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2011 J. Phys.: Conf. Ser. 304 012045

(http://iopscience.iop.org/1742-6596/304/1/012045)

Glutathione and S-nitrosoglutathione in alginate/chitosan nanoparticles: Cytotoxicity

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Abstract. Nitric oxide (NO) is involved in several physiological processes, such as the control of vascular tone, the immune response and the wound healing process. Thus, there is a great interest in the development of NO-releasing drugs and in matrices which are able to stabilize and release NO locally in different tissues. Thiols, such as glutathione (GSH), are ready nitrosated to form the NO donors S-nitrosothiols (RSNOs). In this work, GSH, a precursor of the NO donor S-nitrosoglutathione (GSNO), was encapsulated into a mucoadhesive combination of alginate/chitosan nanoparticles. The encapsulated GSH was nitrosated in the alginate/chitosan nanoparticles by adding sodium nitrite, leading to the formation of encapsulated GSNO. The cytotoxicity characterization of the nanoparticles containing either GSH or GSNO showed that these materials were completely non cytotoxic to cellular viability. These results show that this novel nanostructure biomaterial has a great potential to be use in biomedical applications where NO has a therapeutical effect.

1. Introduction

Nitric oxide (NO) is an endogenous free radical that plays a key role in the regulation of several physiological processes, such as the control of vasodilation, platelet interaction with the vessel wall, immune responses, wound healing and cellular communication [1-5]. The endogenous synthesis of NO is mediated through nitric oxide synthase (NOS) which catalyzes the conversion of *L*-arginine to NO and citrulline. At low concentrations, NO is quite stable and minimally reactive, however, at higher concentrations NO can interact with transition metals, heme-containing proteins, thiol groups leading to oxidation of functional groups on RNA, DNA and proteins [6]. The biological actions of NO have been shown to be dependent on its site and source of production, as well as its concentration.

Due to the multifaceted role of NO *in vivo*, there is a great interest on the developing of NO releasing molecular systems for biomedical applications, especially to treat important diseases [7]. In this context, many different classes of NO donors have been prepared and applied in biological systems [8]. Some studies suggested that cysteine thiols (RSHs) of bioactive peptides such as glutathione (GSH), the most abundant non-protein thiol found *in vivo*, act as NO carriers and donors in tissues [9]. Thiols are directly involved in the metabolism and mobilization of NO, and GSH forms S-nitrosoglutathione (GSNO) *in vivo* [10], which belongs to a class of compounds named S-nitrosothiols (RSNOs). GSNO and others RSNOs exhibit NO-like activities such as the inhibition of platelet adhesion, vasodilation, microbicidal actions, and wound healing [1,2,4,5,11,12]. However, the potential pharmacological applications of RSNOs, and in particular GSNO, require a vehicle since RSNOs are considered unstable in aqueous solution [9].

A promising approach that has been considered to promote the uses of RSNOs as NO donors in biomedical applications is either the direct incorporation of NO donors, such as RSNOs, into biopolymers or nanoparticles [7,13-15]. In both cases, NO can be released directed to the target site for prolonged periods of time and exerts its therapeutic effects, such as the promotion of cutaneous wound healing and dermal vasodilation, upon application *in vivo* [1,2,4,5].

However, a crucial issue with regards to propose the clinical uses of NO-releasing materials is the evaluation of the cytotoxicity of the NO donor molecules (RSNOs) and RSNOs-loaded to the polymers. It was reported that GSNO decreased neutrophil viability in a time and concentration-dependent manner, while GSH exposure alone did not enhance cell death or DNA fragmentation at any concentration studied, as assessed by a fluorescent viability/cytotoxicity assay [16].

In this work the tri-peptide GSH, precursor of the NO donor GSNO, was encapsulated into a mucoadhesive combination of alginate/chitosan. GSH-encapsulated alginate/chitosan nanoparticles were characterized, and the thiol was nitrosated to form encapsulated GSNO. The kinetics of NO release from GSNO-containing alginate/chitosan nanoparticles were monitored at physiological temperature, and compared to free GSNO. Finally, the cytotoxicity of either GSH- or GSNO-encapsulated were evaluated. The results showed that encapsulated GSH can be nitrosated to form GSNO-containing nanoparticles. The results suggest that this formulation is a potent candidate for pharmacological applications that require the release of controlled amounts of NO for prolonged periods of time, where NO can have its physiological actions, without severe side effects.

2. Methods

2.1. Preparation of polymeric nanoparticles with GSH

The alginate/chitosan nanoparticles containing GSH were prepared thorough ionic gelation method [17]. Briefly, it was prepared an aqueous chitosan solution (1.33%) with GSH. A ratio of alginate/chitosan solutions of 0.75 was obtained by dropping wise previous prepared chitosan aqueous solution into the alginate aqueous solution (0.005 g/100 mL) (pH 4.0), under magnetic stirring.

2.2. Characterization, encapsulation efficiency and physical stability of the alginate/chitosan nanoparticles

The average particle size (number average size) and size distribution of prepared nanoparticles as the zeta potential of alginate/chitosan ratio (0.75) were measured by photon correlation spectroscopy (PCS) (Nano ZS Zetasizer, Malvern Instruments Corp). The encapsulation efficiency of GSH in nanoparticles was determined by DTNB titration after ultrafiltration of the nanoparticles solution [12]
The physical stability alginate/chitosan nanoparticles at 0.75 ratio was monitored over 37 days at 4°C, by measuring the average particles sizes and surface charges, as described previously.

2.3. Nitrosation of GSH encapsulated in alginate/chitosan nanoparticles
GSH (400 μM) encapsulated in alginate/chitosan nanoparticles at ratio 0.75 and free GSH (400 μM) were nitrosated by adding equimolar amounts of sodium nitrite directly into polymeric aqueous solution (pH 4.0) at 336 nm (ε = 940 mol/L/cm), using an UV-Vis spectrophotometer [12].

2.4. Kinetic measurements of NO release from encapsulated GSNO and free GSNO
The kinetic curves of GSNO (400 μM) decomposition with NO release were monitored for free GSNO (GSNO in aqueous solution) and encapsulated GSNO in alginate/chitosan nanoparticles from the absorption changes at 336 nm.

2.5. Cytotoxicity of GSH and GSNO free and encapsulated in nanoparticles
The cytotoxicity of GSH and GSNO, free and encapsulated in alginate/chitosan nanoparticles, was assessed in a permanent lung fibroblast cell line (V79) culture derived from Chinese hamster [18]. These cells are commonly used for cytotoxicity studies [19]. The cells were plated at a density of 3×10^4 cells/ml in 96-well plates. Forty-eight hours after cell seeding, semiconfluent cultures were exposed to free GSH, free GSNO, encapsulated GSH and encapsulated GSNO, at different concentrations (0 – 18 μM). The cells were exposed for 24 h to the test medium with or without the compounds studied (control). Each concentration was tested in six replicates in each of three separate experiments. At the end of the incubation, two independent endpoints tests for cytotoxicity (Methylthiazoletetrazolium reduction- MTT and neutral red uptake were evaluated [20,21].

3. Results and Discussion

3.1. Preparation and characterization of GSH-containing alginate/chitosan nanoparticles
Preparation of alginate/chitosan nanoparticles at ratio of 0.75, with and without GSH, showed average particles sizes of 361 and 301 nm, respectively, and positive surface charges with average values of +27 (with GSH) and + 22 mV (without GSH) (Table 1). Alginate/chitosan nanoparticles prepared showed the encapsulation efficiency of 27% (Table 1). These positively charged particles exhibited high stability after 37 days.

Table 1. Particles sizes, surface charge (Zeta potential), and encapsulation efficiency of alginate/chitosan nanoparticles.

Alginate/Chitosan ratios	GSH (mg)	Zeta Potential (mV)	Particle sizes (nm)	PdI	Encapsulation efficiency (%)
0.75	0	+22	301	0.316	---
0.75	5	+27	361	0.330	27

3.2. Nitrosation of GSH-encapsulated in alginate/chitosan nanoparticles
GSH encapsulated in nanoparticles of alginate/chitosan was directly nitrosated by reacting with equimolar amount of sodium nitrite at pH 4.0. The formation of GSNO was confirmed by the detection of the characteristic GSNO absorption band at 336 nm ($\pi \rightarrow \pi^*$ transition) (data not shown). Thus, a NO-donor molecule (GSNO) could be prepared directly in alginate/chitosan suspension by directly nitrosation of encapsulated GSH in the nanoparticles.

3.3. Kinetics of GSNO decomposition with NO release from free GSNO and encapsulated GSNO in polymeric nanoparticles
The kinetic data of GSNO decomposition with NO release at 37°C for free GSNO, which corresponds to aqueous solution of GSNO (400 μM) at pH 4.0 and GSNO (400 μM) encapsulated in alginate/chitosan nanoparticles (alginate/chitosan ratio 0.75) at pH 4.0 were analyzed. The decrease of

the intensity of the absorption band at 336 nm is assigned to the homolytic cleavage of the S-N bond, with GSNO decomposition leading to the release of NO and the formation of oxidized glutathione [9]. Either free GSNO or encapsulated were able to spontaneously decomposes, releasing NO, under physiological temperature, for hours. However, for the first 3 h of the kinetic, the GSNO encapsulate in the nanoparticles can be considered stable (no change in the absorption at 336 nm). Only after 3 h of the kinetic, it was observed an increase in the rate of decomposition of encapsulated GSNO, with NO release. On the other side, the rate of free GSNO decomposition was observed to be continuously and linear from time zero to 7 h. Indeed, the rate of encapsulated GSNO decomposition was found to be $1.2 \times 10^{-2} \pm 0.2 \times 10^{-2}$ h^{-1} for 3 to 7 h. On the other hand, the rate of free GSNO decomposition was found to be $1.5 \times 10^{-2} \pm 0.2 \times 10^{-2}$ h^{-1} for 0 to 7 h. Therefore, encapsulation of GSNO in alginate/chitosan nanoparticles was responsible to decrease the rate of NO release, especially during the initial hours. This delay on the NO release may be due to the low diffusion rates of GSNO from the nanoparticles to the solution.

3.4. Cytotoxicity of free GSH, free GSNO and GSNO and GSH encapsulated in alginate/chitosan nanoparticles

Free glutathione showed to be non cytotoxic to fibroblast V79 cells in the lysosomal (Neutral red) and slightly cytotoxic to mitochondrial (MTT) assays (15-20% around to 10 μM concentration), as is observed in Figure 1A. In the Figure 1B is shown that cytotoxicity in the case of GSNO was also not cytotoxic to fibroblast V79 cells in the lysosomal (Neutral red) and slightly cytotoxic to mitochondrial (MTT) assays (20% around to 16 μM concentration), as is observed in Figure 1B.

Alginate/chitosan nanoparticles with or without GSH or GSNO exhibited non cytotoxicity (till 18 μM concentration).

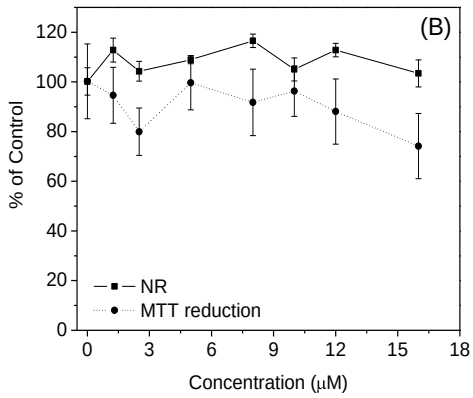

Figure 1. Viability graph in V79 cells with the cellular targets as MTT and NR of the free GSH (A) and free GSNO (B).

4. Conclusions

The kinetic of NO release from free GSNO compared with GSNO-encapsulate in alginate/chitosan reveled that the encapsulation of the NO donor resulted in a thermal and hydrolytic protection of GSNO leading to an enhancement of NO activity by a controlled and sustained NO release from this new biomaterial. The higher stability of encapsulated GSNO, compared to free GSNO enable the use of GSNO in pharmaceutical applications, such as wound healing, or others uses where the biological effects of NO are required.

Acknowledgements

Support from Brazilian Network of Nanobiotechnology, and Nanocosmetics (MCT/CNPq) and FAPESP (process number 2010/14949-4) are acknowledged.

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