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Wen Wu, Caroline Perrin-Sarrado, Hui Ming, Isabelle Lartaud, Philippe Maincent, et al.. Polymer nanocomposites enhance S-nitrosoglutathione intestinal absorption and promote the formation of releasable nitric oxide stores in rat aorta. *Nanomedicine: Nanotechnology, Biology and Medicine*, 2016, 12 (7), pp.1795-1803. 10.1016/j.nano.2016.05.006 . hal-01493332

**HAL Id: hal-01493332**

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Submitted on 3 Apr 2019

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Nanomedicine: Nanotechnology, Biology, and Medicine  
xx (2016) xxx–xxx

**nanomedicine**  
Nanotechnology, Biology, and Medicine

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

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Received 16 October 2015; accepted 5 May 2016

### 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 precontracted 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.

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**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)<sup>1,2</sup> or excessive NO degradation<sup>3,4</sup> (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,<sup>5</sup> pulmonary hypertension,<sup>6</sup> thrombosis,<sup>7</sup>

ischemia<sup>8</sup> and cardiac arrhythmia.<sup>9</sup> To maintain an appropriate level of NO and treat NO deficiency, several NO-related therapeutics have been developed such as nitrosamines, organic nitrates, metal–NO complexes, *N*-diazoniumdiolates. 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.<sup>10</sup> GSNO has been investigated for its powerful antiplatelet activity,<sup>11,12</sup> arterio/venous selective vasodilator effects,<sup>13,14</sup> antimicrobial<sup>15</sup> and antithrombotic effects.<sup>16</sup>

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

Funding and conflicts of interest: This work was supported by the ‘Université de Lorraine and Région Lorraine’. W. Wu and H. Ming acknowledge Chinese Scholarships Council for their doctoral fellowships. X.-M. Hu was a guest professor at the Université de Lorraine, CITHEFOR EA 3452, for three months between 2014 and 2015.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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<http://dx.doi.org/10.1016/j.nano.2016.05.006>

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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<sup>17</sup> and carbonyl reductase 1.<sup>18</sup> For this reason, the bioavailability of GSNO when administrated orally is limited. Two main strategies were described to overcome such limitations, through either inhibiting GSNO reductases activity<sup>19</sup> 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)<sup>20</sup> or solid poly(vinyl alcohol)/poly(vinyl pyrrolidone) film<sup>21</sup> to achieve the controlled release of NO adapted to topical application. de Mel and co-workers passively incorporated GSNO into a polyhedral oligomeric silsesquioxane poly(carbonate-urea)urethane (POSS-PCU) composite to produce a NO realizing implant for cardiovascular diseases treatment.<sup>22</sup> 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.<sup>23,24</sup> 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.<sup>25</sup> 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.<sup>26</sup> However, the release profile we obtained was not long enough for chronic *in vivo* therapeutic applications. In our following work,<sup>27</sup> 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.<sup>28</sup> Microparticles were composed in our case of an alginate or a chitosan matrix including our previously described GSNO-NP.<sup>26</sup> 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.<sup>27</sup>

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.<sup>29–32</sup> As alginate has the capacity to penetrate the mucus layer,<sup>30</sup> this will bring the drug closer to the intestinal cells, while chitosan exerts the property to open cells tight junction.<sup>31,32</sup> 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 ( $>18.2 \text{ m}\Omega\cdot\text{cm}$  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  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  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.<sup>33</sup> 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 ( $\epsilon = 922 \text{ M}^{-1}\text{cm}^{-1}$ ).

#### 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.<sup>26</sup> Briefly, an aqueous solution containing GSNO in 0.1% (w/w) Pluronic® F-68 was emulsified by sonication with an organic phase containing Eudragit® RL PO (100 mg/mL in dichloromethane). Then, sodium alginate and sodium tripolyphosphate (TPP, 220 mg) were fully dissolved in the resulting GSNO-NP suspension. Over an ice bath, calcium chloride solution ( $\text{CaCl}_2$ , 2 M) was added dropwise into this mixture to cross-link alginate 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.

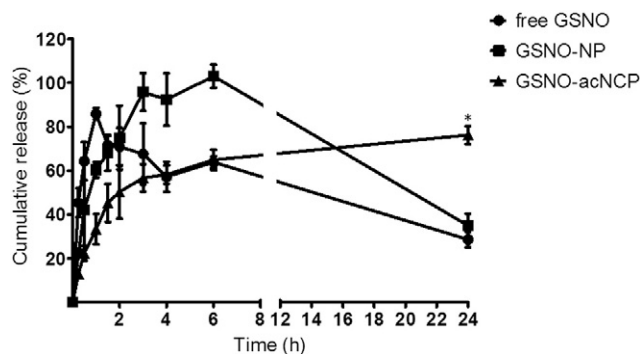


Figure 1. Release kinetic of the payload (GSNO and nitrite ions) from polymer nanocomposite particles in phosphate buffered saline (0.148 M) at 37 °C. Data are shown as mean  $\pm$  sd,  $n = 3$ . \*:  $P < 0.05$  vs free GSNO and GSNO-NP (two-way ANOVA). GSNO-NP: GSNO-loaded nanoparticles, GSNO-acNCPs: GSNO-loaded alginate/chitosan nanocomposite particles.

### 157 Characterization of GSNO-loaded alginate/chitosan 158 nanocomposite particles

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

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

$$EE = m_e / m_i \times 100$$

169 where EE is the encapsulation efficiency in percent (%),  $m_e$  is the  
170 mass of drug entrapped in particles, and  $m_i$  is the initial mass of  
171 drug. The entrapped drug within particles was evaluated by a  
172 two-step-liquid-liquid extraction. The external matrix of particles  
173 composed of sodium alginate and chitosan was disrupted by  
174 mechanical stirring followed by centrifugation and the amount of  
175 GSNO remaining in the supernatant, subtracted by the free nitrite  
176 ions quantified by the Griess assay,<sup>34</sup> was quantified by the Griess–  
177 Saville assay using sulfanilamide and  $\text{HgCl}_2$  in acidic conditions to  
178 cleave the *S*-NO bond, and *N*-(1-naphthyl)ethylenediamine to form a  
179 chromophoric azo product that absorbs at 540 nm.<sup>34</sup>

181 The resulting pellet, which corresponds to GSNO-NPs, was  
182 dissolved in 2 mL dichloromethane and the amount of GSNO in  
183 the nanoparticles was extracted in phosphate buffered saline  
184 (PBS) at pH 7.4 and determined by the Griess–Saville assay as  
185 described above. All the samples were prepared in triplicate.

186 Core loading expressed in mg of GSNO/g of polymer was  
187 estimated from initial GSNO, Eudragit® RL PO, sodium alginate  
188 and chitosan amounts.

189 **In vitro release kinetic.** GSNO-acNCPs were suspended in  
190 1 mL of PBS and were placed in cellulose dialysis tubing  
191 (average flat width 10 mm (0.4 in), cut-off 14 kDa). Release

kinetic was measured as previously described<sup>27</sup> in 200 mL of  
192 PBS at 37 °C protected from light. The GSNO and nitrite ions  
193 released were monitored every 30 min during two hours, every  
194 hour from two to six hours, then at 24 h, and immediately  
195 quantified with a fluorometric method<sup>35</sup> using diaminonaphtha-  
196 lene (added or not with  $\text{HgCl}_2$  to cleave the *S*-NO bond)  
197 producing naphthotriazole (emission 415 nm/excitation 375 nm,  
198 JASCO FP-8300, France).  
199

**Stability of GSNO in GSNO-loaded alginate/chitosan**  
200 **nanocomposite particles.** The GSNO-acNCP or GSNO-NP  
201 suspensions were centrifuged (15,000 g, 20 min, 4 °C) and the  
202 resulting pellets were kept in a freezer at  $-20$  °C, in a fridge at  
203  $+4$  °C or at  $+37$  °C in a humidified incubator. At predetermined  
204 time intervals over 15 days (D) (1D, 2D, 3D, 4D, 5D and 15D),  
205 the GSNO was extracted and analyzed by the Griess–Saville  
206 assay as above to determine the remaining intact and decom-  
207 posed GSNO. All the samples were prepared in triplicate.  
208

### 209 Mucins binding assay

210 Changes in size and zeta potential of GSNO-NPs in contact  
211 with a mucin dispersion were examined.<sup>36</sup> After centrifugation  
212 (42,000 g, 30 min, 4 °C; Heraeus Instruments, France), nano-  
213 particles were resuspended in a mucin dispersion at a  
214 concentration of 5 mg/mL polymer in 1 mg/mL mucin (ratio  
215 5:1). The volume particle size distribution of GSNO-NPs was  
216 determined by the laser diffraction method (Mastersizer 2000,  
217 Malvern Instruments, France) and zeta potential was estimated  
218 by electrophoretic mobility (ZetaSizer NanoZS, Malvern  
219 Instruments, France).

### 220 Cytocompatibility

221 The cytocompatibility of GSNO-acNCPs was evaluated on  
222 intestinal Caco-2 cells ATCC® HTB-37™. Caco-2 cells were  
223 grown in complete medium consisting of Eagle's Minimum  
224 Essential Medium EMEM supplemented with 20% (v/v) fetal  
225 bovine serum, 4 mM of glutamine, 100 U/mL of penicillin,  
226 100 U/mL of streptomycin, 1% (v/v) of non-essential amino  
227 acids. Cells were cultivated at 37 °C under 5%  $\text{CO}_2$  (v/v) in a  
228 humidified incubator. Caco-2 cells were seeded in 96-wells plates  
229 at 20,000 cells/well 24 h before experiment. They were then  
230 exposed to 0.5, 1.0, 5.0, 10.0 or 50.0 mg/mL of GSNO-acNCP for  
231 24 h at 37 °C, complete medium being used as control. 231  
232 Cytocompatibility expressed by metabolic activity was checked  
233 with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-  
234 mide (MTT) assay. The absorbance of extracted formazan crystals  
235 was read at 570 nm with a reference at 630 nm (EL 800  
236 microplate reader, Bio-TEK Instrument, Inc®, France). Metabolic  
237 activity in control condition was considered as 100%.  
238

### 238 In vitro cell permeability

239 The permeability of GSNO across the Caco-2 monolayer  
240 was evaluated in the apical-to-basolateral direction in Hank's  
241 Balanced Salt Solution (HBSS, pH 6.5). After seeding  $10^6$   
242 cells on cell culture inserts (Transwell®, Corning, USA) with  
243  $0.4$   $\mu\text{m}$  pore size disposed in a 12-wells plate, the medium  
244 was replaced every two days during the first week and every  
245 day during the lasting days until the differentiated cell  
246 monolayer was formed (14–18 days,  $588 \pm 44$   $\Omega/\text{cm}^2$ ). For

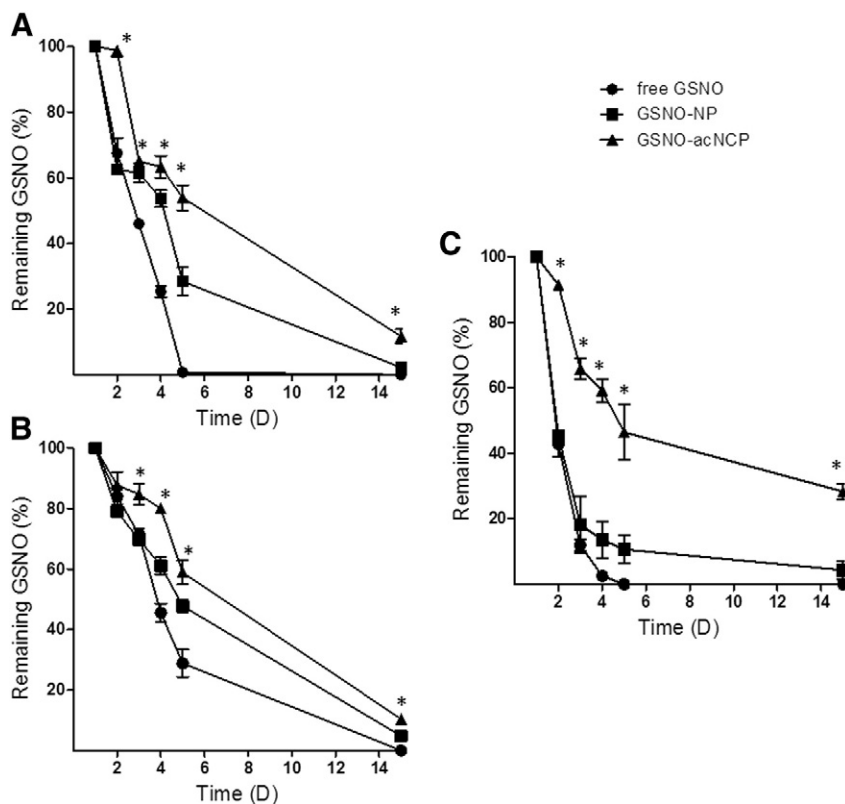


Figure 2. Stability of GSNO in polymer particles. GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) or GSNO-loaded nanoparticles (GSNO-NP) suspensions were centrifuged and the resulting pellets were stored at  $-20\text{ }^{\circ}\text{C}$  (A),  $+4\text{ }^{\circ}\text{C}$  (B) and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

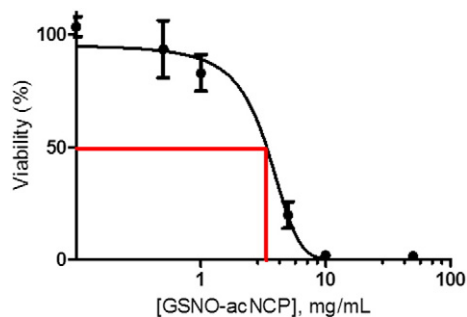


Figure 3. *In vitro* cytotoxicity of polymer nanocomposite particles on Caco-2 cells. Caco-2 cells were treated with the indicated concentrations of GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) for 24 h at  $37\text{ }^{\circ}\text{C}$ . Viability was estimated through mitochondrial activity by the MTT assay. Data are expressed as mean  $\pm$  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<sup>®</sup>-Electrical Resistance system (Millipore, USA). In addition, the permeation of fluorescein sodium ( $5\text{ }\mu\text{M}$ ) 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:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

$dQ/dt$  (mol/s) refers to the permeability rate (mol) of RSNO the basolateral compartment at the time of quantification,  $A$  ( $\text{cm}^2$ ) 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:

$$R = P_{app1}/P_{app2}$$

$P_{app1}$  refers to the apparent permeability coefficient of each treatment, and  $P_{app2}$  corresponds to the apparent permeability coefficient of free GSNO.

the permeation experiments, polymer nanocomposite particles containing  $50\text{ }\mu\text{M}$  of GSNO were suspended in  $500\text{ }\mu\text{L}$  of HBSS and introduced in the apical (donor) compartment, whereas,  $1.5\text{ mL}$  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.<sup>26</sup>

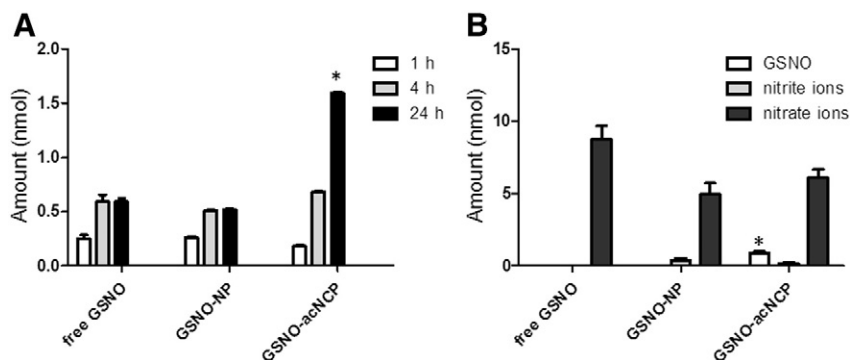


Figure 4. Caco-2 cell permeability of GSNO from polymer particles. Twenty five nmol of free GSNO or equivalent amount of GSNO-loaded nanoparticles (GSNO-NP) or GSNO-loaded alginate/chitosan nanocomposite particles (GSNO-acNCPs) were loaded in the apical compartment of a transwell® system seeded with Caco-2 cells 15 days before. (A) Amount of GSNO permeated from the apical to the basolateral compartment after 1 h, 4 h or 24 h. (B) Amount of the different nitric oxide species remained in the apical compartment after 24 h. Values presented have been corrected from control (cells without treatment for free GSNO, blank formulations for GSNO loaded formulations). Data are shown as mean  $\pm$  sem ( $n = 3$ ). \*:  $P < 0.05$  vs free GSNO and GSNO-NP (two-way ANOVA).

### 279 *Ex vivo pharmacological evaluation*

280 *Animals used and ethical statement.* Vasorelaxation was  
 281 evaluated on endothelium-removed aortic rings isolated from  
 282 12 week-old, male, normotensive, Wistar rats (Janvier Labora-  
 283 tories, Le Genest-St-Isle, France; 400-500 g). All experiments  
 284 were performed in accordance with the European Community  
 285 guidelines (2010/63/EU) for the use of experimental animals in  
 286 the respect of the 3 Rs' requirements for Animal Welfare (C.  
 287 Perrin-Sarrado permit number n°54-72, I. Lartaud n° 54-5; the  
 288 project entitled "Nitro-Vivo" was positively evaluated the 19th of  
 289 December 2014 by the CELMEA (regional ethical committee for  
 290 animal experiments) and approved by the French Ministry of  
 291 Research n°APAFIS1614-2015090216575422v2). Animals  
 292 were kept under standard conditions (temperature:  $21 \pm 1$  °C,  
 293 hygrometry  $60 \pm 10\%$ , light on from 6 am to 6 pm) and had free  
 294 access to standard diet (A04, Safe, Villemoisson-sur-Orge,  
 295 France) and water (reverse osmosis system, Culligan, Brussels,  
 296 Belgium).

297 *Treatment schedule.* GSNO-acNCPs (15 mg of GSNO/kg  
 298 body weight), free GSNO (15 mg of GSNO/kg body weight),  
 299 blank-acNCPs or PBS as controls were administrated by gavage  
 300 (10 mL/kg of rat) to Wistar rats (fasted 8 h before gavage).  
 301 Seventeen hours after gavage, rats were sacrificed by exsanguina-  
 302 tion after anesthesia (sodium pentobarbitone 60 mg/kg,  
 303 intraperitoneal, Sanofi Santé Nutrition Animale, Libourne,  
 304 France) and intravenous administration of heparin (500 U,  
 305 Heparine Choay). A segment (around 3 cm) of the descending  
 306 thoracic aorta was removed and immediately placed in cold  
 307 Krebs' solution. Vessels were cleaned from surrounding  
 308 connective tissues and cut into 2-mm long rings (8 rings per  
 309 rat). The endothelium was removed by rubbing the intimal  
 310 surface of the rings with forceps and immediately used for *ex*  
 311 *vivo* vasoactivity studies.

312 *Measurement of vasoactivity.* Aortic vasoactivity was mea-  
 313 sured using an isometric tension recording system in 10 mL

organ chambers (EMKABATH, Emka Technology, France). 314  
 Baths were filled with Krebs' solution (10 mL, 37 °C, pH 7.4) 315  
 and continuously bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Following 316  
 60-min equilibration with a basal resting tension determined at 317  
 2 g, viability of aortic rings was assessed with potassium 318  
 chloride (KCl,  $6 \times 10^{-2}$  M) added in the baths. The contraction 319  
 rapidly reached a steady state and was expressed, after 15 min 320  
 exposure, as the developed tension from the basal resting tension 321  
 of 2 g. Arteries showing less than 2.5 g of developed tension 322  
 were excluded. After viability test and a 30 min wash-out period so 323  
 that tension returns to baseline, aortic rings were tested with two 324  
 protocols: i) vasoconstriction of aortic rings ( $n = 10-12$  per group, 325  
 from 4 different rats in each group) was measured with increasing 326  
 concentrations of phenylephrine (PHE) ( $10^{-10}$  M to  $3 \times 10^{-5}$  M); ii) 327  
 NO storage in the aorta was evaluated with *N*-acetylcysteine (NAC) 328  
 used to displace NO from cysteine-NO residue. Rings ( $n = 10-12$  per 329  
 group, from 4 different rats in each group) precontracted with  $10^{-6}$  M 330  
 PHE, were added with NAC ( $10^{-5}$  M and  $10^{-4}$  M). Contractile 331  
 effects were expressed as induced developed tension (Delta T, g) and 332  
 relaxant ones in percentage of contraction (100% being the stable 333  
 tension caused by  $10^{-6}$  M PHE). 334

The absence of functional endothelium was confirmed by the 335  
 ability of carbachol  $10^{-5}$  M, a muscarinic receptors agonist, to 336  
 induce less than 10 % of relaxation on  $10^{-6}$  M PHE-precontracted 337  
 aortic rings. 338

### 339 *Statistical analysis*

Results are shown as either mean  $\pm$  standard deviation (sd) or 340  
 mean  $\pm$  standard error of the mean (sem) values. The half 341  
 maximal effective concentration (EC<sub>50</sub>) and maximal response 342  
 (E<sub>max</sub>) were calculated by fitting each concentration response 343  
 curve using the Hill logistic equation. For the comparison of 344  
 Papp, and that of E<sub>max</sub> and EC<sub>50</sub> for PHE, statistical comparisons 345  
 were performed using the one-way ANOVA ( $P < 0.05$ ). 346  
 Other analyses were performed using the two-way ANOVA 347  
 ( $P_{\text{treatment}} < 0.05$ ;  $P_{\text{time}} < 0.05$ ). Statistical analyses were per- 348  
 formed using the GraphPad Prism software (GraphPad Software 349  
 version 5.0, San Diego, USA). 350

t1.1 Table 1  
t1.2 Apparent permeability coefficient (Papp) and enhancement ratio (R) of GSNO permeation across Caco-2 monolayers.

t1.3 Group	Papp ( $10^{-8}$ cm/s)	R
t1.4 Free GSNO	8.3 ± 0.3	1.0
t1.5 GSNO-NP	7.7 ± 3.1	0.9
t1.6 GSNO-acNCPs	34.1 ± 1.5*	4.1

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.

## 351 Results

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

354 The average size of the developed nanocomposite particles  
355 was  $56 \pm 15$   $\mu\text{m}$  (mean ± sd;  $n = 3$ ). GSNO was entrapped  
356 within the nanocomposite particles with a high encapsulation  
357 efficiency ( $76 \pm 10\%$ ), corresponding to an estimated core  
358 loading of  $15.0 \pm 1.9$  mg of GSNO/g of polymer. The inner  
359 core of GSNO loaded nanoparticles presented an estimated core  
360 loading of  $5.7$  mg of GSNO/g of polymer with a zeta potential of  
361  $+40 \pm 6$  mV and a mean diameter of  $0.289 \pm 0.014$   $\mu\text{m}$ .<sup>26</sup> As  
362 shown in Figure 1,  $75 \pm 3\%$  of free GSNO was released from the  
363 dialysis bag during the first hour. Similarly, GSNO-NPs showed  
364 a release profile with a burst release of  $95 \pm 2\%$  of the initially  
365 loaded GSNO within the first 3 h. On the other hand, only  $57 \pm$   
366  $6\%$  of GSNO was released from GSNO-acNCPs within 3 h and  
367 the remaining amount was released in a sustained way over the  
368 next 21 h.

369 *Stability of GSNO in GSNO-loaded alginate/chitosan*  
370 *nanocomposite particles*

371 The stability of GSNO within the polymer nanocomposite  
372 particles was evaluated at  $-20$  °C,  $+4$  °C and  $+37$  °C. After  
373 5 days of storage at  $-20$  °C or  $+4$  °C,  $100 \pm 1\%$  or  $70 \pm 5\%$  of  
374 free GSNO respectively, was decomposed (Figure 2, A, B).  
375 When incorporated into the polymer nanocomposite particles,  
376  $16 \pm 5\%$  ( $-20$  °C) and  $12 \pm 4\%$  ( $+4$  °C) of intact GSNO was  
377 preserved for 15 days, showing improvement of GSNO stability.  
378 At  $+37$  °C, GSNO degraded faster (Figure 2, C):  $57 \pm 4\%$  of  
379 free GSNO had decomposed within two days and  $98 \pm 1\%$  after  
380 4 days. Polymer nanocomposite particles increased the life-time  
381 of GSNO as  $46 \pm 9\%$  and  $12 \pm 4\%$  of intact GSNO were  
382 detected on the 4th and 15th days respectively.

383 *Mucin binding assay*

384 The mucoadhesion property of the polymeric GSNO-NPs  
385 was also evaluated. The diameter of GSNO-NPs in contact with  
386 mucin dispersion immediately increased from  $0.2 \pm 0.0$   $\mu\text{m}$  to  
387  $18.2 \pm 3.2$   $\mu\text{m}$  (100 times more) and estimated zeta potential  
388 value was divided per 3 (from  $+54 \pm 4$  mV to  $+18 \pm 5$  mV,  
389 (mean ± sd;  $n = 3$ )).

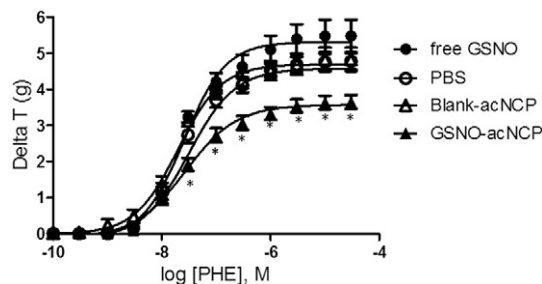


Figure 5. Contractile effect of phenylephrine (PHE) on aortic rings isolated from rats orally treated with free GSNO or GSNO-loaded polymer nanocomposite particles (GSNO-acNCPs) at 15 mg of GSNO/kg of body weight, 17 h before. Blank alginate/chitosan nanocomposite particles (Blank-acNCPs) and phosphate buffered saline (PBS) have been used as controls. Results are expressed as mean ± sem ( $n = 10$ –12 per group, from 4 different rats in each group). \*:  $P < 0.05$  vs PBS (two-way ANOVA).

Cytocompatibility and cell permeability

390

The concentration-response curve built from the MTT 391 metabolic activity assay (Figure 3) gave an  $\text{IC}_{50}$  at  $3.24 \pm 392$   $0.38$  mg/mL for GSNO-acNCPs. Therefore, a concentration of 393  $1$  mg/mL (containing  $50$   $\mu\text{M}$  GSNO) that maintained  $83 \pm 1\%$  394 of cell viability was chosen for Caco-2 permeability studies. 395

Caco-2 cell monolayers were incubated with  $50$   $\mu\text{M}$  396 (representing  $25$  nmol of GSNO in  $0.5$  mL) free or 397 nanoparticle-associated GSNO in the apical compartment to 398 investigate GSNO permeability. After 1 h of incubation, a 399 similar concentration of GSNO was detected in the basolateral 400 compartment for all formulations (Figure 4, A). The 401 permeation kinetics of the GSNO were similar for GSNO 402 and GSNO-NPs: the amount transported rose rapidly to 403  $0.59 \pm 0.07$  nmol and  $0.51 \pm 0.01$  nmol, respectively, over 404 the first 4 h, and then remained at a plateau up to 24 h. 405 GSNO-acNCPs showed a steady penetration rate over the 406 whole 24 h, and promoted 2.7-fold more GSNO crossing the 407 cell monolayer than free GSNO or GSNO-NP (Figure 4, A). 408 The Papp value of GSNO (showing the ability of NPs and 409 acNCPs to help the transport of GSNO across Caco-2 cell 410 monolayer) did not change with NP, but significantly 411 increased after GSNO-NPs incorporation into nanocomposite 412 particles, showing an enhancement ratio of 4.1 (Table 1). In 413 contrast to free GSNO and GSNO-NPs, GSNO-acNCPs 414 retained  $0.9 \pm 0.1$  nmol of intact GSNO in the apical 415 compartment after 24 h of incubation (Figure 4, B) showing 416 that GSNO-acNCPs could have an even longer release profile. 417

At the end of experiments, the test using the low molecular 418 weight hydrophilic tracer sodium fluorescein, showed that the 419 permeation of sodium fluorescein in the treated groups ( $18 \pm 420$   $5\%$ ) was higher than the positive control ( $6.1 \pm 0.5\%$ ) and less 421 than negative control ( $32 \pm 9\%$ ), in which cells were treated with 422 HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Taken together, these results 423 confirmed the integrity of cells monolayer after incubation with 424 the GSNO-acNCPs with a slight opening of tight junction due to 425 chitosan. 426

GSNO-NPs<sup>26</sup> showed a core loading two times under the 427 GSNO-acNCPs core loading ( $5.7$  mg of GSNO/g of polymer 428



t2.1 Table 2

t2.2 Half maximum effective concentration ( $EC_{50}$ ) and maximum effect ( $E_{max}$ ) of phenylephrine measured in isolated aortic rings.

t2.3 Group	$EC_{50}$ ( $10^{-8}$ M)	$E_{max}$ (g)
t2.4 PBS	$3.1 \pm 0.1$	$4.8 \pm 0.1$
t2.5 Blank-acNCPs	$1.9 \pm 0.2$	$4.8 \pm 0.3$
t2.6 Free GSNO	$3.1 \pm 0.1$	$4.9 \pm 0.1$
t2.7 GSNO-acNCPs	$3.1 \pm 0.1$	$3.7 \pm 0.1^*$

Data are shown as mean  $\pm$  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.

t2.8

and  $15.0 \pm 1.9$  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

#### 434 Pharmacological vascular effects

435 The dose-response curves to PHE were similar in aortic rings  
436 from Wistar rats orally treated 17 h earlier with free GSNO,  
437 blank-acNCP or PBS (Figure 5). In aortic rings from rats treated  
438 with GSNO-acNCP (15 mg/kg body weight), the contractile  
439 maximal effect ( $E_{max}$ ) of PHE significantly decreased (from  
440 4.8 g in control groups to 3.7 g in the GSNO-acNCP group, -  
441 25%,  $P < 0.05$ ) with no change in  $EC_{50}$  (Table 2).

442 In all the control groups, NAC failed to relax the vessels.  
443 However, in the group treated with GSNO-acNCP, NAC  
444 induced significant relaxation ( $11 \pm 1\%$  at  $10^{-5}$  M and  $24 \pm$   
445  $3\%$  at  $10^{-4}$  M,  $P < 0.05$  compared to controls, Figure 6).

#### 446 Discussion

447 The encapsulation of the fragile molecule GSNO within drug  
448 delivery systems is still challenging. Different approaches, either  
449 through *S*-nitrosation of free or conjugated GSH<sup>23,25</sup> or direct  
450 encapsulation of GSNO<sup>21,37</sup> are described in the literature. In the  
451 present study, we adopted the second approach with gentle  
452 formulation process to develop polymer nanocomposite particles  
453 loaded with GSNO. The polymer nanocomposites were obtained  
454 by incorporation of GSNO loaded Eudragit® RL nanoparticles  
455 (GSNO-NP)<sup>26</sup> into a polymer matrix prepared from a mixture of  
456 alginate and chitosan by ionic gelation. The challenge was to  
457 control the loading of GSNO within the nanocomposite particles  
458 in order to get potential pharmacological efficiency. We based our  
459 calculations on the minimal concentration of NO required to stimulate  
460 the synthesis of cyclic GMP by soluble guanylate cyclase in vascular  
461 smooth muscle (approximately 10 nmol/L<sup>38</sup>), and the dose of GSNO  
462 that significantly decreases mean arterial blood pressure (*i.e.* 3 mg;  
463 100  $\mu$ g/min for 30 min, intravenous, in human<sup>39</sup>).

464 Compared to our previous published results on GSNO-NP,<sup>26</sup> the  
465 newly formulated composite particles improved the loading capacity  
466 of GSNO by 2.6-fold (from 5.7 to 15.0 mg of GSNO/g of polymer).  
467 This improvement can probably be attributed to the use of alginate  
468 and chitosan, which are hydrophilic and have good affinity for

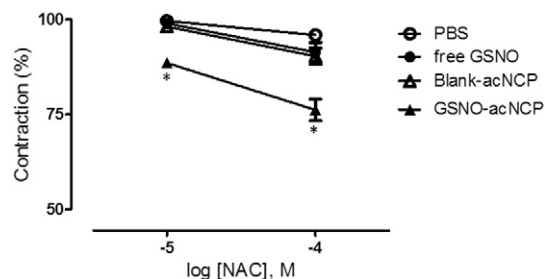


Figure 6. Vascular effect of  $10^{-5}$  M and  $10^{-4}$  M *N*-acetylcysteine (NAC) on precontracted ( $10^{-6}$  M phenylephrine) aortic rings isolated from rats orally treated with free GSNO or GSNO-loaded polymer nanocomposite particles (GSNO-acNCPs) at 15 mg of GSNO/kg of body weight, 17 h before. Blank alginate/chitosan nanocomposite particles (Blank-acNCPs) and phosphate buffered saline (PBS) have been used as controls. Results are expressed as mean  $\pm$  sem ( $n = 10-12$  per group, from 4 different rats in each group). \*:  $P < 0.05$  vs PBS (two-way ANOVA).

GSNO thus allowing the entrapment not only of GSNO-NP but also  
of the free GSNO remaining outside the nanoparticles. This is  
consistent with our previously reported polymer nanocomposites  
based on chitosan or alginate separately.<sup>27</sup>

Our results on *in vitro* release demonstrate that the polymer  
nanocomposite particles prevented the burst release of hydro-  
philic GSNO observed with GSNO-NP alone. This is in  
agreement with the results of Hasan et al<sup>40</sup> showing that  
composite particles were able to reduce the burst release.  
GSNO-acNCPs present two polymer barriers to the diffusion of  
GSNO: the first being formed by the hydrophobic Eudragit® RL  
polymer and the second by the outer hydrophilic matrix  
composed of chitosan and alginate. The combination of both  
leads the diffusion distance to increase and the release rate to  
slow down. Furthermore, as reported by de Seabra et al<sup>20,41,42</sup>  
increased microviscosity of the solvent improve the stability of  
RSNO. The presence of alginate and chitosan, which build a  
hydrophilic matrix for the particles, increases the viscosity  
thereby protecting GSNO from thermal decomposition and  
improved GSNO stability. Moreover, the increased stability we  
obtained at +37 °C for GSNO-acNCPs was in accordance with  
our previous observations using alginate or chitosan in polymer  
nanocomposite particles.<sup>27</sup>

GSNO-acNCPs also improved the permeability of GSNO  
through the Caco-2 cells model, and increased the Papp value, a  
marker of drug absorption. This permeation enhancement may  
be attributed to three major factors: i) the positive charge of  
GSNO-acNCPs (zeta potential approximately equal to  $+18.5 \pm$   
 $0.8$  mV), which allows electrostatic attraction with the nega-  
tively charged cell membrane; ii) the composition of nanocom-  
posite particles based on chitosan and alginate, which have  
mucoadhesive properties and reinforce the interaction with cells;  
iii) the high level of protection of GSNO afforded by the  
nanocomposite particles. While we did not directly measure  
GSNO crossing through intestinal tissue, our results augur a  
promising oral delivery, for which intestinal absorption is one of  
the key factors determining drug bioavailability. The mecha-  
nisms involved in intestinal crossing remain to be evaluated.  
However, the glycosidic bonds of chitosan were shown to be  
rapidly hydrolyzed under gastric conditions,<sup>43</sup> so only a portion

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.<sup>44</sup> 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<sup>26</sup> to reach a final size around 20  $\mu\text{m}$ . Others also showed that the mucus layer strongly increase particle size and modify absorption of particles through the intestine.<sup>45</sup> Indeed, Bajka et al demonstrated that a particle diameter higher than 500 nm largely decreased particles diffusion.<sup>45</sup> 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,<sup>46</sup> 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.<sup>47</sup> 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.<sup>47-50</sup> 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  $\alpha_1$ -adrenergic receptor leading to its decreased affinity for sympathetic ligands.<sup>51</sup> This is unlikely in the present study as EC<sub>50</sub> 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.

## Acknowledgments

Q4

The authors are grateful to Dr. François Dupuis, Patrick Liminana and Isabelle Fries (EA 3452, CITHEFOR, Lorraine University) for the helpful advices and technical support to experimental studies, Pr Gillian Barratt (UMR CNRS 861, Paris-Sud University) for manuscript spelling corrections.

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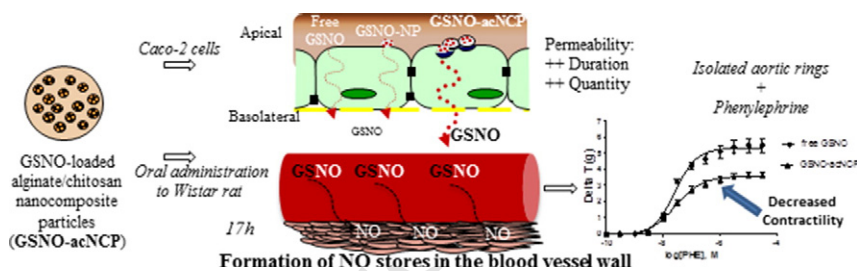
## Graphical Abstract

**Polymer nanocomposites enhance S-nitrosoglutathione intestinal absorption and promote the formation of releasable nitric oxide stores in rat aorta**
*Nanomedicine: Nanotechnology, Biology, and Medicine xxx (2016) xxx–xxx*

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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.



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