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Polymer nanocomposites enhance $S$-nitrosoglutathione intestinal absorption and promote the formation of releasable nitric oxide stores in rat aorta

Wen Wu, PharmD, PhD, Caroline Perrin-Sarrado, PharmD, PhD, Hui Ming, PharmD, Isabelle Lartaud, PharmD, PhD, Philippe Maincent, PharmD, PhD, Xian-Ming Hu, PhD, Anne Sapin-Minet, PhD, Caroline Gaucher, PhD,

**CITHEFOR EA3452 “Drug targets, formulation and preclinical assessment”, Faculté de Pharmacie, Université de Lorraine, Nancy, France
**State Key Laboratory of Virology, Ministry of Education Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Wuhan University School of Pharmaceutical Sciences, Wuhan, China

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Abstract

Alginate/chitosan nanocomposite particles (GSNO-acNCPs), i.e. $S$-nitrosoglutathione (GSNO) loaded polymeric nanoparticles incorporated into an alginate and chitosan matrix, were developed to increase the effective GSNO loading capacity, a nitric oxide (NO) donor, and to sustain its release from the intestine following oral administration. Compared with free GSNO and GSNO loaded nanoparticles, GSNO-acNCPs promoted 2.7-fold GSNO permeation through a model of intestinal barrier (Caco-2 cells). After oral administration to Wistar rats, GSNO-acNCPs promoted NO storage into the aorta during at least 17 h, as highlighted by (i) a long-lasting hyporeactivity to phenylephrine (decrease in maximum vasoconstrictive effect of aortic rings) and (ii) $N$-acetylcysteine (a thiol which can displace NO from tissues)-induced vasodilation of aortic rings preconstricted with phenylephrine. In conclusion, GSNO-acNCPs enhance GSNO intestinal absorption and promote the formation of releasable NO stores into the rat aorta. GSNO-acNCPs are promising carriers for chronic oral application devoted to the treatment of cardiovascular diseases.

Key words: NO-donor; Polymer nanocomposites; Oral delivery; Isolated aorta vasoreactivity

In the cardiovascular system, deficiency of endogenous nitric oxide (NO) is the consequence of either insufficient synthesis (endothelium dysfunction) or excessive NO degradation (increased oxidative or nitrosative stresses, decreased antioxidant enzyme activity). NO depletion is one of the key factors in the initiation and progress of many diseases, such as atherosclerosis, pulmonary hypertension, thrombosis, ischemia and cardiac arrhythmia. To maintain an appropriate level of NO and treat NO deficiency, several NO-related therapies have been developed such as nitrosamines, organic nitrates, metal–NO complexes, $N$-diazeniumdiolates. However, all act at very short term and lead to tolerance phenomena. $S$-nitrosothiols (RSNOs) present the advantage of a longer half-life, with no tolerance nor oxidative stress induction. Under physiological conditions, $S$-nitrosoglutathione (GSNO), a major endogenous RSNO, is one of the main storage forms of NO in tissues. GSNO has been investigated for its powerful antiplatelet activity, arterio/venous selective vasodilator effects, antimicrobial and antithrombotic effects.

Despite such therapeutic potencies, GSNO pharmaceutical forms are still lacking. This may be related to the fast and often unpredictable rate of decomposition of GSNO. In vitro, because of pH-, light- and temperature-dependent sensitivities, GSNO is susceptible to many degradation processes.

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*Corresponding author.
E-mail address: caroline.gaucher@univ-lorraine.f (C. Gaucher).

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including S-NO bond homolysis, metal ion-catalyzed decomposition, and hydrolysis. In vivo, it is subjected to enzymes-induced decomposition such as GSNO reductase and carbonyl reductase. For this reason, the bioavailability of GSNO when administered orally is limited. Two main strategies were described to overcome such limitations, through either inhibiting GSNO reductases activity or improving the stability of GSNO. In respect to the latter, many researchers focused on the protection of GSNO – during storage and in biological media – through the combination with delivery systems to promote pharmaceutical and medical applications. For instance, Seabra and co-workers dispersed GSNO into polyethylene glycol (PEG) or solid poly(vinyl alcohol)/poly(vinyl pyrrolidone) film to achieve the controlled release of NO adapted to topical application. de Mel and co-workers passively incorporated GSNO into a polyhydral oligomeric silsesquioxane poly(carbonate-urea)urethane (POSS-PCU) composite to produce a NO realizing implant for cardiovascular diseases treatment. Encapsulation followed by S-nitrosation of glutathione (GSH, the GSNO precursor) into mucoadhesive polymeric nanoparticles produced GSNO loaded nanoparticles, which slowed down GSNO decomposition at physiological temperature. Similarly, Shah et al described another way to prolong the release of NO by conjugating GSH on chitosan backbone and S-nitrosating the GSH thiol group. In our previous work, polymeric nanoparticles based on poly(methyl)methacrylate were developed to load GSNO through direct encapsulation (GSNO-NP), which protect GSNO and preserve its availability during interaction with smooth muscle cells. However, the release profile we obtained was not long enough for chronic in vivo therapeutic applications. In our following work, we developed polymer nanocomposite particles (NCP), which refer to nanoparticles of nanometric size embedded in a polymer matrix forming a composite particle of micrometric size, as defined by Bhattacharya and coworkers. Microparticles were composed in our case of an alginate or a chitosan matrix including our previously described GSNO-NP. These GSNO-NPs embedded into chitosan or alginate nanocomposites increased the encapsulation efficiency of GSNO compared to GSNO-NPs (from 54% to 69% or 76%, respectively) and sustained the in vitro release of GSNO until 24 h.

From these promising results, in the present study, our aims were to further improve GSNO oral bioavailability, to control its delivery at the absorption site and to prolong the residence time into the gastrointestinal tract, by combining both alginate plus chitosan in the matrix. Alginate and chitosan were chosen as mucoadhesive polymers increasing the residence time on the intestine mucus layer. As alginate has the capacity to penetrate the mucus layer, this will bring the drug closer to the intestinal cells, while chitosan exerts the property to open cells tight junction. Therefore, their combination will lead to increase drug permeability through the intestine. To broke new ground in the field of nanocomposite particles and merge mucopenetration and mucoadhesion properties in one system, we therefore developed GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) formed through the incorporation of GSNO-NPs into a mix of alginate/chitosan matrix. The efficient encapsulation capacity and the sustained release profile of GSNO encouraged us to further investigate GSNO permeability through an intestinal cell barrier model (Caco-2). Finally, for the first time, we administrated GSNO-acNCPs to Wistar rats by gavage in order to evaluate their vascular effects, more particularly their capacity to increase the NO-storage inside the vascular wall.

Methods

Materials

All reagents were of analytical grade and all solutions prepared with ultrapure deionized water (> 18.2 MΩ.cm at 25 °C). Sodium nitrite was purchased from Merck (Germany). Acrylates/ammonium methacrylate copolymer (Eudragit® RL PO) was a generous gift from Evonik industries (Germany). Alginate sodium, chitosan (4-6 kDa), polyoxyethylene-polyoxypropylene block copolymer (Pluronic® F-68), mucin from porcine stomach, HBSS with Ca²⁺ and Mg²⁺ and all other reagents were obtained from Sigma-Aldrich (France). All experiments and assays involving GSNO were conducted under conditions of subdued light and at +4 °C in order to minimize light- and temperature-induced GSNO degradation.

Methods

GSNO synthesis

GSNO was synthesized according to a previously described method. Briefly, reduced GSH was incubated with an equivalent of sodium nitrite under acidic condition (0.626 M HCl); after precipitation, the solid form of GSNO was obtained. The purity of GSNO was assessed by HPLC and UV spectrophotometry using the specific molar absorbance of the S-NO bond at 334 nm (ε = 922 M⁻¹ cm⁻¹).

Preparation of GSNO-loaded alginate/chitosan nanocomposite particles

GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) were constituted by inner cores and external polymer matrix. The inner cores were GSNO-loaded nanoparticles (GSNO-NP) prepared by a double emulsion (water–oil–water) and solvent evaporation method as described in previous work. Briefly, an aqueous solution containing GSNO in 0.1% (w/w) Pluronic® F-68 was emulsified with Ca²⁺ and Mg²⁺ and sonicated with an organic phase containing Eudragit® RL PO (100 mg/mL in dichloromethane). Then, sodium alginate and sodium tripolymethacrylate (TPP, 220 mg) were fully dissolved in the resulting GSNO-NP suspension. Over an ice bath, calcium chloride solution (CaCl₂, 2 M) was added dropwise into this mixture to cross-link alginate and homogenized by sonication (40 W, ultrasonic processor, France). GSNO-loaded alginate/chitosan nanocomposites particles (GSNO-acNCPs) were finally formed by dropwise addition of GSNO-NP-alginate mixture into the chitosan solution (1 mg/mL in 1% (v/v) acetic acid), under mechanical stirring (1300 rpm) over ice bath.
Figure 1. Release kinetic of the payload (GSNO and nitrite ions) from polymer nanocomposite particles in phosphate buffered saline (0.148 M) at 37 °C. Data are shown as mean ± sd, n = 3, * P < 0.05 vs free GSNO and GSNO-NPs (two-way ANOVA). GSNO-NP: GSNO-loaded nanoparticles, GSNO-acNCPs: GSNO-loaded alginate/chitosan nanocomposite particles.

Characterization of GSNO-loaded alginate/chitosan nanocomposite particles

Determination of size. The volume particle size distribution of GSNO-acNCPs was determined by the laser diffraction method (Mastersizer 2000, Malvern Instruments, France). The GSNO-acNCPs were suspended in ultrapure water. The size of GSNO-acNCPs was described by the volume mean diameter measured in triplicate.

Evaluation of GSNO encapsulation efficiency. Encapsulation efficiency (EE) describes the quantity of the drug entrapped within GSNO-NPs compared with the initial drug amount. It was determined according to the following equation:

\[ EE = \frac{m_e}{m_i} \times 100 \]

where EE is the encapsulation efficiency in percent (%), \( m_e \) is the mass of drug entrapped in particles, and \( m_i \) is the initial mass of drug. The entrapped drug within particles was evaluated by a two-step-liquid–liquid extraction. The external matrix of particles composed of sodium alginate and chitosan was disrupted by mechanical stirring followed by centrifugation and the amount of GSNO remaining in the supernatant, subtracted by the free nitrite ions quantified by the Griess assay, \( S \) was quantified with a fluorometric method using diaminonaphthalene (added or not with HgCl2 to cleave the S-NO bond) producing naphthotriazole (emission 415 nm/excitation 375 nm, JASCO FP-8300, France).

Stability of GSNO in GSNO-loaded alginate/chitosan nanocomposite particles. The GSNO-acNCP or GSNO-NP suspensions were centrifuged (15,000 g, 20 min, 4 °C) and the resulting pellets were kept in a freezer at −20 °C, in a fridge at +4 °C or at +37 °C in a humidified incubator. At predetermined time intervals over 15 days (D) (1D, 2D, 3D, 4D, 5D and 15D), the GSNO was extracted and analyzed by the Griess–Saville assay as above to determine the remaining intact and decomposed GSNO. All the samples were prepared in triplicate.

Mucins binding assay

Changes in size and zeta potential of GSNO-NPs in contact with a mucin dispersion were examined. After centrifugation (42,000 g, 30 min, 4 °C; Heraeus Instruments, France), nanocomposite particles were resuspended in a mucin dispersion at a concentration of 5 mg/mL polymer in 1 mg/mL mucin (ratio 5:1). The volume particle size distribution of GSNO-NPs was determined by the laser diffraction method (Mastersizer 2000, Malvern Instruments, France) and zeta potential was estimated by electrophoretic mobility (ZetaSizer NanoZS, Malvern Instruments, France).

Cytocompatibility

The cytocompatibility of GSNO-acNCPs was evaluated on intestinal Caco-2 cells ATCC® HTB-37™. Caco-2 cells were grown in complete medium consisting of Eagle’s Minimum Essential Medium EMEM supplemented with 20% (v/v) fetal bovine serum, 4 mM of glutamine, 100 U/mL of penicillin, 100 U/mL of streptomycin, 1% (v/v) of non-essential amino acids. Cells were cultivated at 37 °C under 5% CO2 (v/v) in a humidified incubator. Caco-2 cells were seeded in 96-wells plates at 20,000 cells/well 24 h before experiment. They were then exposed to 0.5, 1.0, 5.0, 10.0 or 50.0 mg/mL of GSNO-acNCP for 24 h at 37 °C, complete medium being used as control. Cytocompatibility expressed by metabolic activity was checked with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance of extracted formazan crystals was read at 570 nm with a reference at 630 nm (EL 800 microplate reader, Bio-TEK Instrument, Inc®, France). Metabolic activity in control condition was considered as 100%.

In vitro cell permeability

The permeability of GSNO across the Caco-2 monolayer was evaluated in the apical-to-basolateral direction in Hank’s Balanced Salt Solution (HBSS, pH 6.5). After seeding 10⁶ cells on cell culture inserts (Transwell®, Corning, USA) with 0.4 μm pore size disposed in a 12-wells plate, the medium was replaced every two days during the first week and every day during the lasting days until the differentiated cell monolayer was formed (14-18 days, 588 ± 44 Ω/cm²). For
the permeation experiments, polymer nanocomposite particles containing 50 μM of GSNO were suspended in 500 μL of HBSS and introduced in the apical (donor) compartment, whereas, 1.5 mL of HBSS was placed in basolateral (receptor) compartment. After 1, 4 and 24 h, each basolateral compartment was withdrawn and replaced with the same volume of fresh HBSS. The GSNO, nitrite and nitrate ions permeated and presented in basolateral (for each time) and apical (after 24 h only) compartments were detected using the fluorometric method.

Figure 2. Stability of GSNO in polymer particles. GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) or GSNO-loaded nanoparticles (GSNO-NP) suspensions were centrifuged and the resulting pellets were stored at −20 °C (A), +4 °C (B) and +37 °C (C) for 15 days. At regular intervals, the remaining intact GSNO was determined by the Griess–Saville assay. Data are expressed as mean ± sd (n = 3). *: P < 0.05 vs free GSNO (two-way ANOVA).

Figure 3. In vitro cytotoxicity of polymer nanocomposite particles on Caco-2 cells. Caco-2 cells were treated with the indicated concentrations of GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) for 24 h at 37 °C. Viability was estimated through mitochondrial activity by the MTT assay. Data are expressed as mean ± sem (n = 3).

The integrity of the cell monolayer was checked before the permeability studies by measuring the transepithelial electrical resistance (TEER) using a Millicell®-Electrical Resistance system (Millipore, USA). In addition, the permeation of fluorescein sodium (5 μM) was also used to verify the integrity of monolayer.

The cumulative amounts of GSNO crossing the Caco-2 monolayer were calculated from the concentrations measured in basolateral compartments. The apparent permeability coefficient (Papp) values were calculated using the following equation:

\[
P_{\text{app}} = \frac{dQ/dt}{A} \times \frac{1}{C_0}
\]

\(dQ/dt\) (mol/s) refers to the permeability rate (mol) of RSNOS in the basolateral compartment at the time of quantification, \(A\) (cm²) refers to membrane diffusion area, and \(C_0\) (mol/mL) refers to the initial concentration in the apical compartment.

The enhancement ratio (R) was calculated through the following equation:

\[
R = \frac{P_{\text{app1}}}{P_{\text{app2}}}
\]

Papp1 refers to the apparent permeability coefficient of each treatment, and Papp2 corresponds to the apparent permeability coefficient of free GSNO.
Measurement of vasoactivity. Aortic vasoactivity was measured using an isometric tension recording system in 10 mL organ chambers (EMKABATH, Emka Technology, France). Baths were filled with Krebs’ solution (10 mL, 37 °C, pH 7.4) and continuously bubbled with 95% O₂ and 5% CO₂. Following 60-min equilibration with a basal resting tension determined at 2 g, vasoactivity of aortic rings was assessed with potassium chloride (KCl, 6 × 10⁻² M) added in the baths. The contraction rapidly reached a steady state and was expressed, after 15 min exposure, as the developed tension from the basal resting tension of 2 g. Arteries showing less than 2.5 g of developed tension were excluded. After viability test and a 30 min wash-out period so that tension returns to baseline, aortic rings were tested with two protocols: i) vasorelaxation of aortic rings (n = 10-12 per group, from 4 different rats in each group) preconstricted with 10⁻⁶ M and 10⁻⁴ M phenylephrine (PHE, were added with N-acetylcysteine (NAC) (10⁻⁴ M); ii) NO storage in the aorta was evaluated with N-acetylcysteine (NAC) was used to displace NO from cysteine-NO residue. Rings (n = 10-12 per group, from 4 different rats in each group) preconstricted with 10⁻⁶ M PHE, were added with NAC (10⁻⁵ M and 10⁻⁴ M). Contractile effects were expressed as induced developed tension (Delta T, g) and relaxant ones in percentage of contraction (100% being the stable tension caused by 10⁻⁶ M PHE).

The absence of functional endothelium was confirmed by the inability of carbachol 10⁻⁵ M, a muscarinic receptors agonist, to induce less than 10 % of relaxation on 10⁻⁶ M PHE-preconstricted aortic rings.

Statistical analysis

Results are shown as either mean ± standard deviation (sd) or mean ± standard error of the mean (sem) values. The half maximal effective concentration (EC₅₀) and maximal response (Emax) were calculated by fitting each concentration response curve using the Hill logistic equation. For the comparison of Papp, and that of Emax and EC₅₀ for PHE, statistical comparisons were performed using the one-way ANOVA (P < 0.05). Other analyses were performed using the two-way ANOVA (P<sub>treatment</sub> < 0.05; P<sub>time</sub> < 0.05). Statistical analyses were performed using the GraphPad Prism software (GraphPad Software version 5.0, San Diego, USA).
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Papp (10⁻⁶ cm/s)</th>
<th>R</th>
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<tbody>
<tr>
<td>Free GSNO</td>
<td>8.3 ± 0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>GSNO-NP</td>
<td>7.7 ± 3.1</td>
<td>0.9</td>
</tr>
<tr>
<td>GSNO-acNCPs</td>
<td>34.1 ± 1.5*</td>
<td>4.1</td>
</tr>
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</table>

Data are shown as mean ±SEM, n = 3. All data sets were compared to free GSNO. *: P < 0.05 vs free GSNO (one-way ANOVA). GSNO-NP: GSNO-loaded nanoparticles, GSNO-acNCPs: GSNO-loaded alginate/chitosan nanocomposite particles.

Results

Physico-chemical characterization of GSNO-loaded alginate/chitosan nanocomposite particles

The average size of the developed nanocomposite particles was 56 ± 15 μm (mean ± sd; n = 3). GSNO was entrapped within the nanocomposite particles with a high encapsulation efficiency (76 ± 10%), corresponding to an estimated core loading of 15.0 ± 1.9 mg of GSNO/g of polymer. The inner core of GSNO-loaded nanoparticles presented an estimated core loading of 5.7 mg of GSNO/g of polymer with a zeta potential of +40 ± 6 mV and a mean diameter of 0.289 ± 0.014 μm. As shown in Figure 1, 75 ± 3% of free GSNO was released from the dialysis bag during the first hour. Similarly, GSNO-NPs showed a release profile with a burst release of 95 ± 2% of the initially loaded GSNO within the first 3 h. On the other hand, only 57 ± 6% of GSNO was released from GSNO-acNCPs within 3 h and the remaining amount was released in a sustained way over the next 21 h.

Stability of GSNO in GSNO-loaded alginate/chitosan nanocomposite particles

The stability of GSNO within the polymer nanocomposite particles was evaluated at −20 °C, +4 °C, and +37 °C. After 5 days of storage at −20 °C or +4 °C, 100 ± 1% or 70 ± 5% of free GSNO, respectively, was decomposed (Figure 2, A, B). When incorporated into the polymer nanocomposite particles, 16 ± 5% (−20 °C) and 12 ± 4% (+4 °C) of intact GSNO was preserved for 15 days, showing improvement of GSNO stability. At +37 °C, GSNO degraded faster (Figure 2, C): 57 ± 4% of free GSNO had decomposed within two days and 98 ± 1% after 4 days. Polymer nanocomposite particles increased the life-time of GSNO as 46 ± 9% and 12 ± 4% of intact GSNO were detected on the 4th and 15th days respectively.

Mucin binding assay

The mucadhesion property of the polymeric GSNO-NPs was also evaluated. The diameter of GSNO-NPs in contact with mucin dispersion immediately increased from 0.2 ± 0.0 μm to 18.2 ± 3.2 μm (100 times more) and estimated zeta potential value was divided per 3 (from +54 ± 4 mV to +18 ± 5 mV, (mean ± sd; n = 3)).

Cytocompatibility and cell permeability

The concentration-response curve built from the MTT metabolic activity assay (Figure 3) gave an IC₅₀ at 3.24 ± 0.38 mg/mL for GSNO-acNCPs. Therefore, a concentration of 1 mg/mL (containing 50 μM GSNO) that maintained 83 ± 1% of cell viability was chosen for Caco-2 permeability studies.

Caco-2 cell monolayers were incubated with 50 μM (representing 25 nmol of GSNO in 0.5 mL) free or nanoparticle-associated GSNO in the apical compartment to investigate GSNO permeability. After 1 h of incubation, a similar concentration of GSNO was detected in the basolateral compartment for all formulations (Figure 4, A). The permeation kinetics of the RSNO were similar for GSNO and GSNO-NPs: the amount transported rose rapidly to 0.59 ± 0.07 nmol and 0.51 ± 0.01 nmol, respectively, over the first 4 h, and then remained at a plateau up to 24 h. GSNO-acNCPs showed a steady penetration rate over the whole 24 h, and promoted 2.7-fold more GSNO crossing the cell monolayer than free GSNO or GSNO-NP (Figure 4, A). The Papp value of GSNO (showing the ability of NPs and acNCPs to help the transport of GSNO across Caco-2 cell monolayer) did not change with NP, but significantly increased after GSNO-NPs incorporation into nanocomposite particles, showing an enhancement ratio of 4.1 (Table 1). In contrast to free GSNO and GSNO-NPs, GSNO-acNCPs retained 0.9 ± 0.1 nmol of intact GSNO in the apical compartment after 24 h of incubation (Figure 4, B) showing that GSNO-acNCPs could have an even longer release profile.

At the end of experiments, the test using the low molecular weight hydrophilic tracer sodium fluorescein, showed that the permeation of sodium fluorescein in the treated groups (18 ± 20%) was higher than the positive control (6.1 ± 0.5%) and less than negative control (32 ± 9%), in which cells were treated with HBSS without Ca²⁺ and Mg²⁺. Taken together, these results confirmed the integrity of cells monolayer after incubation with the GSNO-acNCPs with a slight opening of tight junction due to chitosan.

GSNO-NPs showed a core loading two times under the GSNO-acNCPs core loading (5.7 mg of GSNO/g of polymer. Therefore, a concentration of 1 mg/mL (containing 50 μM GSNO) that maintained 83 ± 1% of cell viability was chosen for Caco-2 permeability studies.
and 15.0 ± 1.9 mg of GSNO/g of polymer, respectively). Furthermore, the permeability studies failed in showing a higher and long lasting absorption of GSNO for the GSNO-NPs through intestinal cells compared to free GSNO. For these two reasons, the GSNO-NPs were moved aside from the in vivo studies.

Pharmacological vascular effects

The dose–response curves to PHE were similar in aortic rings from Wistar rats orally treated 17 h earlier with free GSNO, blank-acNCP or PBS (Figure 5). In aortic rings from rats treated with GSNO-acNCP (15 mg/kg body weight), the contractile maximal effect (Emax) of PHE significantly decreased (from 4.8 g in control groups to 3.7 g in the GSNO-acNCP group, −25%, P < 0.05) with no change in EC50 (Table 2).

In all the control groups, NAC failed to relax the vessels. However, in the group treated with GSNO-acNCP, NAC induced significant relaxation (11 ± 1% at 10−5 M and 24 ± 3% at 10−4 M, P < 0.05 compared to controls, Figure 6).

Discussion

The encapsulation of the fragile molecule GSNO within drug delivery systems is still challenging. Different approaches, either through S-nitrosation of free or conjugated GSH23,25 or direct encapsulation of GSNO21,37 are described in the literature. In the present study, we adopted the second approach with gentle formulation process to develop polymer nanocomposite particles loaded with GSNO. The polymer nanocomposites were obtained by incorporation of GSNO loaded Eudragit® RL nanoparticles (GSNO-NP)26 into a polymer matrix prepared from a mixture of alginate and chitosan by ionic gelation. The challenge was to control the loading of GSNO within the nanocomposite particles in order to get potential pharmacological efficiency. We based our calculations on the minimal concentration of NO required to stimulate the synthesis of cyclic GMP by soluble guanylate cyclase in vascular smooth muscle (approximately 10 nmol/L 38), and the dose of GSNO that significantly decreases mean arterial blood pressure (i.e. 3 mg; 100 μg/min for 30 min, intravenously, in human 39). Compared to our previous published results on GSNO-NP,26 the newly formulated composite particles improved the loading capacity of GSNO by 2.6-fold (from 5.7 to 15.0 mg of GSNO/g of polymer). This improvement can probably be attributed to the use of alginate and chitosan, which are hydrophilic and have good affinity for

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<th>Group</th>
<th>EC50 (10−5 M)</th>
<th>Emax (g)</th>
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<tr>
<td>PBS</td>
<td>3.1 ± 0.1</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Blank-acNCPs</td>
<td>1.9 ± 0.2</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td>Free GSNO</td>
<td>3.1 ± 0.1</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>GSNO-acNCPs</td>
<td>3.1 ± 0.1</td>
<td>3.7 ± 0.1*</td>
</tr>
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</table>

Data are shown as mean ±sem, (n = 10-12 per group, from 4 different rats in each group). *: P < 0.05 vs PBS (one-way ANOVA). PBS: phosphate buffered saline. Blank-acNCPs: blank alginate/chitosan nanocomposite particles, GSNO-acNCPs: GSNO-loaded alginate/chitosan nanocomposite particles.
of this polymer and the alginate of the complex matrix will be able to adhere on the mucus layer and to open tight junctions in the intestinal compartment. Moreover, alginate will help the inner core made of GSNO-NPs to penetrate the mucus layer. GSNO-NPs will no longer stay at the nanometer size; as we showed they adsorb proteins like mucins or albumin to reach a final size around 20 μm. Others also showed that the mucus layer strongly increase particle size and modify absorption of particles through the intestine. Indeed, Bajka et al demonstrated that a particle diameter higher than 500 nm largely decreased particles diffusion. Therefore, it is probable that only GSNO or other NO species will diffuse within the intestinal tight junctions, already opened by chitosan, and reach the vascular compartment.

Whatever the mechanisms involved in intestinal crossing, oral administration of GSNO-acNCPs to Wistar rats reveals NO-related vascular effects attesting that NO effectively reaches the blood stream. The decreased contractile response of aorta to PHE is the proof that the absorbed GSNO is transported from the intestine to the aortic wall by itself or through transnitrosation process of albumin or hemoglobin in the blood stream. Then, close to the vascular wall, transnitrosation processes occur again to generate a NO store inside the vascular wall. The final storage of NO in vascular smooth muscle cells was highlighted by aortic hyporeactivity to PHE and vasorelaxation in response to NAC. Indeed, in the absence of NO storage, NAC is not able to relax aortic rings, whereas, in presence of NO stores (S-nitrosation of proteins/peptides cysteine residues) within the vessel wall, NAC after entering into the cells displaces NO from vascular stores, which finally activates the guanylate cyclase/GMPc pathway inducing relaxation. Finally, the transnitrosation reactions with S-nitrosated proteins/peptides, leading to the formation of unstable S-nitrosothiols may be involved, both in the storage processes during the 17 hours following gavage, and in the release of NO induced by NAC. Similar results were obtained ex vivo in previous experiments where NO donors applied directly on the aortic rings, sustained hyporeactivity to vasoconstrictors from 1 to 4 hours. Oral administrations of PBS or blank-acNCPs, as that of free GSNO – due to its limited stability – were unable to form a reservoir of NO in vascular tissue, and NAC has no vasorelaxant effect.

Another hypothesis for the decreased response to PHE may relate to the S-nitrosation of the alpha1-adrenergic receptor leading to its decreased affinity for sympathetic ligands. This is unlikely in the present study as EC50 remained similar in all groups of treatment.

In conclusion, we demonstrated the therapeutic potential of polymer nanocomposite particles based on poly(methyl)methacrylate inside an alginate plus chitosan matrix for oral delivery of GSNO. The efficient loading, protection and sustained release of GSNO provided by these formulations promote GSNO absorption through the intestinal barrier, allow GSNO to reach the blood stream and contribute to form a reservoir of NO by transnitrosation inside the vascular wall. Seventeen hours following oral administration, vascular hyporeactivity to the vasoconstrictor remained. These new drug delivery systems of NO donors may be particularly adapted for oral treatment of cardiovascular diseases.

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References


Polymer nanocomposites enhance $S$-nitrosoglutathione intestinal absorption and promote the formation of releasable nitric oxide stores in rat aorta

Wen Wu, PharmD, PhD, Caroline Perrin-Sarrado, PharmD, PhD, Hui Ming, PharmD, Isabelle Lartaud, PharmD, PhD, Philippe Maincent, PharmD, PhD, Xian-Ming Hu, PhD, Anne Sapin-Minet, PhD, Caroline Gaucher, PhD

$^a$CITHEFOR EA3452 “Drug targets, formulation and preclinical assessment”, Faculté de Pharmacie, Université de Lorraine, Nancy, France

$^b$State Key Laboratory of Virology, Ministry of Education Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Wuhan University School of Pharmaceutical Sciences, Wuhan, China

Novel alginate/chitosan nanocomposite particles were successfully developed for the delivery of $S$-nitrosoglutathione (GSNO), a physiological nitric oxide (NO) donor, with improved encapsulation efficiency, sustained-release. The developed nanocomposite particles enhanced intestinal absorption of GSNO and thus promoted the formation of NO stores in the blood vessels after oral administration to Wistar rats.