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Research paper

Time lasting *S*-nitrosoglutathione polymeric nanoparticles delay cellular protein *S*-nitrosation

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ABSTRACT

Physiological *S*-nitrosothiols (RSNO), such as *S*-nitrosoglutathione (GSNO), can be used as nitric oxide (NO) donor for the treatment of vascular diseases. However, despite a half-life measured in hours, the stability of RSNO, limited by enzymatic and non-enzymatic degradations, is too low for clinical application. So, to provide a long-lasting effect and to deliver appropriate NO concentrations to target tissues, RSNO have to be protected. RSNO encapsulation is an interesting response to overcome degradation and provide protection. However, RSNO such as GSNO raise difficulties for encapsulation due to its hydrophilic nature and the instability of the S-NO bound during the formulation process. To our knowledge, the present study is the first description of the direct encapsulation of GSNO within polymeric nanoparticles (NP). The GSNO-loaded NP (GSNO-NP) formulated by a double emulsion process, presented a mean diameter of 289 ± 7 nm. They were positively charged (+40 mV) due to the methacrylic acid and ethylacrylate polymer (Eudragit® RL) used and encapsulated GSNO with a satisfactory efficiency (i.e. 54% or 40 mM GSNO loaded in the NP). In phosphate buffer (37 °C; pH 7.4), GSNO-NP released 100% of encapsulated GSNO within 3 h and remained stable still 6 h. However, in contact with smooth muscle cells, maximum protein nitrosation (a marker of NO bioavailability) was delayed from 1 h for free GSNO to 18 h for GSNO-NP. Therefore, protection and sustained release of NO were achieved by the association of a NO donor with a drug delivery system (such as polymeric NP), providing opportunities for vascular diseases treatment.

1. Introduction

Since the discovery of the pivotal role of nitric oxide (NO) in several physiological processes in 1999 by Ignarro [1], NO has rapidly emerged as a promising candidate for the treatment of numerous disorders, mainly in cardiovascular function but also in stroke [2], asthma [3] and erectile dysfunction [4]. Nitric oxide is a second messenger *in vivo*, produced endogenously by three distinct nitric oxide synthases via L-arginine conversion [5–7] and it corresponds to the major endothelial relaxing factor that relaxes smooth muscle not only in the vasculature, but also in the gastrointestinal tract [8–10].

The biological activity of NO can be explained by its high chemical reactivity. It is a free radical species, carrying a single unpaired electron in its outer shell. The substances that are known to react with NO include other radicals, transition metal ions and nucleophiles such as thiols (RSH) and amines [11]. NO acts by two main signaling pathways to regulate vascular function *in vivo*. The first is nitrosylation, corresponding to reversible bounding of NO to transition metal ions, such as ferrous (FeII) heme prosthetic groups within proteins (such as soluble guanylyl cyclase); this leads to enzyme activation and increased conversion of guanosine-3,5′-triphosphate to cyclic guanosine monophosphate (cGMP). The elevated cGMP activates specific kinases and finally vasorelaxation [12–14]. The second is *S*-nitrosation, targeting sulfhydryl-containing proteins and resulting in NO being covalently bound to cysteine. The formation of a mixed disulfide between a thiol group on an effector protein or peptide and a low molecular mass thiol, is able to modulate the function of the former. This posttranslational modification of proteins is as important as phosphorylation [15–17].

The imbalance of NO production and bioavailability is at the center of many cardiovascular diseases such as atherosclerosis,

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pulmonary hypertension, thrombosis, ischemia and cardiac arrhythmia [18]. The direct application of gaseous NO (such as in the treatment of pulmonary hypertension) is limited by its high reactivity. Expense, complex operational conditions and potential toxicity are also reported [19]. In this context, over the past few decades, several NO-related therapeutics based on more complex chemical system have emerged, such as nitrosamines [20], organic nitrates [21], metal–NO complexes [22], N-diazeniumdiolates [23], and *S*-nitrosothiols (RSNO) [24]. Organic nitrates and nitrate esters have been used in therapy of cardiovascular diseases [25–29], for example in the treatment of *angina pectoris*. However, these compounds induce undesirable effects such as oxidative stress, tolerance, thunderclap headache, and hypotension [30]. Without any recorded side effects in preclinical studies, RSNO (such as *S*-nitrosoglutathione (GSNO), *S*-nitroso-*N*-acetylcysteine, NACNO) therefore represent an especially promising class of NO donors for *in vivo* applications [31]. Endogenous RSNO, such as GSNO, *S*-nitrosoalbumin and *S*-nitrosocysteine, are formed by the nitrosation of free thiols by reactive nitrogen species (e.g., N_2O_3) [32] and constitute a physiological pool of NO. Many investigations relating to the therapeutic potential of RSNO in the cardiovascular system have focused on GSNO, which is a powerful antiplatelet agent [33] with arterioselective vasodilator effects and also with well-documented antimicrobial [34] and antithrombotic effects [35,36].

With half-lives measured in hours [37], the stability of RSNO is actually too low for clinical applications. Therefore, the combination of RSNO with a delivery system represents a very promising strategy for the pharmaceutical and medical applications of NO [38]. Three different strategies to achieve this have been described in the literature. The first is the development of new macromolecular RSNO (thiomers), assembled as nanostructures [39,40]. For example, NO has been covalently bound to PEG-conjugated bovine serum albumin (PEG-poly SNO-BSA) via a *S*-nitrosothiol linkage (by nitrosation of cysteine) in the study of Katsumi et al. [41], which increased *in vivo* stability and prolonged NO release. A possible second strategy is the nitrosation of encapsulated free thiols, thereby constructing a *S*-nitrosothiol-loaded carrier. Marcato et al. [42] have developed polymeric NP based on alginate/chitosan to encapsulate GSH. After nitrosation of GSH, they obtained the GSNO-loaded NP. As a rarer third strategy, the direct encapsulation of *S*-nitrosothiol in liposomes [43], inorganic NP delivery system [44] and polymeric films [45,46] has also been described. This third option is often hampered by the sensitivity of RSNO to many factors (such as light, temperature, and oxygen) and to the biological environment, making it difficult to maintain a therapeutic concentration. Taking into account the fragility of RSNO, mild pharmaceutical processes would be preferred. In the current study, polymeric nanocarriers based on poly (methyl)methacrylate were elaborated as potential delivery systems of GSNO, able to preserve S-NO bound throughout the formulation process. Drastic handling conditions were conducted to efficiently encapsulate this small hydrophilic and labile molecule. The platform showed its ability to protect GSNO from physicochemical and enzymatic degradations *in vitro*. Protein *S*-nitrosation in cell culture gave proof of concept of NO donor activity from encapsulated GSNO. To our knowledge, this report represents the first description of polymeric NP for efficient encapsulation and prolonged release of effective GSNO, as a pharmaceutical drug.

2. Materials and methods

All reagents were of analytical grade and all solutions prepared with ultrapure deionized water (>18.2 mΩ cm). Sodium nitrite was purchased from Merck (Germany). GSNO was synthesized according to a previously described method [42]. Eudragit® RL PO was a generous gift from Evonik industries (Germany). The BCA Protein Assay Kit was purchased from Pierce. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, 2,3-diaminonaphthalene (DAN), sulfanilamide, N-(1-naphthyl)ethylenediamine and all other reagents were obtained from Sigma–Aldrich (France). All manipulations and assays involving GSNO were conducted under conditions of subdued light and at 4 °C, in order to minimize light-induced GSNO degradation.

2.1. Preparation of GSNO-loaded NP

GSNO-loaded NP (GSNO-NP) were prepared by a double emulsion (water–oil–water) and solvent evaporation method. Briefly, an aqueous solution of GSNO (5 mg) in 500 µL of 0.1% (w/w) Pluronic® F-68 solution was emulsified by sonication for 60 s (11 W, 80% amplitude, Vibra cell™ 72434, France) over an ice bath in 5 mL of methylene chloride containing 500 mg of Eudragit® RL PO. This primary emulsion was further emulsified in 20 mL of 0.1% (w/w) Pluronic® F-68 solution by sonication (30 W, Vibra cell™ 75022, France) for 30 s, over an ice bath to form a water–oil–water emulsion. An opalescent emulsion was obtained. The NP were hardened by solvent evaporation. Finally the GSNO-NP were collected by ultracentrifugation at 287,000*g*, 30 min, 4 °C (Optima™ TLX ultracentrifuge, USA) before use.

2.2. Physicochemical characterization of NP

The hydrodynamic diameter, size distribution and polydispersity index (PDI) of the NP were measured in 1 mM NaCl by dynamic light scattering (DLS) (Zetasizer® Nano ZS, Malvern® Instrument, France). All DLS measurements were performed at 25 °C with an angle detection of 173° backscatter (NIBS default). The samples were measured after 30 s autocorrelation and 15 runs were performed on each sample. For zeta potential measurements by electrophoretic migration (Zetasizer® Nano ZS, Malvern® Instrument, France), samples were diluted with 1 mM NaCl. All measurements were performed in triplicate.

2.3. Determination of GSNO encapsulation efficiency and core loading

Encapsulation efficiency (EE) describes the quantity of the drug entrapped within NP compared with the total amount of initial drug. It was determined according to the following equation: $EE = m_e/m_i \times 100$, where EE is encapsulation efficiency (%), m_e is the mass of drug entrapped in NP, and m_i is the mass of initial drug. The mass of encapsulated GSNO was determined by liquid–liquid extraction. The mass of initial GSNO was calculated by the addition of the quantity of encapsulated GSNO and that remaining in the supernatant. The concentrations of GSNO and nitrite ions (a product of GSNO decomposition) were quantified by Griess-Saville and Griess reaction, respectively [20]. The concentration of loaded GSNO describes the capacity of NP matrix to carry GSNO. It was determined with the following equation: $C = n_e/v_f$, where C is the concentration of loaded GSNO (mM), n_e is the quantity (mol) of entrapped GSNO in the NP, and v_f is the volume of opalescent nanosuspension (mL). Both the encapsulation efficiency and the concentration of loaded GSNO were detected immediately after the GSNO-NP preparation.

2.4. In vitro release of GSNO from NP

GSNO-NP were suspended in 1 mL of 0.148 M phosphate buffered saline (PBS, pH 7.4) and were laid in dialysis tubing cellulose membrane (average flat width 10 mm (0.4 in), cut-off is 14,000 Da). Release kinetics were studied in 200 mL of PBS at 37 °C, protected from light. Released GSNO and nitrite ions were