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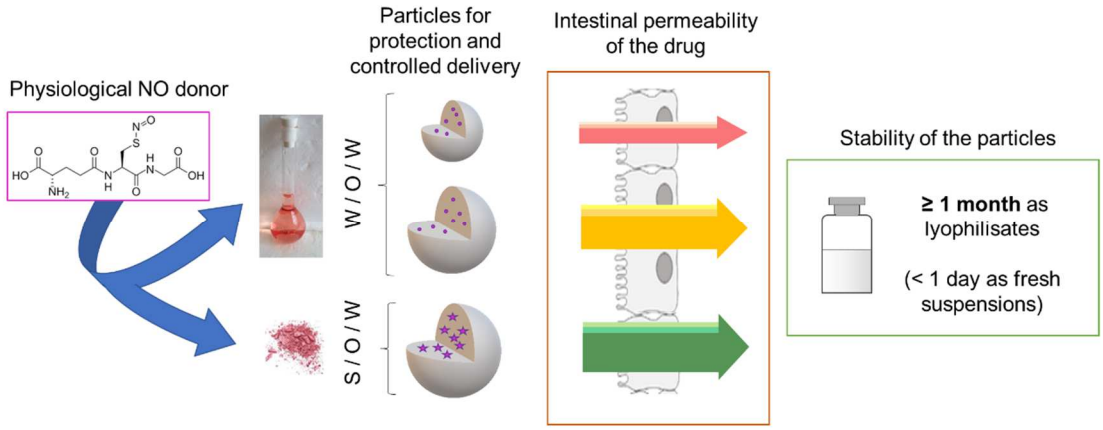
Graphical Abstract:



Abstract:

Nitric oxide (NO) deficiency is often associated with several acute and chronic diseases. NO donors and especially *S*-nitrosothiols such as *S*-nitrosoglutathione (GSNO) have been identified as promising therapeutic agents. Although their permeability through the intestinal barrier have recently be proved, suitable drug delivery systems have to be designed for their oral administration. This is especially challenging due to the physico-chemical features of these drugs: high hydrophilicity and high lability. In this paper, three types of particles were prepared with an Eudragit polymer: nanoparticles and microparticles obtained with a water-in-oil-in-water emulsion/evaporation process *versus* microparticles obtained with a solid-in-oil-in-water emulsion/evaporation process. They had a similar encapsulation efficiency (around 30%), and could be freeze-dried then be stored at least one month without modification of their critical attributes (size and GSNO content). However, microparticles had a slightly slower *in vitro* release of GSNO than nanoparticles, and were able to boost by a factor of two the drug intestinal permeability (Caco-2 model). Altogether, this study brings new data about GSNO intestinal permeability and three ready-to-use formulations suitable for further preclinical studies with oral administration.

Keywords: nitric oxide, *S*-nitrosoglutathione, nanoparticles, microparticles, oral delivery

Highlights:

- GSNO is a promising drug, but challenging to formulate (hydrophilicity, lability)
- Nano and microparticles suitable for GSNO oral delivery were produced and compared
- Their critical attributes were maintained after freeze-drying and stable upon storage
- They improved the drug permeability through a Caco-2 model of intestinal barrier

1. Introduction

Nitric oxide (NO) is a radical messenger with a variety of physiological roles, *e.g.* control of vascular tone, neurotransmission at both central and peripheral levels, mediation of immune response, wound healing, tissue regeneration and anti-cancer defense [1]. As a result of these pleiotropic actions, many pathological conditions are accompanied by a NO deficiency (*e.g.* atherosclerosis, pulmonary hypertension, thrombosis, ischemia… [2-5]). As these conditions are becoming increasingly frequent in the population with ageing, a treatment able to restore the physiological NO levels would represent a major therapeutic advance for a growing number of patients.

NO itself cannot be used directly in many cases because of its very short half-life (< 1 s) [6]. Moreover, its gaseous nature makes it difficult to store, to transport and to administer. In this context, NO donors have been developed over the past decades, including organic nitrates [7-8], nitrosamines [9], metal-NO complexes [10], diazenium-diolates (NONOates, [11]) and *S*-nitrosothiols (RSNO, [12-13]. As RSNO are the physiologic forms of NO transport and storage in our body, they are considered as "hot candidates" with no efficacy nor toxicity concerns. In particular, *S*-nitrosoglutathione (GSNO) has gained a lot of attention in preclinical and clinical trials for different biomedical applications [14-15]. GSNO is a physiological low-molecular-weight RSNO, with arterioselective vasodilating and antiplatelet effects. Additionally, the NO moiety in GSNO is grafted to glutathione (GSH), which is the main redox buffer with antioxidant properties in our cells. Thus, GSNO will release both NO and GSH: this could be beneficial as NO deficiency is generally associated to oxidative stress in many conditions. However, pharmacokinetics data are drastically limited by the fact that GSNO is a very hydrophilic (clogP = - 2.7) and labile active drug, highly reactive to its environment. GSNO is rapidly degraded or metabolized by several (*e.g.* protein disulfide isomerases [16], thioredoxins [17], GSNO reductase [18], gamma-glutamyl transpeptidase [19]). RSNO metabolism leads to the formation of multiple NO-derived species (NOx, including nitrite and nitrate ions), therefore difficult to follow throughout the entire organism. Only few studies have attempted to define RSNO pharmacokinetic parameters, especially after oral administration, as this require technically demanding methods (radioisotope tracking of NO [20-21]). Furthermore, little is known on the crossing of the intestinal barrier by RSNO. Bonetti et al. [22] revealed the ability of 3 RSNO (including GSNO) to cross the intestinal barrier (Caco-2 model) with a medium apparent permeability rate, using a passive mode. As the therapeutic effects of NO depend not only on the location of production/release, but also on the rate and quantity of available NO, it is a real pharmaceutical challenge to propose a formulation of GSNO able to protect and to deliver it properly. As already mentioned, to the great distress of formulators, GSNO is very sensitive: to light, oxygen, heat, pH changes, or presence of thiols or metallic ions even at low concentrations. As a drug, GSNO can be stored only as powder, at -20°C, protected from light and under an inert atmosphere. Precautions (ultrapure water, light protection) have to be taken to prepare GSNO solutions, which should be discarded after few hours. These characteristics make it very challenging and raise many hurdles to develop GSNO formulations adapted to oral delivery, the most attractive route of administration to manage patients in the context of chronic diseases.

Among the few examples in the literature, conjugation of GSNO to diblock polymers was proposed [23] as well as direct encapsulation of GSNO into various hydrophilic (alginate, chitosan…) or hydrophobic polymeric matrices [24-29]. Hydrophilic polymers have generally less standardized characteristics because of their natural origin. Their hydrophilic nature is favorable for GSNO encapsulation, but the resulting loose hydrogels might lead to a fast drug release. Among hydrophobic polymers, the Eudragit ones are especially interesting, as they are mucoadhesive excipients already approved for human oral administration.

Wu et al. [30] used Eudragit RLPO, commonly used for tablet coating, to prepare nanoparticles which were incorporated into an alginate/chitosan matrix to enhance GSNO loading and release profile [31]. The resulting composite microparticles generated a NO store in rat aorta after a single oral administration [32]. Despite of these very interesting results, the preclinical development of these composites formulations is hindered by several points. First, the multi-step preparation and the adequate characterization are too time-consuming. Second, these formulations are not stable and should be used immediately after preparation (no possibility to be sent to other labs to be tested in preclinical models).

Following these precedents, the aims of this work were to: (i) propose a new drug delivery system that enables GSNO stabilization and controlled release; (ii) provide information on GSNO intestinal absorption from these developed delivery systems.

To overcome these barriers, GSNO-loaded Eudragit RLPO particles adapted for oral delivery were prepared, with a reasonably simple protocol, at least similar encapsulation efficiency and release profile than the previously described particles, and with a sufficient stability to be stored and/or sent before use. According to the hydrophilic nature of the drug, microparticles (MP) were prepared by double emulsion/solvent evaporation, either with a water-in-oil-in water (W/O/W) process (GSNO-MPW) or a solid-in-oil-in-water (S/O/W) process (GSNO-MPS). They were compared to nanoparticles obtained with a W/O/W process (GSNO-NP) in terms of size, encapsulation efficiency and *in vitro* release. Moreover, to get particles in a stable solid state, a lyophilization process was applied. Potential modifications of critical product attributes (GSNO content and particle size) were assessed immediately after lyophilization and during refrigerated storage. Afterwards, the cytocompatibility of the particles was evaluated on Caco-2 cells. Finally, intestinal permeability of NOx species was evaluated using an *in vitro* model of intestinal barrier.

2. Material and methods

All solutions were prepared with ultrapure deionized water (> 18.2 MΩ.cm). GSNO powder was synthesized, purified and stored as previously described [31]. Eudragit® RLPO was a gift from Evonik industries (Germany). $HgCl_2$ was purchased from Prolabo (Switzerland), $NaNO_2$ from Merck (Germany), NaOH from VWR Chemicals (Czech Republic), methanol from Carlo Erba Reagents (France). All other reagents were obtained from Sigma–Aldrich (France).

2.1. GSNO powder synthesis

GSNO powder was synthesized, purified and stored as previously described [33]. Briefly, sodium nitrite and reduced glutathione (GSH), equivalent amount, were incubated under acidic condition (0.626 M HCl). The solid form of GSNO was obtained by cold acetone precipitation, then washed, dried under vacuum and stored at -20°C under inert dinitrogen atmosphere. GSNO purity > 95% is required for batch release (see [33] for details about control).

2.2 Quantification of GSNO, nitrite ions and nitrate ions

Nitrite ions and GSNO were quantified using a colorimetric (Griess and Griess-Saville reactions) or a fluorimetric method (diaminonaphthalen (DAN) and DAN-Hg^{2+}) with standard curves of sodium nitrite and GSNO, respectively. Briefly, N_2O_3 generated from acidified nitrite ions reacts with Griess reagent (Sulfanilamide and N-(1-naphtyl)ethylenediamine) or DAN in the presence (for GSNO) or absence (for nitrite ions) of $HgCl_2$ producing a diazonium salt or 2,3-naphthotriazole that either absorbs at 540 nm or emits fluorescence at 415 nm after excitation at 375 nm (JASCO FP-8300,

France). GSNO concentration was obtained by subtracting Griess to Griess-Saville quantification or DAN to DAN-Hg^{2+} quantification. Nitrate ions quantification (using a standard curve of sodium nitrate) included a reduction step of nitrate in nitrite ions using a nitrate reductase and its cofactors before the addition of DAN reagent (fluorometric kit nitrite/nitrate Cayman Chemical Ref. 780051, USA). Nitrate concentration was obtained by subtracting DAN-Hg^{2+} quantification to nitrate quantification using nitrate reductase.

2.3. Preparation and lyophilization of GSNO-loaded particles

2.3.1. Preparation of GSNO-loaded nanoparticles and microparticles

GSNO-loaded nanoparticles (GSNO-NP) were prepared by a water-in-oil-in-water (W/O/W) emulsion/solvent evaporation technique [30]. Briefly, 500 µL of 0.1% (w/v) Kolliphor P188 solution containing 10 mg of GSNO was emulsified by sonication for 60 s (11 W, 100% amplitude, Vibra cell™ 72434, France) in 5 mL of methylene chloride containing 500 mg of Eudragit, over an ice bath. The resulting primary emulsion was then poured in 19.5 mL of 0.1% (w/v) Kolliphor P188 solution with ultrasonication (30 W, amplitude maximum, Vibra cell™ 75022, France) for 30 s, to get the W/O/W emulsion. The final GSNO-NP suspension was obtained after 10 min of solvent evaporation.

GSNO-loaded microparticles were prepared by W/O/W process (GSNO-MPW) or by S/O/W process (GSNO-MPS). GSNO-MPW preparation followed the same process as GSNO-NP, except for the second emulsification, which was performed with a vortex (VV3, VWR, USA) at maximum speed for 60 s, rather than with ultrasonication. The whole process was conducted at controlled temperature (24 ± 0.5 °C). GSNO-MPS were prepared as GSNO-MPW, except that 10 mg of GSNO powder (sieved at 40 µm) were directly suspended in the Eudragit/DCM solution. Attempts to reduce the size of GSNO powder grain were made with supercritical fluid technology (see Table 1).

Table 1. **Conditions used in supercritical trials to decrease the size of GSNO powder grains.** Gaseous antisolvent (GAS) and supercritical antisolvent (SAS) processes rely on the use of the supercritical CO_2 as an anti-solvent. The crystallization of GSNO is induced by the mass transfer of the solvent (DMSO) in the supercritical phase.

Treatment	Crystallization conditions	Drying conditions
GAS	Crystallization pressure: 300 bar Crystallization temperature: 40°C GSNO 1% wt in DMSO 10.9 g of solution injected CO_2 pressurization: 150 bar/min Equilibrium: agitation 54 rpm 10 min before drying 20 mL autoclave	300 bar/40°C CO_2 flow rate: 0.6 kg/h Duration: 3 h
SAS (1st trial)	Crystallization pressure: 300 bar Crystallization temperature: 40°C GSNO 1% wt in DMSO 15.8 g of solution injected Molar CO_2 fraction: 0.984 2.2 kg_{CO2}/h – 1.0 g_{sol}/min) Coaxial nozzle (100 µm i.d.) 300 mL autoclave	300 bar/40°C CO_2 flow rate: 1.0 kg/h Duration: 2 h 30

SAS (2^{nd} trial)	Crystallization pressure: 160 bar Crystallization temperature: 40°C GSNO 1% wt in DMSO 22.1 g of solution injected Molar CO_2 fraction: 0.971 1.1 kg_{CO2}/h – 1.0 g_{sol}/min) Coaxial nozzle (130 µm i.d.) 300 mL autoclave	160 bar/40°C CO_2 flow rate: 1.1 kg/h Duration: 3 h

2.3.2. Lyophilization of GSNO-loaded particles

After particles preparation, the suspensions were centrifuged (42,000 g, 4°C, 30 min for NP or 10 min for MP) and the pellets were re-suspended in 10 mL of 10% (w/v) sucrose solution. The re-suspensions were frozen at -80°C for 15 h ± 1 h then lyophilizated for 24 h ± 1 h (FreeZone6 LABCONCO USA, setups: condenser at -50°C and pressure at 0.04 mbar). A preliminary study was conducted to optimize the lyophilization of GSNO-NP and to select the best and lowest-cost conditions such as -80 °C freezing of the particles (instead of liquid nitrogen) and using sucrose as protectant (instead of trehalose, and no mannitol). The residual water content in the lyophilizate was immediately tested after freeze-drying using a Karl-Fischer apparatus (756 KF coulometer, France). All powders were placed under nitrogen and stored at 4°C protected from light.

2.4. Physicochemical characterization of particles

The hydrodynamic diameter, polydispersity index (PDI) and zeta-potential of the GSNO-NP were measured in triplicate in 1 mM NaCl by dynamic light scattering (Zetasizer Nano ZS, Malvern Instrument, France). All measurements were performed at 25°C after 30 s of equilibration with an angle detection of 173° backscatter.

The size of GSNO-MP was also measured in triplicate in 0.001 M NaCl by a Mastersizer (hydro 2000 SM, Malvern Instruments, France). Span was calculated as follows:

Span = (Dv90 – Dv10) /Dv50

where Dv10, Dv50, Dv90 are values of size below which 10%, 50% or 90% of the particles are contained.

The surface morphology of the particles was investigated by scanning electron microscopy (SEM, Hitachi S4800, Japan, accelerating voltage 1 kV). Briefly, fresh suspension of GSNO-NP was diluted 10^6-fold. Then, a drop was deposited and dried overnight at room temperature, and flash carbon coated (4 s). In the case of GSNO-MP, lyophilizates were observed after carbon coating.

2.5. Determination of GSNO encapsulation efficiency (direct method)

The encapsulation efficiency (EE) was determined by quantifying the GSNO contained inside the particles. After particles centrifugation (42,000 g, 30 min for NP, 10 min for MP), the pellets were collected and destroyed by methylene chloride. GSNO was then extracted with phosphate buffer saline (0.148 M PBS, pH=7.4) (ratio methylene chloride/PBS 1:10) using high speed shaking (2,000 rpm, Heidolph Vibramax 110, Germany). After centrifugation (2,500 g for 10 min), GSNO and nitrite ions were quantified in the supernatant using Griess-Saville and Griess reactions. GSNO

concentration was deduced by subtracting Griess quantification to Griess-Saville quantification. Total recovery of GSNO during the extraction process has been verified and potential matrix effect with polymer residues have been ruled out in preliminary experiments. The encapsulation efficiency was assessed immediately after particles preparation and calculated according to:

$EE = (m_e / m_i) * 100\%$

Where EE is encapsulation efficiency (%), m_e is the mass of drug entrapped in particles, and m_i is the mass of initial drug. The determination of GSNO content inside the particles after lyophilization and during storage was performed using the same protocol (the residual water content of the lyophilizates was considered in the calculations).

2.6. Stability of particles before and after lyophilization

After preparation, fresh suspensions of particles were centrifuged (42,000 g, 4°C, NP for 30 min and MP for 10 min). The pellets were re-suspended in 1 mL water and stored at 5±3°C protected from light. After 1, 2, 3, or 4 days, size and GSNO and potential nitrite content of separated aliquots were measured as previously described.

After lyophilization, the particle powders were stored at 5±3°C under nitrogen and protected from light. After 1, 2, 3, 4, 5, 8 and 12 weeks, size and GSNO content of separated aliquots were checked as previously described and compared to the values obtained immediately after lyophilization.

2.7. Kinetics of GSNO release

Immediately after preparation, 1 mL of fresh particles suspensions were centrifuged (42,000 g, 4°C, 30 min for NP and 10 min for MP). The pellets were suspended in 1 mL of 0.148 M PBS (pH 7.4) and transferred into dialysis cellulose membrane (average flat width 10 mm (0.4 in), cut-off 14,000 Da). The membrane was immerged in 200 mL PBS at 37°C protected from light with magnetic agitation at 200 rpm. The GSNO and nitrite ions released in the medium were quantified using the DAN and DAN-Hg^{2+} methods at different time intervals (every 30 min during two hours and every hour from two to six hours) [30].

2.8. Cytocompatibility of particles

Intestinal Caco-2 cells (ATCC® HTB-37™) were grown in complete medium consisting of Eagle's Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum (FBS), 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% CO_2 (v/v) in a humidified incubator. Cells were then seeded in 96-well plates at 10^5 cells/well. After 24 h, cells were exposed to free GSNO (from 25 µM to 2500 µM) or to lyophilizate particles (3 batches, in duplicate, at equivalent GSNO concentrations from 25 µM to 2500 µM) for 24 h in complete medium. After incubation, cytocompatibility was checked by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [22]. The absorbance was read at 570 nm with a reference at 630 nm using EL 800-microplate reader (Bio-TEK Instrument, France). Metabolic activity in the presence of treatments was compared to the control condition, i.e. medium alone (as 100%).

2.9. Intestinal Permeability

Caco-2 cells were seeded at 2 ×10^6 cell/cm^2 on cell culture inserts (Transwell®, Corning, USA) with 0.4 µm pore size disposed in a 12-wells plate. Complete medium was replaced every two days during the first week and every day during the second week. The intestinal barrier was validated when the differentiated cell monolayer was obtained after 14-15 days of culture with a transepithelial electrical resistance (TEER) value > 500 Ω.cm^2. Intestinal permeability of the 3 different lyophilizated particles (GSNO concentration 100 µM in all cases, 3 batches, in triplicate) was compared to free GSNO in HBSS containing Ca^{2+} and Mg^{2+} (HBSS+). Two controls of permeability and integrity of the intestinal barrier model using HBSS+ and HBSS without Ca^{2+} and Mg^{2+} (HBSS-) were performed. After 1 h of incubation, the amounts of NOx species (i.e. RSNO + nitrite ions + nitrate ions) were quantified using DAN-Hg^{2+}, DAN methods with or without nitrate reductase, in the apical compartment both free in the medium and inside the particles, and in the basolateral compartment. A TEER value higher than 300 Ω.cm^2 and a fluorescein permeability lower than 5% at the end of the test validated the integrity of the intestinal monolayer [22].

The apparent permeability coefficients (Papp) were calculated as follows:

$$Papp = (dQ/dt) \times (1/(A \times C_0))$$

Where dQ/dt (mol.s^{-1}) refers to the permeability rate (mol) of RSNO or NOx in the basolateral compartment at the time of quantification, A (cm^2) to membrane diffusion area, and C_0 (mol.mL^{-1}) to the initial concentration in the apical compartment.

The recovery rate of NOx species (mass balance of GSNO) was checked by comparing the amount of NOx species quantified in the different compartments (apical, basolateral, in the particles) with the initial amount of GSNO deposited.

2.10. Statistical analysis

All the results are shown as either mean ± standard deviation (sd, for characterization) or mean ± standard error of mean (sem, for cells results). In all cases, three independent batches (preparation and lyophilization) were used.

The one-way ANOVA or two-way ANOVA (Dunnett's multiple comparisons test or Tukey's multiple comparisons test) were used for the analysis of particles stability, release and Caco-2 cells experiments. $p < 0.05$ was considered as significantly different. Statistical analyses were performed using the GraphPad Prism software (GraphPad Software, USA).

3. Results and discussion

Currently, pharmacokinetic data of the orally administered GSNO are lacking while its pharmacodynamic effects are well documented and indicate a great therapeutic potential. For example, a recent literature review from Liu et al. [15] highlighted the results obtained with GSNO in clinical trials and preclinical models of stroke: repeated oral administration is used in several of the preclinical studies with promising cerebro-protective or regenerative effects against ischemic lesions, without deciphering the drug transport across the intestinal nor the haemato-encephalic barriers. This absence of information limits the use of GSNO as a therapeutic drug. A pioneer work has been done by Wu et al. [31], whose composite particles promoted GSNO intestinal permeation and led to the formation of releasable NO store in rat aorta 17 h after a single oral administration. However, a

lot of work remains to be done to implement data and identify the "best" way to present the drug (*i.e.* drug delivery system) to the intestinal barrier and how it will impact the drug permeability.

3.1. Particles size and encapsulation efficiency

The double emulsion/solvent evaporation method is generally suitable for hydrophilic drugs and was previously successfully applied for GSNO encapsulation into Eudragit RLPO [30]. The process applied with two successive ultrasonic emulsifications and methylene chloride evaporation led to a submicronic formulation. However, the encapsulation was still limited and no sustained release was described. The optimization of this formulation process is the challenge of our present study. Starting from the same raw materials (and the same drug/polymer ratio), the initial W/O/W protocol was modified in two ways. On the one hand, the second emulsion step was modified to produce microparticles (GSNO-MPW) instead of nanoparticles (GSNO-NP), as the lower energy emulsification might protect GSNO from degradation. Additionally, the bigger size of the emulsion droplets and resulting particles might both hinder drug leakage during particles preparation and slow down its release from hardened particles [34]. On the second hand, the S/O/W protocol was tested (GSNO-MPS), using GSNO powder instead of GSNO solution in the first emulsion. In comparison to W/O/W processes, S/O/W methods generally improve encapsulation efficiency of hydrophilic or sensitive drugs and can also lead to better release profiles [35-37].

In our case, particles size was indeed increased, from 0.225 ± 0.003 µm for GSNO-NP to 69 ± 7 µm for GSNO-MPW and 165 ± 14 µm for GSNO-MPS. However, there was no improvement in the encapsulation efficiency (28 ± 2% for GSNO-MPS, 26 ± 3% for GSNO-MPW, versus 33 ± 6% for GSNO-NP), corresponding to a drug loading around 0.7% (w drug/w polymer). In all cases, the amount of nitrite ions was negligible (< limit of quantitation 0.5 µM), meaning that the formulation process is suitable for this fragile drug, and that the mild encapsulation efficiency of GSNO is more likely caused by its low entrapment into the polymeric matrices rather than by its degradation.

In the first step of the S/O/W method, the solid drug is dispersed into an organic solvent. A smaller size of the grain powder facilitates the homogeneous dispersion of the drug, thus improving its final encapsulation [38-39]. Grain size reduction is generally achieved with conventional grinding techniques, but the sensitivity of GSNO to thermal degradation, requires rather innovative supercritical fluid processes [40]. At first, a supercritical antisolvent (SAS 1st trial Table 1) process using DMSO was used. However, it led to a non-acceptable degradation of the drug (> 70% loss). So, at second, a gaseous anti-solvent (GAS) process was selected, which failed to obtain any solid product, probably because of the too high solubility of GSNO in the CO_2/DMSO mixture, and a too high fraction of DMSO. Finally, the SAS process adapted with milder conditions (2^{d} trial) led to a pink powder (yield 72%), with more than 80% purity. However, this GSNO powder was too hygroscopic and turned into a sticky product impossible to characterize. This could be due either to a high residual content of DMSO in the powder and/or to the specific features of GSNO, amplified by the higher surface are (reduction of powder particles size with the process). As a result, we used directly the raw GSNO powder in our experiments and dichloromethane as traditional solvent for the 3 formulations tested (GSNO-NP, -MPW, -MPS) to compare GSNO intestine absorption in order to conduct a pharmacokinetic approach.

3.2. Stability: fresh suspensions versus lyophilized particles

According to the International Conference on Harmonization (ICH) guidelines (Q1A (R2)), a drug product should be evaluated in studies where “the storage conditions and the lengths of studies chosen should be sufficient to cover storage, shipment and subsequent use”. For our studies, we choose to store our products at 5 ± 3°C, as a compromise between the thermal fragility of GSNO and

an easier storage/shipment. Moreover, due to the very challenging development of GSNO stable particles, we considered that a 10% (and not 5% as in ICH) change from the initial value will be considered significant for the critical product attributes, *i.e.* size and GSNO content.

When stored as suspensions, the size of all particles remained stable during three days (not shown). This can be explained by the electrostatic repulsion between particles allowed by positive charges (quaternary ammonium groups) brought by Eudragit RLPO polymer (zeta potential of GSNO-NP at +38 ± 1 mV). However, particles GSNO content dropped to less than 90% of the initial value after one day, and fell around 60% after two days (not shown) for all suspensions. These results are consistent with those reported previously for GSNO-NP [30-31].

Lyophilization using 10% w/v sucrose as cryoprotectant was successfully implemented to stabilize the three particles types (GSNO-NP, -MPW, -MPS) without modifying their critical attributes (Figure 1A, B, and C). A cryoprotectant was imperative to retain the GSNO content of the particles (no cryoprotectant = more than 50 % of GSNO lost during freeze-drying), but sucrose was as effective as trehalose. SEM images show that the particles are roughly spherical, with a more or less smooth surface and no visible pores at the surface (Figure 1D). It was not possible to observe freeze-dried powder of GSNO-NP (instead, fresh particles were observed), because the sucrose included in the lyophilizate generated structures in the same range of size than NP.

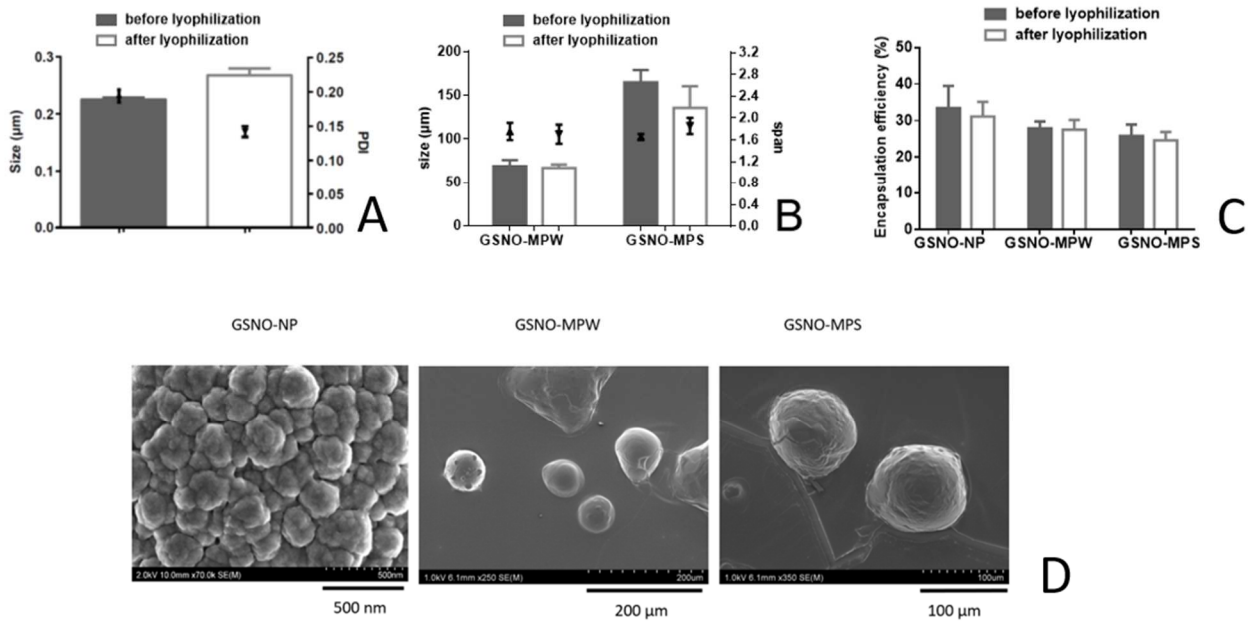


Figure 1: Characterization of GSNO-loaded particles before and after lyophilization. Size and polydispersity index (PDI) of the GSNO-NP (A) as well as size and span of the GSNO-MP (B), encapsulation efficiencies (C) are presented as mean ± sd (n=3). Representative Scanning Electron Microscopy images are also presented (D).

After lyophilization, size remain stable for the three types of particles during storage for the three months of study (Figure 2). In contrast, GSNO content decreased significantly after four weeks of storage for both kinds of microparticles, and more slowly for GSNO-NP (90% of initial content remaining after three months). These results might be linked to the different residual water content (w/w) of the lyophilizates, namely 7 ± 5% for GSNO-NP, 9 ± 5% for GSNO-MPW and 26 ± 11% GSNO-MPS. These values are rather high for freeze-dried formulations (usually < 2%). Either the freeze-

drying process was not sufficiently efficient, or the formulations have recaptured water before the residual water content was measured (this was done as fast as possible, using dessicant to transport the formulations, but it was not technically feasible to do it in a moisture-controlled environment). This hygroscopic behavior echoes the one observed previously in the supercritical fluid experiments. Further investigation of the freeze-drying process might be warranted.

However, more than 80% of the initial GSNO amount remained inside GSNO-MPS after one month and inside GSNO-MPW after two months. This result offers a promising approach for oral delivery system development.

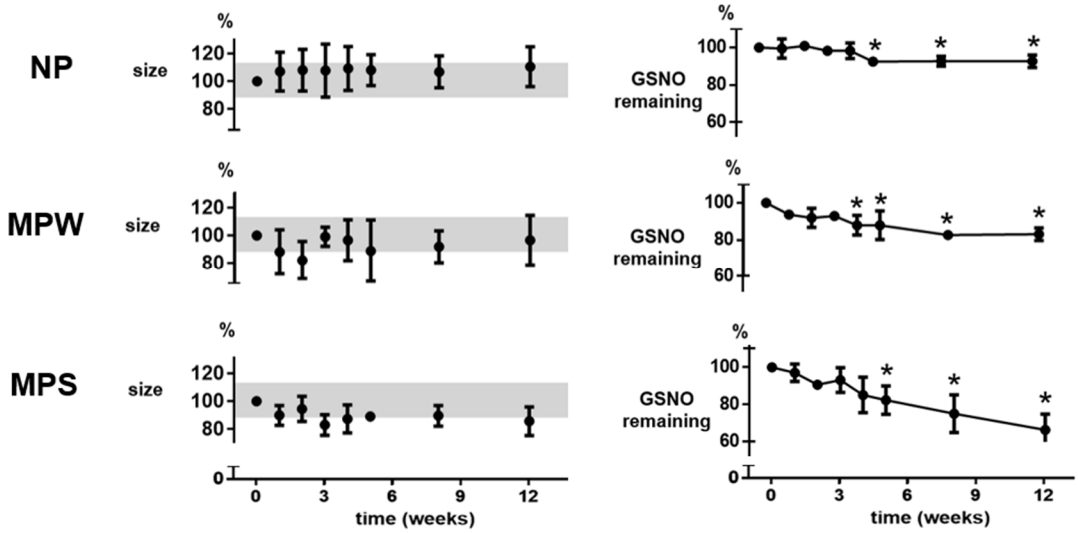


Figure 2: Evolution of size and GSNO remaining for the GSNO-NP, GSNO-MPW and GSNO-MPS particles stored at 4 °C under inert atmosphere after lyophilization. Values measured immediately after lyophilization are considered as 100%. Results are presented as mean ± sd (n = 3). The grey areas delimit the sizes between 90% and 110% of the initial values. For GSNO remaining contents: one-way ANOVA $p < 0.05$ for the three formulations (Dunnett's multiple comparisons test* $p < 0.05$ *versus* week 0).

3.3. In vitro release

The kinetic release study was conducted under discriminating conditions (phosphate buffer), with no ambition of *vitro/vivo* correlation but to observe the ability of developed drug delivery systems to protect and release GSNO in a controlled manner. The *in vitro* GSNO release profiles (Figure 3) showed an initial burst in the first 1.5 h for all types of particles. At 2 h, the cumulative release from microparticles was lower (around 70-75%) than for free GSNO and GSNO-NP (around 90% released). Complete release was achieved after 6 h.

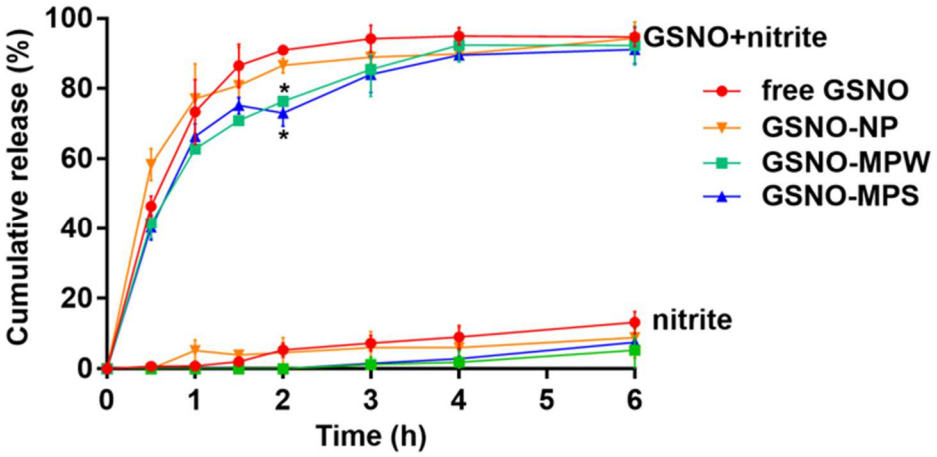


Figure 3: *In vitro* drug release. Results are presented as mean ± sd (n = 3, free GSNO as control), two-way ANOVA with p< 0.05 for treatments, time and interaction (Tukey's multiple comparisons test: * p < 0.05 *versus* free GSNO and GSNO-NP). The cumulative release is presented as total release ("GSNO + nitrite", *i.e.* corresponding to the DAN-Hg^{2+} assay, upper curves) or as nitrite ions ("nitrite", lower curves, DAN assay).

In the recent literature, Lautner et al. [41] and Hlaing et al. [25] used S/O/W processes to encapsulate RSNO into PLGA microparticles. With these polymers, they achieved higher drugs loadings (respectively 12% for *S*-nitroso-*N*-acetylpenicillamine, SNAP, and 5% for GSNO) and sustained release (several days). With our polymer especially adapted for oral administration, we obtained a 0.7% drug loading, and a shorter release time, more suited for interaction with the gastrointestinal tract. These results clearly highlight the challenge to encapsulate GSNO directly into hydrophobic particles obtained by double emulsion/evaporation. Modifications of the drug (to increase its molecular weight, for example using phytochelatins, natural polymers of GSNO analogues [42]) or of the polymer (to graft GSNO directly on the polymer backbone) seem necessary to achieve high drug loading/sustained release. An attractive alternative strategy mimicking the physiology could be to encapsulate high molecular weight *S*-nitrosothiols, which will generate GSNO *in situ* by transnitrosation of physiological GSH. Previous studies with encapsulation of *S*-nitroso-N-acetylcysteine [43] and *S*-nitroso-captopril [44] proved the effectiveness of this transnitrosation approach to elicit vasodilation or anti-infectious effects after parenteral or local administration, respectively.
However, despite the lower encapsulation and duration of drug release with our Eudragit particles, the release rate itself, around 150 pmol. $mg^{-1}.min^{-1}$ for the first 1.5h, is in the range of previously described PLGA systems encapsulating NO donors (*e.g.* from 40 pmol.$mg^{-1}.min^{-1}$ with SNAP [41] to 400-800 pmol.$mg^{-1}.min^{-1}$ with DETA NONOate [45]). So, the interaction of particles with a cell model of intestinal barrier was evaluated.

3.4. Cytocompatibility

The 24-h cytocompatibility of the three particle types and free GSNO decreased while increasing the GSNO concentration (Figure 4). Concentrations tested in this study were deliberately far beyond the ones needed for physiological effect of GSNO, in order to compare IC_{50} values of free *versus* encapsulated drug. The cell metabolic activity went below 80% for concentrations above 500 µM for

free GSNO, and 50 µM of eq [GSNO] for all particle types. The IC_{50} value of free GSNO (1718 ± 1 µM) decreased with its encapsulation in GSNO-MPS (310 ± 1 µM), GSNO-MPW (198 ± 1 µM) and GSNO-NP (140 ± 1 µM).

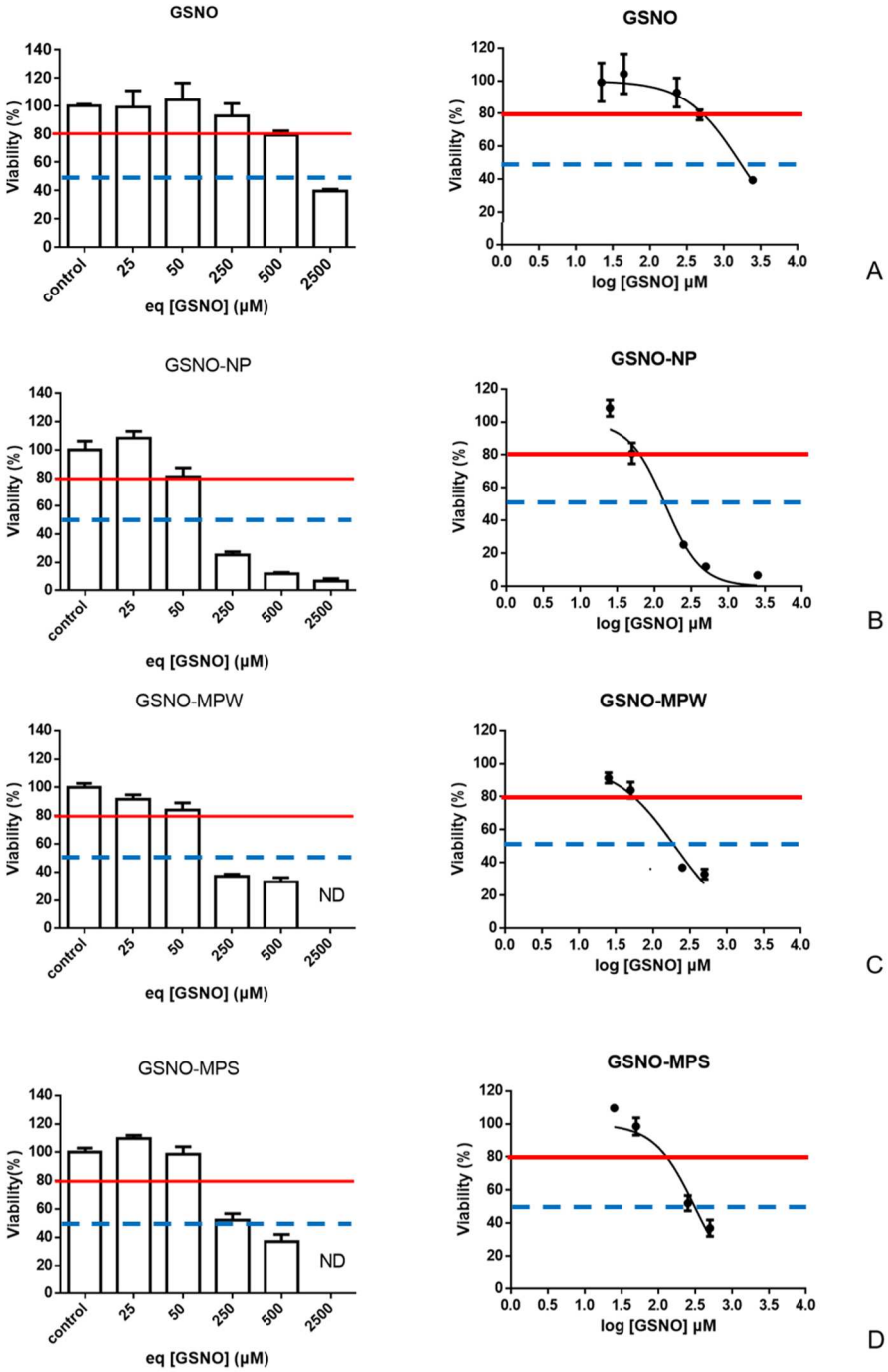


Figure 4: Cytocompatibility of Caco-2 cells after 24 h of incubation with GSNO (A), GSNO-NP (B), GSNO-MPW (C), GSNO-MPS (D). Control condition = culture medium without fetal bovine serum. Results are presented regarding GSNO loading, in equivalence of free GSNO from 25 µM to 2500 µM. Results are shown as means ± sem, n = 3 in duplicate. ND = not determined

Eudragit RL (R for Retard) are poly(ethylacrylate,methyl methacrylate and methylammonio ethylmethacrylate copolymers, bearing 8.8−12% quaternary ammonium groups. This material is commonly employed for pH-independent coating of solid oral drug dosage forms. However, since it is insoluble in water at physiological pH values and undergoes a certain degree of swelling, it may also be suitable for the dispersion and controlled oral delivery of actives [46]. The cytocompatibility of drug delivery based on this polymer is well documented. In this context, the results obtained were not surprising. However, after oral administration, the concentration of 50 µM (corresponding roughly to 2.5 g/L of particles) should not be locally achieved in 24 h but distributed and spread throughout the length of the intestine. In addition, it is important to note that the study conducted here was voluntarily conducted on undifferentiated Caco-2 cells, known to be more sensitive than the cells used for permeability studies (cultured for 15 days prior to the study) [47].

3.5. Intestinal permeability

The intestinal permeability of NOx species released from particles compared to free GSNO was evaluated using an *in vitro* intestinal barrier model (Figure 6). Bonetti et al. [22] demonstrated recently that free RSNO (GSNO, *S*-nitroso-*N*-acetylcysteine NACNO and SNAP), without drug delivery systems, can cross this barrier (Caco-2 model) with a medium apparent permeability rate, using a passive mode. In the present study, we wanted to explore the ability of Eudragit RL-based-drug delivery systems to improve GSNO intestine permeability. To follow the future of NO through the intestinal barrier, it is essential to follow the RSNO but also the degradation products (nitrite ions and nitrate ions).

The mass balance (Table 2) and the values of apparent permeability coefficient (Papp) (Table 3) were then calculated for all NOx species. The mass balance calculated at the end of the permeability study showed that most of the GSNO initially encapsulated or added (free) was recovered under RSNO, nitrite ions and mostly nitrate ions species. Therefore, no NOx species were produced by cells attesting for non-stress conditions imposed by the presence of particles.

Table 2. **Mass balance of NOx species** for GSNO non-formulated or formulated, after 1 h of permeability study (GSNO initial amount 50 nmol). Results are presented as mean ± sem, n=3 in duplicate, one-way ANOVA on each compartment (Tukey's multiple comparisons test): *p < 0.05 *versus* GSNO.

	Amount in nmol			
	Apical		**Basolateral**	**Total**
	Free	**In particles**		
GSNO	45 ± 2 *(90 ± 4%)*	-	0.8 ± 0.3 *(1.6 ± 0.6%)*	45 ± 2 *(91 ± 4%)*
GSNO-NP	38 ± 2 *(75 ± 4%)*	5 ± 1 *(11 ± 3%)*	1.5 ± 0.8 *(3 ± 2%)*	44 ± 1 *(89 ± 2%)*
GSNO-MPW	32 ± 3* *(62 ± 6%)*	10 ± 2 *(20 ± 4%)*	3 ± 1 *(5 ± 2%)*	43 ± 1 *(85 ± 2%)*

GSNO-MPS	$30 \pm 2^{*}$ *(60 ± 5%)*	11 ± 2 *(21 ± 4%)*	2 ± 1 *(5 ± 2%)*	43 ± 2 *(86 ± 4%)*

At the end of incubation, the amount of GSNO in the apical media was significantly lower for GSNO-MPW and GSNO-MPS conditions (circa 60% of initial load) than for GSNO-NP (~75%) and free GSNO (~90%). While around 10% of GSNO remained inside NP, almost 20% were still inside MP. This confirms the slower release of GSNO for MP than NP, previously observed in the *in vitro* experiment, which is even slower than in the *in vitro* release study. This can be explained by the bioadhesion phenomenon, already observed for Eudragit RL, and the establishment of electrostatic interactions with plasma membranes of Caco-2 cells [46]. In the meantime, both types of MP led to a drastically higher concentration of nitrate ions (the most permeable NO species) in the basolateral side compared to free GSNO or NP conditions (Figure 5). Despite the high local concentration of polymer proposed in this experiment in order to deliver a quantity of RSNO that can be measured, the cells do not endogenously produce nitrates, as shown by the mass balance (Table 2). As the same amount of GSNO is contained in the different formulations, this result demonstrates that the same dose of GSNO delivered by MPs, more localized with a more limited point of contact, results in greater permeability of the naturally more permeable NO species.

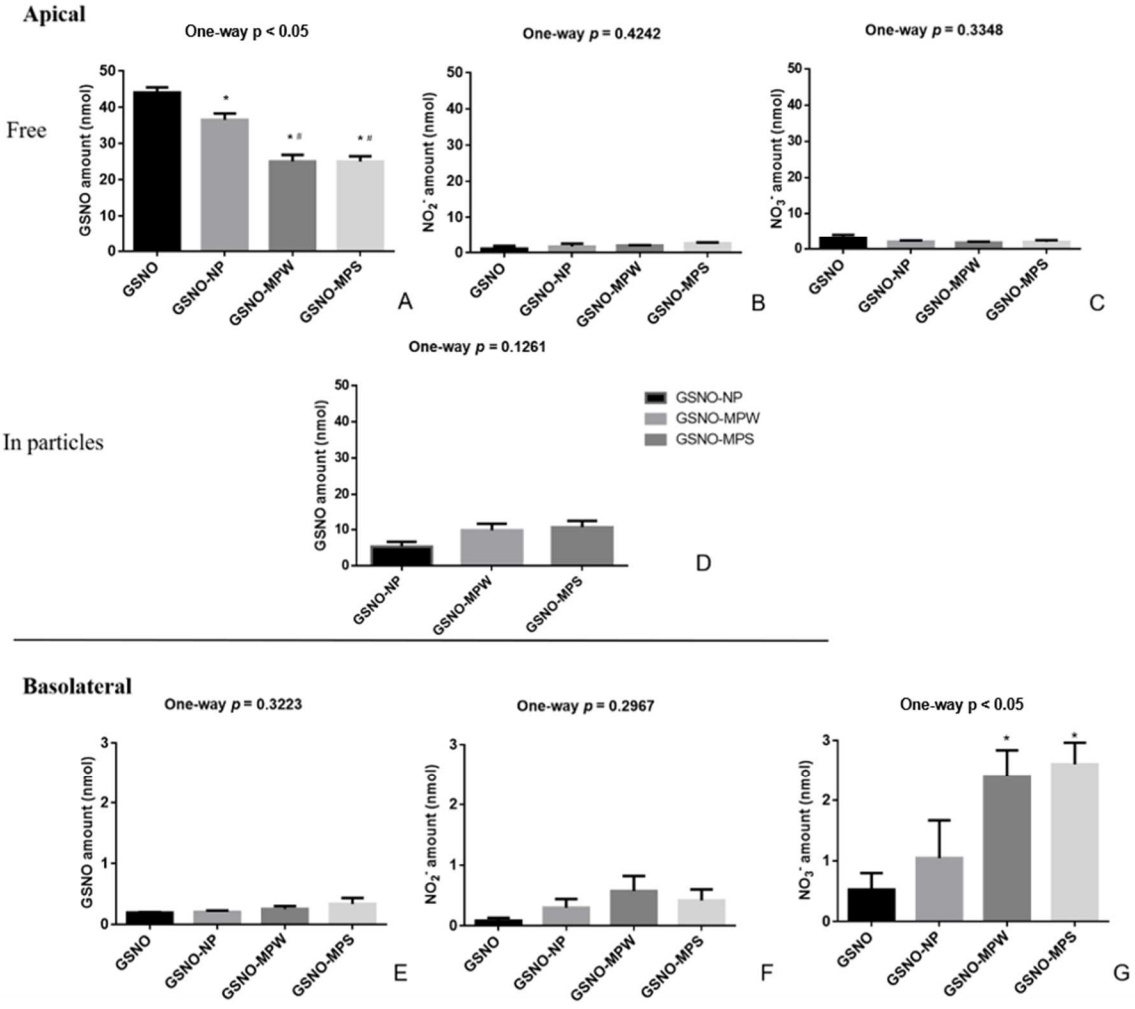


Figure 5: Quantity of GSNO, nitrite ions nitrate ions remaining in the apical medium (A, B and C, respectively), as well as in the basolateral medium (E, F and G, respectively), and GSNO remaining inside the particles (D) after 1 h of permeability study (GSNO initial amount = 50 nmol in all cases). Results are shown as means ± sem, n=3 in duplicate, one-way ANOVA (Tukey's multiple comparisons test): *p < 0.05 *versus* GSNO and # p < 0.05 *versus* GSNO-NP).

The Papp values of each NO species are higher for the two types of GSNO-MP than for GSNO-NP or free GSNO (Table 3). This led to a global (NOx species) Papp value at least two times higher for GSNO-MPW and GSNO-MPS than for GSNO-NP and free GSNO. This situation is mainly driven by a higher permeability of nitrate ions than nitrite ions and RSNO, as previously observed for non-formulated RSNO [22]. As previously mentioned, this could be simply explained by the different distribution of particles on the cell surface (between NP or MP). The formation of an embolus, where the dose of GSNO is focalized on a more limited point of contact with the cell, is favorable to absorption in this *in vitro* model. This phenomenon will probably be different *in vivo* because the intestinal barrier is not limited to a cell monolayer and mucus also participates in bioadhesion phenomena.

Table 3. Values of apparent permeability coefficients (Papp) for RSNO, nitrite ions, nitrate ions and NOx (sum of all species) after 1 h of free GSNO, GSNO-NP or GSNO-MPs incubation. Results are presented as mean ± sem, n=3 in duplicate.

	Papp values ($\times 10^{-6}$ cm.s^{-1})			
Treatment	**RSNO**	**NO_2^-**	**NO_3^-**	**NOx**
GSNO	0.8 ± 0.4	0.3 ± 0.2	2.0 ± 0.2	3.1 ± 0.6
GSNO-NP	0.6 ± 0.2	0.9 ± 0.3	4 ± 1	6 ± 2
GSNO-MPW	1.1 ± 0.7	1.8 ± 0.3	7 ± 1	10 ± 2
GSNO-MPS	1.1 ± 0.6	1.3 ± 0.3	8 ± 2	11 ± 2

Indeed, the three types of particles showed an improvement of the intestinal permeability of NOx species (GSNO-MPS > GSNO-MPW > GSNO-NP) after only 1 h and without damaging the cells monolayer (Figure 6). The Papp parameter characterizes the intestinal permeability of a drug in *in vitro* or *ex vivo* models. Drugs could have low permeability (Papp < 1 $\times 10^{-6}$ cm.s^{-1}) or high permeability (≥ 10 $\times 10^{-6}$ cm.s^{-1}), or be in a medium permeability class [48]. As a result, considering all NOx species, GSNO is in the medium permeability class when administered as free GSNO or GSNO-NP, and reaches the high permeability class using GSNO-MPs (≥ 10 $\times 10^{-6}$ cm.s^{-1}). This data is relevant and the study shows that, depending on the drug delivery system used, a modulation of GSNO absorption can be observed.

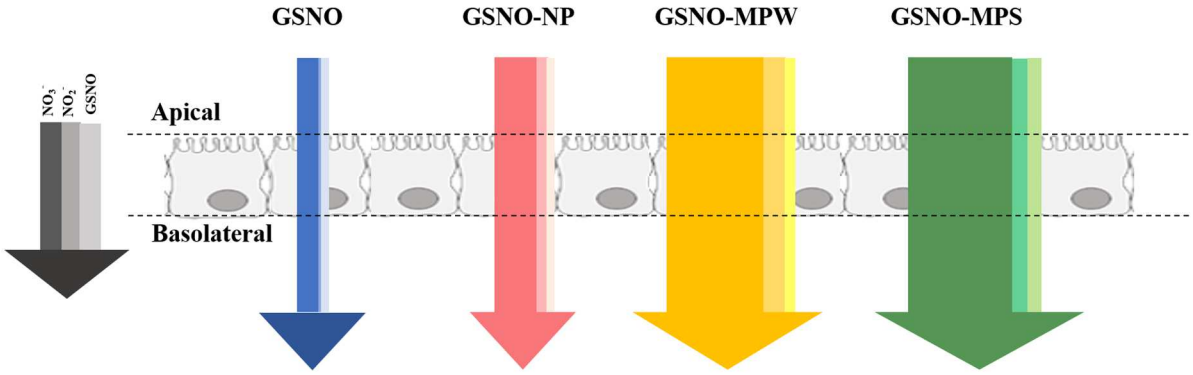


Figure 6: Graphical summary of NOx species permeability for each condition (free GSNO, GSNO-NP, GSNO-MPW and GSNO-MPS). The width of each section of the arrows is correlated with the amounts (from left to right) of NO_3^-, NO_2^- and GSNO.

4. Conclusion

In this study, we overcame the challenge of getting stable and easy-to transport particles for oral administration of GSNO. Critical parameters of the particles (i.e. size and GSNO content) were maintained after freeze-drying, and the resulting products were stable at last one month stored at 4°C protected from oxygen. In this way, the lyophilization process has brought a very significant improvement, since initial suspensions had to be used right after preparation. The discrepancies in water residual content of the particles after drying indicate that MP (especially the GSNO-MPS ones) still retain a high amount of water. This might be due to the hygroscopic properties of GSNO powder. As GSNO is less stable in solution than in dry state, optimization of the lyophilization process could be conducted in the future in order to reduce residual water content and therefore to improve the stability of these GSNO-MPS. Microparticles offered a slightly slower *in vitro* release of the drug than nanoparticles, and led to a better enhancement of NOx permeability through a model intestinal barrier. This work confirms the importance of presenting the drug with a suitable delivery system to the intestine. The NP and MP compared in this study could have different behaviors in *in vivo* situations and therefore constitute tools for future GSNO oral administration in chronic conditions.

References

[1] Ignarro, L.J., 1999. Nitric oxide as a signaling molecule in the vascular system: an overview. J. Cardiovasc. Pharmacol. 34, 879-86. doi:10.1097/00005344-199912000-00016.

[2] Kuhlencordt P.J., Gyurko R., Han F., Scherrer-Crosbie M., Aretz T.H., Hajjar R., Picard M.H., Huang P.L., 2001. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. Circulation 104, 448-54. doi:10.1161/hc2901.091399.

[3] Tonelli A.R., Haserodt S., Aytekin M., Dweik R.A., 2013. Nitric oxide deficiency in pulmonary hypertension: Pathobiology and implications for therapy. Pulm. Circ. 3, 20-30. doi:10.4103/2045-8932.109911.

[4] Momi S., Caracchini R, Falcinelli E, Evangelista S, Gresele P., 2014. Stimulation of platelet nitric oxide production by nebivolol prevents thrombosis. Arterioscler .Thromb. Vasc. Biol. 34, 820-9. doi:10.1161/ATVBAHA.114.303290.

[5] Bienvenu L.A., Morgan J., Reichelt M.E., Delbridge L.M.D., Young M.J., 2017. Chronic in vivo nitric oxide deficiency impairs cardiac functional recovery after ischemia in female (but not male) mice. J. Mol. Cell. Cardiol. 112, 8-15. doi:10.1016/j.yjmcc.2017.08.012.

[6] Hakim T.S., Sugimori K., Camporesi E.M., Anderson G., 1996. Half-life of nitric oxide in aqueous solutions with and without haemoglobin. Physiol. Meas. 17, 267-77. doi: 10.1088/0967-3334/17/4/004.

[7] Zhuge Z., Paulo L.L., Jahandideh A., Brandão M.C.R., Athayde-Filho P.F., Lundberg J.O., Braga V.A., Carlström M.1., Montenegro M.F., 2017. Synthesis and characterization of a novel organic nitrate NDHP: Role of xanthine oxidoreductase-mediated nitric oxide formation. Redox. Biol. 13, 163-9. doi: 10.1016/j.redox.2017.05.014.

[8] Münzel T., Daiber A., 2018. Inorganic nitrite and nitrate in cardiovascular therapy: A better alternative to organic nitrates as nitric oxide donors? Vascul. Pharmacol. 102, 1-10. doi: 10.1016/j.vph.2017.11.003.

[9] Messin R., Boxho G., De Smedt J., Buntinx I.M., 1995. Acute and chronic effect of molsidomine extended release on exercise capacity in patients with stable angina, a double-blind cross-over clinical trial versus placebo. J. Cardiovasc. Pharmacol. 25, 558-63. Doi: 10.1097/00005344-199504000-00008.

[10] Pereira A.C., Araújo A.V., Paulo M., Andrade F.A., Silva B.R., Vercesi J.A., da Silva R.S., Bendhack L.M., 2017. Hypotensive effect and vascular relaxation in different arteries induced by the nitric oxide donor RuBPY. Nitric Oxide 62, 11-6. doi: 10.1016/j.niox.2016.11.001.

[11] Kauser N.I., Weisel M., Zhong Y.L., Lo M.M., Ali A., 2020. Calcium Dialkylamine Diazeniumdiolates: Synthesis, Stability, and Nitric Oxide Generation. J. Org. Chem. 85, 4807-12. doi: 10.1021/acs.joc.0c00020.

[12] Al'Sadoni H., Ferro A., 2000. S-Nitrosothiols: a class of nitric oxide-donor drugs. Clin. Sci. (Lond). 98, 507-20. https://doi.org/10.1042/cs0980507

[13] Perrin-Sarrado C., Zhou Y., Salgues V., Parent M., Giummelly P., Lartaud I., Gaucher C., 2020. S-Nitrosothiols as potential therapeutics to induce a mobilizable vascular store of nitric oxide to counteract endothelial dysfunction. Biochem. Pharmacol. 173, 113686. doi: 10.1016/j.bcp.2019.113686.

[14] Gaucher C., Boudier A., Dahboul F., Parent M., Leroy P., 2013. S-nitrosation/denitrosation in cardiovascular pathologies: facts and concepts for the rational design of S-nitrosothiols. Curr. Pharm. Des. 19, 458-72. **doi**: 10.2174/1381612811306030458.

[15] Liu S., Zheng H., Yu W., Ramakrishnan V., Shah S., Gonzalez L.F., Singh I., Graffagnino C., Feng W., 2020. Investigation of S-Nitrosoglutathione in stroke: A systematic review and meta-analysis of literature in pre-clinical and clinical research. Exp. Neurol. 328, 113262. doi: 10.1016/j.expneurol.2020.113262.

[16] Sliskovic I., Raturi A., Mutus B., 2005. Characterization of the S-denitrosation activity of protein disulfide isomerase. J. Biol. Chem. 280, 8733-41. doi:10.1074/jbc.M408080200.

[17] Stoyanovsky D.A., Tyurina Y.Y., Tyurin V.A., Anand D., Mandavia D.N., Gius D., Ivanova J., Pitt B., Billiar T.R., Kagan V.E., 2005. Thioredoxin and lipoic acid catalyze the denitrosation of low molecular weight and protein S-nitrosothiols. J. Am. Chem. Soc. 127, 15815-23. doi: 10.1021/ja0529135.

[18] Barnett S.D., Buxton I.L.O., 2017. The role of S-nitrosoglutathione reductase (GSNOR) in human disease and therapy. Crit. Rev. Biochem. Mol. Biol. 52, 340-54. doi: 10.1080/10409238.2017.1304353.

[19] Angeli V., Tacito A., Paolicchi A., Barsacchi R., Franzini M., Baldassini R., Vecoli C., Pompella A., Bramanti E., 2009. A kinetic study of gamma-glutamyltransferase (GGT)-mediated S-nitrosoglutathione catabolism. Arch. Biochem. Biophys. 481, 191-6. doi: 10.1016/j.abb.2008.10.027.

[20] Warnecke A., Luessen P., Sandmann J., Ikic M., Rossa S., Gutzki F.M., Stichtenoth D.O., Tsikas D., 2009. Application of a stable-isotope dilution technique to study the pharmacokinetics of human 15N-labelled S-nitrosoalbumin in the rat: possible mechanistic and biological implications. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877, 1375-87. doi: 10.1016/j.jchromb.2008.11.035.

[21] Yu H., Chaimbault P., Clarot I., Chen Z., Leroy P., 2019. Labeling nitrogen species with the stable isotope 15N for their measurement by separative methods coupled with mass spectrometry: A review. Talanta 191, 491-503. doi: 10.1016/j.talanta.2018.09.011.

[22] Bonetti J., Zhou Y., Parent M., Clarot I., Yu H., Fries-Raeth I., Leroy P., Lartaud I., Gaucher C., 2018. Intestinal absorption of S-nitrosothiols: Permeability and transport mechanisms. Biochem. Pharmacol. 155, 21-31. doi: 10.1016/j.bcp.2018.06.018.

[23] Duong H.T., Kamarudin Z.M., Erlich R.B., Li Y., Jones M.W., Kavallaris M., Boyer C., Davis T.P., 2013. Intracellular nitric oxide delivery from stable NO-polymeric nanoparticle carriers. Chem. Commun. (Camb). 49, 4190-2. doi : 10.1039/c2cc37181b.

[24] Parent M., Boudier A., Perrin J., Vigneron C., Maincent P., Violle N., Bisson J.-F., Lartaud I., Dupuis F., 2015. In Situ Microparticles Loaded with S-Nitrosoglutathione Protect from Stroke. PLoS One 10, e0144659. doi: 10.1371/journal.pone.0144659.

[25] Hlaing S.P., Kim J., Lee J., Hasan N., Cao J., Naeem M., Lee E.H., Shin J.H., Jung Y., Lee B.L., Jhun B.H., Yoo J.W., 2018. S-Nitrosoglutathione loaded poly(lactic-co-glycolic acid) microparticles for

prolonged nitric oxide release and enhanced healing of methicillin-resistant Staphylococcus aureus-infected wounds. Eur. J. Pharm. Biopharm. 132, 94-102. doi: 10.1016/j.ejpb.2018.09.009.

[26] Pelegrino M.T., de Araújo D.R., Seabra A.B., 2018. S-nitrosoglutathione-containing chitosan nanoparticles dispersed in Pluronic F-127 hydrogel: Potential uses in topical applications. J Drug Deliv Sci Technol 43, 211-20. https://doi.org/10.1016/j.jddst.2017.10.016.

[27] Shah S.U., Martinho N., Socha M., Pinto Reis C., Gibaud S., 2015. Synthesis and characterization of S-nitrosoglutathione-oligosaccharide-chitosan as a nitric oxide donor. Expert Opin. Drug Deliv. 12, 1209-23. doi: 10.1517/17425247.2015.1028916.

[28] Shah S.U., Socha M., Fries I., Gibaud S., 2016. Synthesis of S-nitrosoglutathione-alginate for prolonged delivery of nitric oxide in intestines. Drug Deliv. 23, 2927-35. doi: 10.3109/10717544.2015.1122676.

[29] Shah S.U., Socha M., Sejil C., Gibaud S., 2017. Spray-dried microparticles of glutathione and S-nitrosoglutathione based on Eudragit® FS 30D polymer. Ann. Pharm. Fr. 75, 95-104. doi: 10.1016/j.pharma.2016.09.001

[30] Wu W., Gaucher C., Diab R., Fries I., Xiao Y.L., Hu X.M., Maincent P., Sapin-Minet A., 2015. Time lasting S-nitrosoglutathione polymeric nanoparticles delay cellular protein S-nitrosation. Eur.J. Pharm. Biopharm. 89, 1-8. doi: 10.1016/j.ejpb.2014.11.005.

[31] Wu W., Gaucher C., Fries I., Hu X.M., Maincent P., Sapin-Minet A., 2015b. Polymer nanocomposite particles of S-nitrosoglutathione: A suitable formulation for protection and sustained oral delivery. Int. J. Pharm. 495, 354-61. doi: 10.1016/j.ijpharm.2015.08.074.

[32] Wu W., Perrin-Sarrado C., Ming H., Lartaud I., Maincent P., Hu X.M., Sapin-Minet A., Gaucher C., 2016. Polymer nanocomposites enhance S-nitrosoglutathione intestinal absorption and promote the formation of releasable nitric oxide stores in rat aorta. Nanomedicine 12, 1795-803. doi: 10.1016/j.nano.2016.05.006.

[33] Parent M., DahboulF., Schneider R., Clarot I., Maincent P., Leroy P., Boudier A., 2013. A complete physicochemical identity card of S-nitrosoglutathione. Curr. Pharm. Anal. 9, 31-42. doi: 10.2174/1573412911309010006.

[34] Pagels R.F., Prud'homme R.K., 2015. Polymeric nanoparticles and microparticles for the delivery of peptides, biologics, and soluble therapeutics. J. Control. Release 219, 519-35. https://doi.org/10.1016/j.jconrel.2015.09.001

[35] Chesa-Casalengua P., Jiang C., Bravo-Osuna I., Tucker B.A., Molina-Martinez I.T., Young M.J., Herrero-Vanrell R., 2012. Preservation of biological activity of glial cell line-derived neurotrophic factor (GDNF) after microencapsulation and sterilization by gamma irradiation. Int. J. Pharm. 436, 545-54. doi: 10.1016/j.ijpharm.2012.07.019

[36] He J., Li H., Liu C., Wang G., Ge L., Ma S., Huang L., Yan S., Xu X., 2015. Formulation and evaluation of poly(lactic-co-glycolic acid) microspheres loaded with an altered collagen type II peptide for the treatment of rheumatoid arthritis. J. Microencapsul. 32, 608-17. doi: 10.3109/02652048.2015.1065924.

[37] Liu J., Xu Y., Liu Z., Ren H., Meng Z., Liu K., Liu Z., Yong J., Wang Y., Li X., 2019. A modified hydrophobic ion-pairing complex strategy for long-term peptide delivery with high drug

encapsulation and reduced burst release from PLGA microspheres. Eur. J. Pharm. Biopharm. 144, 217-29. doi: 10.1016/j.ejpb.2019.09.022

[38] Zhang Y., Zhang Y., Guo S., Huang W., 2009. Tyrosine kinase inhibitor loaded PCL microspheres prepared by S/O/W technique using ethanol as pretreatment agent. Int. J. Pharm. 369, 19-23. doi: 10.1016/j.ijpharm.2008.10.032.

[39] Han Y., Tian H., He P., Chen X., Jing X., 2009. Insulin nanoparticle preparation and encapsulation into poly(lactic-co-glycolic acid) microspheres by using an anhydrous system. Int. J. Pharm. 378, 159-66. doi: 10.1016/j.ijpharm.2009.05.021.

[40] Jung J., Perrut M., 2001. Particle design using supercritical fluids: Literature and patent survey. J. Supercrit. Fluids 20, 179-219. https://doi.org/10.1016/S0896-8446(01)00064-X.

[41] Lautner G., Meyerhoff M.E., Schwendeman S.P., 2016. Biodegradable poly(lactic-co-glycolic acid) microspheres loaded with S-nitroso-N-acetyl-D-penicillamine for controlled nitric oxide delivery. J. Control. Release 225, 133-9. doi: 10.1016/j.jconrel.2015.12.056.

[42] Heikal L., Starr A., Martin G.P., Nandi M., Dailey L.A., 2016. In vivo pharmacological activity and biodistribution of S-nitrosophytochelatins after intravenous and intranasal administration in mice. Nitric Oxide 59, 1-9. doi: 10.1016/j.niox.2016.06.006 .

[43] Nacharaju P., Tuckman-Vernon C., Maier K.E., Chouake J., Friedman A., Cabrales P., Friedman J.M., 2012. Nitric Oxide 27, 150-60. doi: 10.1016/j.niox.2012.06.003.

[44] Mordorski B., Pelgrift R., Adler B., Krausz A., Batista da Costa Neto A., Liang H., Gunther L., Clendaniel A., Harper S., Friedman J.M., Nosanchuk J.D., Nacharaju P., Friedman A.J., 2015. Nanomedicine 11, 283-91. doi: 10.1016/j.nano.2014.09.017 .

[45] Yoo J.W., Lee J.S., Lee C.H., 2010. Characterization of nitric oxide-releasing microparticles for the mucosal delivery. J. Biomed. Mater. Res. A 92, 1233-43. doi: 10.1002/jbm.a.32434.

[46] Paolino D. Vero A., Cosco D., Pecora T.M.G., Ciancolo S., Fresta M., Pignatello R., 2016. Improvement of oral bioavailability of curcumin upon microencapsulation with methacrylic copolymers. Front. Pharmacol. 7, 485. doi: 10.3389/fphar.2016.00485.

[47] Gerloff K., Pereira D.I.A., Faria N., Boots A.W., Kolling J., Förster I., Albrecht C., Powell J.J., Schins R.P.F., 2013. Influence of simulated gastro-intestinal conditions on particle-induced cytotoxicity and interleukin-8 regulation in differentiated and undifferentiated Caco-2 cells. Nanotoxicology 7, 353-66. doi: 10.3109/17435390.2012.662249.

[48] Peng Y., Yadava P., Heikkinen A.T., Parrott N., Railkar A., 2014. Applications of a 7-day Caco-2 cell model in drug discovery and development. Eur. J. Pharm. Sci. 56, 120-30. doi: 10.1016/j.ejps.2014.02.008.