Cytotoxicity of hyaluronic acid coated chitosan nanoparticles containing nitric oxide donor against cancer cell lines

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Abstract. The incidence and mortality rates of all types of cancer have a global burden. The lack of specificity of chemotherapy is one of the major problems in cancer treatment and it is related with moderate to severe adverse effects. CD44 is a protein overexpressed in certain types of cancer cells and can be used as an anchor of attachment for nanoparticles decorated with hyaluronic acid (HA). HA is a natural polymer with high affinity for CD44 receptors. In this work, chitosan nanoparticles (CS NPs) were coated with HA to delivery nitric oxide (NO) donor to cancer cells. NO is a small molecule involved in several physiological processes and it has cytotoxicity against cancer cells. HA-coated and uncoated NO-releasing CS NPs were synthesized and characterized by different techniques. Uncoated and HA-coated NO-releasing CS NPs have hydrodynamic diameters of 142.80 ± 2.22 nm and 170.80 ± 0.14 nm, respectively, polydispersity index of 0.282 ± 0.010 and 0.37 ± 0.04, respectively, and a zeta potential values of + 25.20 ± 0.85 mV and + 15.60 ± 0.15 mV, respectively. As expected, the presence of HA layer on the surface of NO-releasing CS NPs increased hydrodynamic size ca. 20%. The encapsulation efficiency of the NO donor into CS NPs was found to be higher than 90%. The kinetic of NO release from uncoated and HA-coated CS NPs has two distinct phases, an initial burst followed by the establishment of a plateau, with a maximum of NO released at 40 mmol·L⁻¹. At this concentration, NO is expected to have cytotoxic effects. The cytotoxicity of uncoated and HA-coated NO releasing CS NPs showed a concentration-dependent toxicity against human prostatic carcinoma (PC-3) and human uterine cervix carcinoma (HeLa) cell lines. Taken all together, uncoated and HA-coated NO-releasing CS NPs might find important biomedical applications, including cancer in treatment.

1. Introduction
The incidence and mortality rates of all types of cancer are growing worldwide. Bray et al. provided a report of global burden of cancer and they estimated 18.1 million new cancer cases and 9.6 million deaths in 2018 [1]. Chemotherapy is an important component of treatment of many cancers and the development of new drugs represents one of the largest areas of pharmaceutical industry [2]. The main disadvantage of the chemotherapy treatment is the lack of specificity, since the treatment might damage cancer cells as well as health cells. This feature is related to adverse effects of the treatment that affects the quality of life, physical health and emotional state of patients, which can negatively contribute to survival rates [2]. CD44 is a protein expressed in larger quantity on surface of certain cancer cells [3-7]. CD44 is a multifunctional cell surface molecule involved in cell proliferation and angiogenesis [3]. In addition, CD44 can help mediate the cellular uptake process [5].
Hyaluronic acid (HA) is a natural polymer with the ability to specifically target CD44 receptors [3]. HA is a glycosaminoglycan polysaccharide composed of G-glucuronic acid and N-acetyl-D-glucosamine and it is a critical component of the extracellular matrix [5,8]. HA is involved in the angiogenesis and inflammation processes and its administration is related to wound healing, relieve pain and reduction of inflammation in arthritis cases [3,6]. CD44 affinity to HA is used as an anchor of attachment for physically and chemically HA-decorated nanocarriers increasing specificity of cancer treatments [5].

Nanoparticles (NPs) are effective in delivering drugs for target cells, there are numerous studies confirming the role of NPs to enhance drug accumulation at the tumor site towards the enhanced permeability and retention (EPR) effect [9,10] and to maintain a sustainable drug release to prolong curing times [3,11]. Chitosan (CS) is a natural polymer that has been frequently employed in pharmaceutical and biomedical applications. CS is composed of D-glucosamine and N-acetyl-D-glucosamine, which is derived from the partial de-acetylation of chitin obtained from crustaceans. The main features of CS are porous structure, hemostatic properties, antibacterial activity and biodegradability [6]. In addition, CS stimulates cell proliferation and histoarchitectural tissue organization [6]. Chitosan nanoparticles (CS NPs) are easy to synthesize and they have been successfully used for drug delivery of proteins [5], chemotherapy agents [3] and DNA plasmid [12].

Nitric oxide (NO) is an important endogenous molecule related to a selective toxicity toward cancer cells [13]. It is well known that high levels of NO (micro to milli molar range) have toxicity due to the formation of peroxynitrite (ONOO⁻) and direct membrane damage with cytochrome release, among other mechanisms [14]. NO has a small half-life of 1-5 s, which impairs its application. For practical applications, NO donors, such as S-nitrosothiols (RSNOs), have been employed due to their superior stability and ability to spontaneously release free NO [15]. The use of HA and NO donors might create a synergetic effect to increase the selectivity of CS NPs to tumor cells.

Therefore, this study describes the synthesis and characterization of uncoated and HA-coated NO-releasing CS NPs (Figure 1) and their cytotoxicity towards cancer cell lines. To this end, in the first step, the NO donor precursor molecule, mercaptosuccinic acid (MSA), was encapsulated into uncoated and HA-coated CS NPs. The obtained nanoparticles were characterized by different techniques. Free thiol groups of MSA molecule were nitrosated by leading to the formation of NO-releasing CS NPs upon the incorporation of S-nitroso-mercaptosuccinic acid (S-nitroso-MSA) (Figure 1). This process led to the formation of NO-releasing CS NPs upon the incorporation of S-nitroso-MSA into the NPs.

![Figure 1](image.png)

Figure 1. Schematic representation of (A) CS NPs containing MSA, precursor of the NO donor. (B) HA layer (grey circle) was added on the surface of MSA-CS NPs (black circle). (C) Nitrosation of MSA leading to the formation of S-nitroso-MSA (NO donor) into uncoated and HA-coated CS NPs.

2. Methods

2.1. Synthesis of uncoated and HA-coated CS NPs
Firstly, the NO donor precursor molecule, mercaptosuccinic acid (MSA), was incorporated into uncoated and HA-coated CS NPs. To this end, an aqueous solution containing 1.0 mg·mL⁻¹ of CS and 66.67 mmol·L⁻¹ of MSA was dissolved in 1% acetic acid at room temperature. After 90 min of
magnetic stirring, a solution of sodium tripolyphosphate (TPP) at 0.6 mg·mL\(^{-1}\) was dropwise added to the CS/MSA previous prepared solution. The final mixture was magnetic stirred for at least 30 min leading to the formation of MSA-containing CS NPs (uncoated NPs). To coat the surface of MSA-CS NPs, 5 mL of aqueous suspension of MSA-CS NPs were mixture with 1.58 mg of HA for 1 hour at room temperature.

2.2. Dynamic light scattering measurements (DLS)
The hydrodynamic size diameter, polydispersity index (PDI) and zeta potential of uncoated and HA-coated MSA-CS NPs were evaluated by DLS (Nano ZS Zetasizer, Malvern Instruments Co, UK) [16]. Measurements were performed at a fixed angle of 173°, using a disposable capillary cuvette. The results were reported as the average of three independent experiments with the error bar values expressed by their standard error of the mean (SEM).

2.3 Nanoparticle tracking analysis (NTA)
The hydrodynamic size and concentration of uncoated and HA-coated CS NPs were evaluated by NTA LM-20 (NanoSight Ltd. UK). The size distribution of the NPs was obtained on particle-by-particle basis. NTA enables separation of particle population by size and intensity, microscopical visualization of individual NPs in suspension and simultaneously determining their Brownian motion [17].

2.4 Atomic force microscopy (AFM)
Topography and phase contrast images of HA-coated CS NPs containing MSA were simultaneously obtained in an atomic force microscope (Agilent, AFM/STM Series 5500) using non-contact mode tip (Nanoworld, 320 kHz, 42 Nm\(^{-1}\)). Images generated by the AFM were treated in the WSxM 5.0 Develop 8.2 and OriginPro 8 software.

2.5. Encapsulation efficiency of MSA into CS NPs
The encapsulation efficiency of the NO donor precursor molecule (MSA) into uncoated and HA-coated CS NPs was measured by the titration of free thiols groups of MSA molecule with the thiol reagent 5,50-dithiobis-(2-nitrobenzoic acid) (DTNB), as already described [16, 18]. Non-encapsulated (free) MSA was separated from encapsulated MSA by using a Microcon centrifugal filter device (MWCO 10,000, Millipore) and titrated with DTNB. The absorption band at 412 nm (\(\varepsilon = 14.15\text{ mmol} \cdot\text{L}^{-1} \cdot\text{cm}^{-1}\)), assigned to the formation of 2-nitro-5-thiobenzoate anion, which is generated in the reaction of DTNB with MSA, was measured in the ultra violet-visible spectrophotometer (Agilent 8454, Palo Alto, CA, USA). The analysis was performed in duplicate.

2.6. Nitrosation of MSA incorporated into CS NPs
Free thiol group of MSA encapsulated into uncoated and HA-coated CS NPs was nitrosated by the addition of equimolar amount of sodium nitrite (NaNO\(_2\), 50 mmol·L\(^{-1}\)), related to MSA, to the aqueous suspension of MSA-CS NPs and HA-MSA-CS NPs (pH ~ 3.0). The final suspensions were transferred to an ice bath (5°C), protected from the ambient light, leading to the formation of uncoated and HA-coated containing S-nitroso-MSA-CS NPs (NO-releasing NPs). The confirmation of the nitrosation of MSA yielding S-nitroso-MSA inside the NPs was performed by the appearance of the characteristic S-NO group absorption bands at 336 nm (\(\varepsilon = 980.0\text{ mmol} \cdot\text{L}^{-1} \cdot\text{cm}^{-1}\)) and at 545 nm (\(\varepsilon = 18.4\text{ mmol} \cdot\text{L}^{-1} \cdot\text{cm}^{-1}\)) by using the UV–vis spectrophotometer (Agilent 8454, Palo Alto, CA, USA).

2.7. NO release profile from uncoated and HA-coated S-nitroso-MSA-CS NPs at physiological temperature
The kinetics of NO release from uncoated and HA-coated S-nitroso-MSA-CS NPs were determined by monitoring the spectral changes at 545 nm (nN \(\rightarrow\) \(\pi^*\) transition) [13,19]. The decay of this absorption band is associated with the decomposition of S-nitroso-MSA with free NO release. The initial
concentration of S-nitroso-MSA was 50 mmol·L$^{-1}$ and the kinetic data were collected in 30 min intervals at 37°C for 6 h of monitoring. The amount of S-nitroso-MSA decomposed is related to the amount of NO released, since the decay of the absorption band at 545 nm over time is solely assigned to the cleavage of the S–N bond and NO release. 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).

2.8. Cytotoxicity of S-nitroso-MSA- containing uncoated CS NPs and HA coated- CS NPs

The cytotoxicity of uncoated and HA-coated S-nitroso-MSA-CS NPs was evaluated towards human prostatic carcinoma (PC-3) and human uterine cervix carcinoma (HeLa) cell lines and compared to HA-coated MSA-CS NPs. Different concentrations of the NPs were added to 96 wells plates containing 1 x 10$^4$ cells/well followed by incubation for 24 h at 37°C in a 5% CO$_2$ atmosphere. After, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.3 mg·mL$^{-1}$) was added to each well followed by 2 h incubation at 37°C. Then, the MTT solution was removed from the wells and the formazan crystals were dissolved in 150 μL of dimethyl sulfoxide (DMSO) and the plates were shaken at room temperature and the absorbance was measured at 570 nm (BiochromAsys Expert Plus Microplate Reader, Biochrom Ltd., UK). The control was performed in the absence of NPs and considered as 100% of viable cells. Each point represents the average of two independent experiments performed in sextuplicate, with the error bar values expressed by their standard error of the mean (SEM).

3. Results and discussion

3.1. Synthesis and characterization of uncoated and HA-coated MSA-CS NPs

CS NPs have been used for drug delivery [3,5], tissue engineering, with particular interest in cartilage regeneration [3, 12] and wound dressing [6, 20]. In addition, there is a patent of HA crosslinked materials for cosmetic and therapeutical applications [21]. In this work, initially, the NO donor precursor molecule, MSA, was encapsulated into CS NPs. The synthesis of MSA-containing CS NPs is based on the electrochemical interaction of protonated amino groups (NH$_3^+$) of CS and negatively charged TPP ions [16]. The coating with HA is also based on a strong electrochemical interaction between HA and CS (Figure 1).

DLS measurements revealed that the hydrodynamic diameter of MSA-CS NPs and HA-MSA-CS NPs were found to be 142.80 ± 2.22 nm and 170.80 ± 0.14 nm, respectively, with PDI values of 0.282 ± 0.010 and 0.37 ± 0.04, respectively. NTA measurements showed that the hydrodynamic diameter of MSA-CS NPs and HA-MSA-CS NPs were found to be 139 nm and 154 nm, respectively, with similar concentrations of 9.29·10$^5$ and 0.75·10$^5$ particles·L$^{-1}$, for uncoated and HA-coated CS NPs, respectively. The coating of HA increased the hydrodynamic size by 20% (as assayed by DLS measurements) and by 10% (as assayed by NTA measurements). This increase indicated the presence of HA layer on the surface of MSA-containing CS NPs. Our results are in accordance with previous reports. For instance, Chiesa et al. showed that everolimus, a key molecule to avoid organ rejection, containing HA-coated CS NPs have a hydrodynamic size of ca. 181-300 nm [5]. Similarly, Wang et al. showed that uncoated and HA-coated 5-fluracil containing CS NPs have hydrodynamic sizes of 98.5 ± 5.4 and 118.9 ± 9.5 nm, respectively, an increase in the hydrodynamic size by 20% after the HA coating [3].

The nanoparticle size distribution at solid state and nanoparticle morphology of HA-coated MSA-containing CS NPs were assayed by AFM. Figure 2A indicates an average diameter of 22.07 ± 0.74 nm for HA coated MSA-CS NPs. The nanoparticle size distribution at solid state has a smaller size compared with the size distribution in aqueous suspension, as expected. In addition, AFM images showed that the NPs have spherical shape and good dispersity (Figure 2B).
Figure 2. (A) Size distribution at solid state of HA-coated MSA-containing CS NPs, and (B) representative image of HA-coated MSA-containing CS NPs acquired using AFM.

The zeta potential values for MSA-CS NPs and HA-MSA-CS NPs were found to be $+25.20 \pm 0.85$ mV and $+15.60 \pm 0.15$ mV, respectively, indicating a good stability of the NPs in an aqueous environment, which is necessary for biomedical applications. The positive values of zeta potential are due to the presence of CS, since CS has positive NH$_3^+$ groups. In addition, the magnitude of zeta potential decreased by 40% after HA coating, indicating the presence of HA chains on the surface of coated NPs. HA is known to have a negative potential due to the presence of carboxyl groups [4,5]. Our results are in accordance with published papers. Indeed, Wang et al. showed zeta potential values of $+21.9 \pm 2.6$ and $+15.6 \pm 3.7$ mV for CS NPs and HA coated CS NPs, respectively [3].

The encapsulation efficiency values of MSA into uncoated and HA-coated CS NPs were found to be 90.3 ± 1.4 and 91.1 ± 0.7 %, respectively, indicating a high affinity of MSA to CS NPs, as previously described [22-23]. In addition, the presence of HA coating on the surface of CS NPs did not significantly affect the NO donor precursor encapsulation efficiency into the nanoparticle.

3.2 Nitrosation of MSA leading to S-nitroso-MSA
Free thiol group of encapsulated MSA was nitrosated by the reaction with nitrous acid (HNO$_2$) leading to formation of S-nitroso-MSA containing CS NPs (uncoated and HA-coated NPs) (Figure 1C). The nitrosation reaction was performed by the generation of nitrous acid (HNO$_2$), which is the thiol nitrosating agent, generated by the presence of sodium nitrite (NaNO$_2$) in acid solution. This process led to the formation of S-nitroso-MSA (NO donor) containing uncoated and HA-coated CS NPs. The formation of S-nitroso-MSA was confirmed by the detection of S-NO absorption bands at 336 nm or 545 nm.

3.3. NO release profile from S-nitroso-MSA-containing uncoated and HA-coated CS NPs at physiological temperature
The kinetics of NO release from uncoated and HA-coated S-nitroso-MSA-CS NPs in aqueous solution were monitored by following the spectral changes at 545 nm absorption band, associated the S-N bound cleavage with free NO release (Figure 3) [13, 19, 22]. S-nitroso-MSA undergoes a spontaneous decomposition leading to free NO release. Figure 3 shows the kinetic curves of NO release from encapsulated S-nitroso-MSA into uncoated and HA-coated CS NPs. It can be observed the presence of two distinct phases: (a) an initial burst, in the first 4 h of monitoring, and (b) a second phase of NO release that is characterized by a decrease in the rates of NO release from 4 to 6 h and the establishment of a plateau. The initial rates of NO release at the first phase were found to be 15.44 ±
0.77 and 13.90 ± 0.93 mmol·L$^{-1}$ for S-nitroso-MSA encapsulated into uncoated and HA-coated CS NPs, respectively. The rates of NO release at the second phase from S-nitroso-MSA encapsulated into uncoated and HA-coated CS NPs were 47.38 ± 0.60 and 42.32 ± 0.45 mmol·L$^{-1}$, respectively. Thus, the addition of HA layer on the surface of CS NPs slightly decreased the rates of NO release at both phases, indicating a sustained NO release, as expected. Moreover, the concentration of NO release, in both cases, is in the mmol/L rage, at this concentration NO is known to have antitumor and antimicrobial activities [18,19,23]. This behaviour is similar to other reports for our group [18,19,23].

![Figure 3](image.png)

**Figure 3.** Kinetics of NO release from S-nitroso-MSA (NO donor) encapsulate into uncoated (i) and HA-coated (ii) CS NPs. In both cases, initial S-nitroso-MSA concentration was 50 mmol·L$^{-1}$. The results are presented as mean ± standard error of three independent experiments.

3.4. **Cytotoxicity of S-nitroso-MSA encapsulated into uncoated and HA-coated CS NPs**

Figure 4 shows the cell viabilities of HeLa (Figure 4A) and PC3 (Figure 4B) cells incubated with S-nitroso-MSA containing uncoated and HA-coated CS NPs. For both cases, it can be observed a decrease in the cell viability upon the increase of the nanoparticle concentration. The results indicate a cytotoxicity at higher nanoparticle concentrations (higher than 25 µg·mL$^{-1}$). In addition, S-nitroso-MSA encapsulated into HA-coated CS NPs showed a slight enhance in the decrease of HeLa and PC3 viabilities compared with S-nitroso-MSA containing uncoated CS NPs. In addition, MSA containing HA-coated CS NPs have lower cytotoxicity compared with S-nitroso-MSA containing HA-coated CS NPs against HeLa cell line in the range of 0 to 100 µg·mL$^{-1}$. This result indicates a slightly higher toxicity to HA-coated NPs, compared with uncoated NPs, which might be assigned to the superior interactions of HA with CD44 receptors present on the cell membranes, since cancer cells are known to super-express CD44. Chiesa at al. showed similar results for CS NPs coated with HA, and confocal microscopy results of marked NPs with fluorescein isothiocyanate (FITC) indicated a higher internalization of HA-coated CS NPs [5].
Figure 4. Percentages of cell viability of (A) HeLa upon incubation with S-nitroso-MSA (NO donor) encapsulated into uncoated (black line) and HA-coated CS NPs (red line), compared to MSA encapsulated into HA-coated CS NPs (blue line) and (B) PC3 upon incubation with S-nitroso-MSA (NO donor) encapsulated into uncoated (black line) and HA-coated CS NPs (red line) at different concentrations. Cell viability was estimated by a tetrazolium-based (MTT) reduction assay. The results are presented in percentage of control (absence of NPs) as mean ± standard error of two independent experiments.

4. Conclusion
In this work, the NO donor, S-nitroso-MSA, was encapsulated into uncoated and HA-coated CS NPs. The obtained NPs have a small size with good polydispersity and spherical shape. In addition, the results demonstrated a high encapsulation efficiency of the NO donor into the NPs. Uncoated and HA coated S-nitroso-MSA CSNPs were able to spontaneously release free NO, in a sustained manner, at concentrations suitable for biomedical applications. Uncoated and HA coated NO-releasing CS NPs demonstrated a concentration dependent toxicity towards HeLa and PC3 cell lines. As expected, the presence of HA on the surface of CS NPs slightly increased the toxicity of the NPs to tumor cells. Uncoated and HA coated NO-releasing CSNPs might find important applications in drug delivery therapies.

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