temperatures. A sustained NO release is observed for at least 10 h. The NO-release profile increased with the increase of the temperature, since the stability of this NO donor is dependent on the temperature, as expected [25-27]. Initial rates of NO released were calculated from the kinetic curves of Figure 2. The initial rates of NO release from S-nitroso-MSA-nanoparticles were found to be $0.13 \pm 0.01$ and $0.19 \pm 0.01 \text{ mmolL}^{-1}\text{h}^{-1}$ for 28 and 37°C, respectively. Higher rates are reported for higher temperatures [27]. The NO release from S-nitroso-MSA encapsulated in polymeric nanoparticles is reported to occur mainly through diffusion process over the pores or wall and disintegration of the hydropolymeric structure of chitosan [20].

3.3. Antitrypanosomal activity of NO-releasing polymeric nanoparticles

The inhibitory effects of S-nitroso-MSA chitosan nanoparticles on insect replicating epimastigote forms of *T. cruzi* Y strain are presented in Figure 3(A). Similarly, S-nitroso-MSA chitosan nanoparticles decreased the number of motile trypomastigotes, the non-replicating and infective form, after 24 h (Figure 3(B)). In both assays, a dose-dependent antitrypanosomal effect was observed and the IC$_{50}$ and EC$_{50}$ calculated for these nanoparticles were 252 µg/mL and 91 µg/mL, respectively.

![Figure 3](image)

*Figure 3.* Antitrypanosomal activity of S-nitroso-MSA-containing chitosan/TPP nanoparticles in different concentrations, as indicated in the Figure, for 48 h of incubation with: (A) epimastigote form of *T. cruzi*, (B) trypomastigote form of *T. cruzi*. Temperatures of incubation: 28°C and 37°C for epimastigote and trypomastigote forms, respectively.

Figure 4 shows the antitrypanosomal activity of S-nitroso-MSA chitosan nanoparticles, at final concentration of 200 µg/mL, after 48 h of incubation with *T. cruzi* epimastigote cultures. Control groups were performed by incubating epimastigote cultures with only the medium solution, medium solution plus MSA-chitosan nanoparticles (nanoparticles without the NO donor S-nitroso-MSA), and medium with plus sodium nitrite (NaNO$_2$), the nitrosating agent of MSA to form S-nitroso-MSA. The inhibitory effect is due to the presence of S-nitroso-MSA in the chitosan nanoparticles, since MSA-nanoparticles and the sodium nitrite were not toxic (Figure 4). Therefore, the potent antitrypanosomal activity observed can be attributed to the presence of S-NO groups incorporated in the polymeric nanoparticles. S-NO groups act as spontaneous NO donor due to the homolytic bound cleavage of S-N with free NO release. The sustained NO release from S-nitroso-MSA incorporated into chitosan nanoparticles over several hours as shown in Figure 2 demonstrated potent toxic effects against *T. cruzi*. Thus, free NO release from the nanoparticles is the toxic molecule.
Figure 4. Antitrypanosomal activity of S-nitroso-MSA chitosan nanoparticles at final concentration of 200 µg/mL, after 48 h of incubation with *T. cruzi* epimastigote forms. Control groups were performed by incubating epimastigote cultures with only the medium solution, medium solution plus MSA-chitosan nanoparticles (nanoparticles without the NO donor S-nitroso-MSA), and medium plus sodium nitrite (NaNO$_2$), the nitrosating agent of MSA to form S-nitroso-MSA. Temperature of incubation: 28°C. The asterisks indicate significant difference in the antitrypanosomal activity of the group treated with NO-releasing polymeric nanoparticles in comparison with the control groups.

3.4. Interaction of NO-releasing polymeric nanoparticles with macrophage cells

Because macrophages serve as effector cells in protecting against intracellular parasites, the interaction of *T. cruzi* and macrophages was studied in vitro. Figure 5 shows the antitrypanosomal activity of S-nitroso-MSA incorporated in chitosan nanoparticles (final concentration of 200 µg/mL) during *T. cruzi* interaction with murine peritoneal macrophages. As can be observed, S-nitroso-MSA-chitosan/TPP nanoparticles decreased the number of intracellular amastigotes, which is the replicating form found in mammalian hosts. Interestingly, NO-releasing polymeric nanoparticles were found to have a potent antitrypanosomal activity in all tested treatment conditions: before infection, during infection and after infection. Before infection means that macrophages were pre-treated with S-nitroso-MSA chitosan nanoparticles for 3 h, washed and then infected with *T. cruzi*. During infection means that macrophages were infected with *T. cruzi* and simultaneous incubated with S-nitroso-MSA nanoparticles. After infection means that macrophages were infected with *T. cruzi* and then incubated with S-nitroso-MSA nanoparticles. These results indicate that NO-releasing nanoparticles may interfere with the adhesion and/or invasion of host cells, or replication of *T. cruzi* in mammalian cells, suggesting their use in the treatment and prevention of Chagas disease.

In addition, NO is a versatile molecule in the biological system, involved in multiple defense mechanism pathways. Indeed, NO has been implicated in multiple cellular processes of the immune system and play multiple functions in infected host. The antimicrobial action of NO against pathogens is reported to occur via multiple pathways, which include DNA damage/mutation, alteration of proteins by S-nitrosation, inhibition of protein synthesis, tyrosine nitration, lipid membrane peroxidation [13]. As NO is a free radical, in the biological system, it readily reacts with superoxide (O$_2^•$), leading to the formation of the harmful peroxynitrite (ONOO$^-$). Tyrosine nitration by peroxynitrite is one of the major mechanism of the microbicidal action of NO [13].
Figure 5. Antitrypanosomal activity of S-nitroso-MSA incorporated in chitosan nanoparticles (final concentration of 200 µg/mL) in T. cruzi interaction with murine peritoneal macrophages. Control: medium; Before infection: macrophages were pre-treated with S-nitroso-MSA chitosan nanoparticles for 3 h, washed and then infected with T. cruzi; During infection: macrophages were infected with T. cruzi and simultaneous incubated with S-nitroso-MSA nanoparticles; After infection: macrophages were infected with T. cruzi and then incubated with S-nitroso-MSA nanoparticles. Temperature of incubation: 37 °C.

4. Conclusions
This work describes the preparation and characterization of S-nitroso-MSA-chitosan/TPP nanoparticles and their important effect as potent antitrypanosomal agent against different life cycle forms of T. cruzi protozoa. The results indicate that the encapsulation of the NO donor S-nitroso-MSA represents an interesting approach to control Chagas disease.

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6. References
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